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Invertebrate Pathology and Microbial Control (ICIPMC)

XXXV Annual Meeting of the SIP

VI International Conference on Bacillus thuringiensis (ICBt)

Proceedings

August 18 to 23, 2002

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VIII International Colloquium on Invertebrate Pathology and Microbial Control

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35th Annual Meeting of the SIP

Foz do Iguassu, Brazil
18 - 23 August 2002

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Table of Contents

Plenary Session
Baculoviruses and the Bonus of Biotechnology

Baculovirus genetics and gene regulation

D.A. Theilmann

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Baculoviruses are actively being used as biocontrol agents and biotechnology holds great promise in enhancing the utility of these viruses in both agriculture and medicine. Essential for the development of enhanced baculoviruses is a complete understanding at the molecular level of the remarkable pathology of baculovirus infections. Determining the mechanisms of the baculovirus infection process at both a whole animal and cellular level will lead to new insights of gene regulation and host-pathogen interactions. The life cycle of baculoviruses involves a cascade of gene expression that involves early genes which are involved in trans-regulatory functions and genome (DNA) replication, followed by late gene expression that is required for viral structural proteins and virion packaging. Essential to the whole infection process are the early regulatory proteins, such as IE0, IE1, IE2, and PE38 (p34), and LEF proteins, that have roles directly or indirectly in the regulation of both early and late gene expression, and DNA replication. These proteins are key elements for designing genetically modified baculoviruses and harvesting the fruits of the biotechnology revolution. The study of regulatory genes highlights the complexity of baculovirus replication and will identifies points in the replicative cycle that can affect the host range or virulence.

Improvements in insect cell culture for recombinant protein production

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Introduction

The insect cell culture-based baculovirus expression vector (BEV) system is a threefold process composed of the virus vector, the host cell, and the cell culture media. While numerous virus vectors have been developed and many types of cell growth media have been commercially formulated, progress in the establishment or improvement of insect cell lines has been somewhat neglected. Fortunately, three excellent cell lines, the *Spodoptera frugiperda*, IPLB SF21 (and its clone SF 9) and the *Trichoplusia ni* BTI TN5B1-4 cell lines were available for recombinant protein expression studies and production. While it is appreciated that the BEV system has been used successfully for expression many different foreign gene products for almost two decades, it is also recognized that this system has some limitations that need to be addressed through further research. Some of these limitations include variation in production levels of protein, different cell protein processing pathways, and the transient expression of foreign gene products. In this report I will present an overview of the recent developments that have helped to overcome some of these limitations and discuss our studies on new growth parameters and the engineering of insect cells for enhanced protein production.

Cell lines and culture media

Numerous types of insect tissue culture media formulations have been developed for the optimization of cell growth. In general, these media are serum-free and /or protein- free formulations used to culture cell lines which routinely reach densities greater than 1×10^7 cells/ml with viability's above 95%. In many cases, media are optimized for specific cell lines such as Express Five (Life Technologies) or Ex Cell 405 (JRH Biosciences) media for culture of BTI TN5B1-4 (High Five) cells or ExCell 420 (JRH Biosciences) for Sf 21 or SF 9 cells. In other instances, media such as HyQ SFX-Insect MP (HyClone) are formulated for optimum growth of many different cell lines. Almost all of these media formulations are based on Graces medium or specific nutritional requirements of insect cells. Although it has been known for many years that insect cells can grow in mammalian cell culture media (McIntosh et al., 1973) little progress has been reported in the exploitation of mammalian media for insect cell growth and recombinant protein production. Significant progress has been made in the optimization of large-scale culture of insect cells (Taticek et al., 1995).

Historically, there are three cell lines which have been widely used for recombinant protein production. These cells were established from lepidopteran species in the family Noctuidae and include the established cell lines, IPLB SF21 AE (Fig. 1) derived from *Spodoptera frugiperda* ovarian tissue (Vaughn et al., 1977) and its clonal derivative, SF9 (Summers and Smith, 1987). Both of these Spodopteran cell lines have excellent properties and are widely used for protein expression. More recently, Protein Sciences Inc. (Meridian, CT.) reported the development of a new patented *Spodoptera frugiperda* cell line (Express SF+) which is claimed to be superior to SF 9 cells.

In recent years it has been demonstrated in many laboratories that BTI TN5B1-4 (Fig. 2), an insect cell line established from *Trichoplusia ni* eggs (Granados et al. 1994), provides higher levels of foreign protein production than other lines grown in serum-containing or serum-free

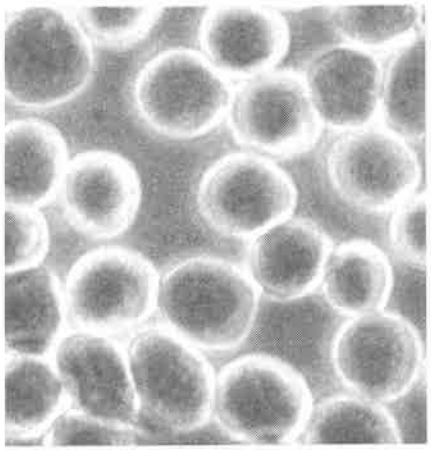

FIG. 1. *Spodoptera frugiperda* (SF21) cells

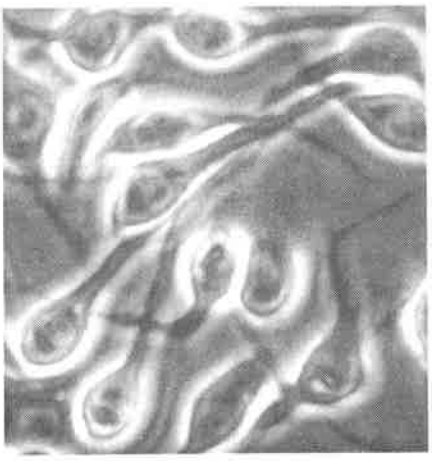

FIG. 2. *Trichoplusia ni* (TN5B1-4) cells

medium (Wickham, et al., 1992). As a result, BTI TN5B1-4 cells, commonly known as "High Five™" cells are marketed by Invitrogen, Corp., Carlsbad, CA. for research purposes and have become a widely used host cell for BEVs (Fig 3 A and B). High Five cells are being used by research and commercial laboratories for the production of pharmaceuticals and animal vaccines.

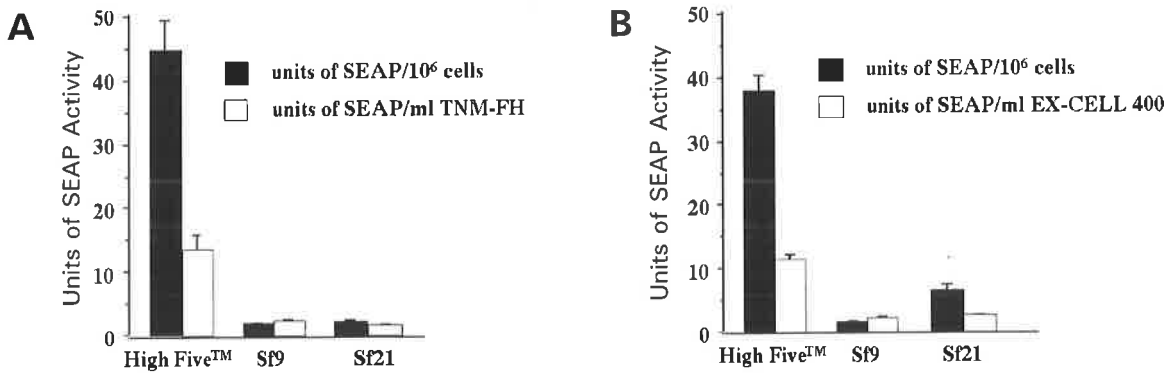

FIG. 3. Expression of recombinant SEAP by three insect cell lines grown in serum-containing medium:TNMFH (A) or serum-free medium: EX-CELL 400(B). Davis et al. (1993).

Another commercial (Novagen, Inc.) insect cell line, Ea4, a clone of an established insect cell line, BTI EAA (Granados and Naughton, 1976) produces carbohydrate side chains which can serve as a substrate for the addition of complex carbohydrates. Many new cell lines have recently been established from different lepidopteran species (McKenna et al., 1998, Goodman et al., 2001) but none have shown superior properties for protein expression than High Five cells. In the absence of new more productive insect cell lines for recombinant protein production, researchers have turned to the engineering of existing cell lines to optimize their efficiency.

Host Cell Engineering

While it is recognized that the BEV system has been used with much success to produce different recombinant proteins, this system still has some important limitations. One such limitation is that insect cell protein processing pathways are not necessarily identical to those of higher eukaryotes, and as a result, chemical modifications of recombinant proteins produced in this system may differ from those on the native protein. For example, insect cells normally fail to glycosylate proteins in precisely the same manner as mammalian cells and this is widely

accepted as a major limitation of BEVs. Several recent studies have used metabolic engineering approaches to improve N-glycosylation in SF9 and High Five cells (Hollister and Jarvis, 2001; Breitbach and Jarvis, 2001). In these studies, insect cell lines were genetically transformed with mammalian genes encoding b1,4-galactosyltransferase and a2,6-sialyltransferase under the transcriptional control of a baculovirus immediate early promoter. New engineered cell lines were produced that could express both genes, served as hosts for baculovirus infection, and produced glycoproteins with terminally sialylated N-glycans. Since insect cells generally fail to glycosylate proteins precisely the same manner as mammalian cells, this accomplishment suggests a means of overcoming a widely recognized major limitation of the BEV system.

Another disadvantage of the BEV system is that it provides only transient expression since the foreign gene is delivered into the host cell by a virus that eventually kills the cell. Furthermore, it is believed that certain protein processing pathways may be compromised by baculovirus infection well before the cell dies. The health, growth, and propagation of host cells prior to infection by recombinant baculoviruses are an important practical concern for small and large-scale protein production. The baculovirus P35 protein is a caspase inhibitor that prevents the induction of apoptosis during infection of SF21 cells by *Autographa californica* nucleopolyhedrovirus (AcMNPV). P35 inhibits the induction of apoptosis in a broad range of cells. In our lab we examined the effects of constitutive cellular P35 expression on the response of cells to stress in culture conditions and on recombinant protein expression. Expression vectors with the P35 gene were used to transform SF9 cells and clonal cell lines were generated and examined from nutrient deprivation and expression of secreted alkaline phosphatase (SEAP) and b-galactosidase (Lin et al., 2001). When compared to wild-type cells, the transformed cells exhibited increased resistance to actinomycin D-induced apoptosis and significant resistance to nutrient deprivation. Expression of SEAP from transformed cells exceeded that from the parental SF9 cells and was comparable to expression levels obtained from TN5B1-4 cells, the best available cell line for high-level expression. It was concluded that increased levels of protein expression appeared to result from a prolonged infection cycle and accumulation of the secreted glycoprotein. Clearly the engineering of cells with genes (metabolic pathway enzymes or P35) that confer resistance of cells to adverse culture conditions or alter their protein expression ability is a significant advancement in this area.

Host Cell Passage and Temperature Effects

Previous studies indicated that the infection of certain insect cell lines with baculoviruses was not affected by long-term passage of host cells in culture in either serum-containing or serum-free media. However, others have noted detrimental affects of long-term passaging of cells. Donaldson and Shuler (1998) demonstrated that long term passaging of BTI-TN5B1-4 cells had a detrimental affect on their growth and recombinant protein expression. Early passage TN5B1-4 cells (130 passages) were superior in expression of the glycoprotein, SEAP than were late passage commercial High five cells (360 passages). Furthermore, the low passage cell lines are smaller in diameter and have a greater propensity to clump in suspension compared to High Five cells. In our laboratory we have confirmed that low passage TN5B1-4 cells are also more efficient than late passage cells that we have grown under similar conditions. Furthermore we found that early passage cells are more tolerant to stress conditions than late passage cells. These studies confirm previous observations that the health, growth, and careful propagation of host cells prior to infection by baculoviruses are important practical concern. Clearly, detrimental effects can be associated with long term culture of insect cells and shows the benefit of using early passage cells for recombinant protein production.

Insect cells are normally cultured at temperatures ranging from 25 to 30° C, unlike vertebrate cells which are normally grown at 37° C. The adaptation and growth of lepidopteran cell lines (Sf9 and Sf21 cells) to 37° C has been reported (Gerbal et al., 2000). We have succeeded in the adaptation of several different lepidopteran cell lines to high temperatures and their susceptibility to baculovirus and /or recombinant protein expression has been demonstrated.

Summary and conclusions

New media formulations and culture conditions have been developed and have facilitated the growth of cells and production of foreign proteins by BEVs. Although numerous host insect cell lines have been developed for recombinant protein production, the most widely used established lines continue to be the SF21 (and derivatives) and TN5B1-4 (High Five) cells. Significant progress has been made in host cell engineering. The stable transformation of cells with mammalian or insect genes can alter the metabolic pathways of cells or increase the tolerance of cells to stressful culture conditions. These studies have demonstrated that genetic transformation of insect cells holds considerable promise for the future improvement of BEVs.

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Engineered baculovirus insecticides

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Dilemma: Agriculture or preservation of the natural environment

The practice of agriculture is a significant shift from nature preservation, displacing natural flora from its place and replacing it with man-altered plants. Since the old ages of agriculture, genetically modified plants were produced using parental varieties as black boxes without control over the process of gene mixing. Later on significant advances in plant genetics made it possible to use more solid scientific background information to recombine genomes and select the most valuable offspring. Now that plant genomics is a reality and as recombinant DNA techniques became more sophisticated, the production of transgenic plants with improved yields and resistance to environmental stresses, infectious agents and pests, can be designed on more rational grounds. Such transgenics do not inherently impose a greater risk on the environment (including human population) than that caused by the more traditional agricultural practice and contribute to important aspects of crop management.

- Protecting yields
- Increasing yields
- Improving yield quality

In general, concurrent strategies are use to address these aspects including cultural practices, development of new varieties (using genetic engineering or molecular markers-assisted plant breeding), integrated pest management, rapid diagnosis of infectious agents, etc.

In this context, a major risk (for humans and other species) associated with agriculture is the widespread use of chemical insecticides that are toxic to a varying and, usually, wide range of species, depending on the particular product. With this in mind, we shall discuss here the approaches that improve the competitiveness of baculoviruses in pest control.

Baculoviruses for pest control: there is room for improvement

Baculoviridae include two genera (*Nucleoplyhedrovirus*, NPV, and *Granulovirus*, GV) of arthropod-specific pathogens and the majority of the baculoviruses are infectious only for insect species within the order Lepidoptera, with no adverse effect on members of other orders. In addition, most of the baculoviruses exhibit a very narrow, mostly single-species, host range (Blissard *et al.*, 2000). Target specificity makes them good candidates for use in integrated pest management systems, and to this date quite a few baculoviruses have been registered as commercial products. However, in general (with few exceptions), they have not gained widespread use in intensive agronomic systems (Moscardi, 1999; Moscardi & Lobo de Souza, 2002). The ingestion of a lethal dose by the target insect and the length of time required to kill or prevent the damage of the crop are the major limitations.

The presence of a sufficient quantity of active virus on the crop can usually be improved by adjusting the formulation of the bioinsecticide to increase its resistance to UV and other environmental injuries (Shapiro, 1992, 1995). The lethal time, on the other hand, is dependent on the virus interaction with its host and the mechanism of infection, determined by its gene content and expression. In this presentation we shall discuss how genetic engineering has been used and can be further applied to enhance the pesticidal properties of baculoviruses,

while maintaining their desirable pest-specificity, and, in this way address the problem of improving their capacity to compete on the market with the fast killing natural and synthetic chemical insecticides.

Strategies for the modification of baculovirus genomes

Due to the large size of the viral genome (80-180 kb) the most widely used methods for the introduction of site-directed changes rely on homologous recombination with a foreign gene flanked by sequences corresponding to a particular locus (*e.g., polyhedrin, p10, egt*). Usually, the cloned gene is inserted in a transfer plasmid-vector, cotransfected with viral DNA in a susceptible cell line and viable virions are recovered. The mixed viral population consists mainly of parental virus and a very small percentage (0.01-1.0 %) of recombinant virus (O'Reilly *et al.*, 1992; King & Possee, 1992). To improve the proportion of recombinants, viral DNA is linearized with a selected restriction endonuclease before cotransfection, dramatically decreasing the recovery of parental virus (Kitts & Posse, 1993). The circularization of the viral genome occurs mainly by recombination with the transfer plasmid containing sequences homologous to those at the ends of the linear DNA. As an alternative to the use of transfer vectors, the gene to be inserted can be flanked by sequences necessary for homologous recombination by PCR amplification using conveniently designed primers (Gritsun *et al.*, 1997). Another strategy has been successfully used to generate the recombinant baculoviral genomes within a bacterial background and rescuing the virus by transfection into insect cells. This method relies on the replication of the viral DNA modified by insertion of a bacterial single copy origin of replication and the use of transposition to transfer the foreign gene (Luckow *et al.*, 1993;). Also, a direct *in vitro* ligation procedure has been reported (Ernst *et al.*, 1994).

Since these procedures are strongly dependent upon susceptible cell lines, more progress has been achieved in engineering NPVs than GVs. Nevertheless, transfection of insect larvae has also been used for recombination of baculovirus DNA with a transfer vector (Hajós *et al.*, 1998); eventually, the selection of recombinants can be done by *in vivo* cloning instead of by plaque assay.

At least, theoretically, two types of genetic modifications can be considered: those that should improve the efficacy of the insecticide and those aimed at expanding the host range.

Enhancement of the biological effects of baculoviruses on their hosts

Improvement of the insecticidal activity of a particular baculovirus on its natural host can be addressed through genetic modifications aimed at reducing the **dose** of virus necessary to kill the insect (LC_{50}, LD_{50}) and/or reducing the **time** to kill the pest (LT_{50}) or to stop damage caused by the pest (feeding time 50: FT_{50}).

In order to insure the desired effect, the following variables should be considered when designing the genetic modification:

- promoters: timing and level of expression (early-weak vs. very late-strong),
- translation efficiency, codon usage
- protein stability, ubiquitinilation
- site of action of the foreign gene product: fusion proteins (Bt: midgut), secretion/ signal peptides (neurotoxins)

The modifications can have an effect on the infectious process or exert an added toxic effect.

Enhancement of events related to the virus entry into the organism (efficiency of infection)

Synergistic and virus enhancing factors (SF and VEF, Tanada, 1985; Derksen & Granados, 1988) have been described as GV proteins responsible for the enhancement of susceptibility to NPV in some mixed infections. SF causes an increase in virus attachment to the cells, whereas VEF (also called enhancin) is a metalloprotease that degrades peritrophic membrane - the first barrier for virus infection. More recently, it became apparent that *vef* genes are not only found in certain GVs, but are also present in some NPVs (LdMNPV, Bischoff & Slavicek, 1997, Popham *et al.*, 2001). There is low homology between the different VEF proteins and different viruses might have gene products belonging to several families of synergistic factors.

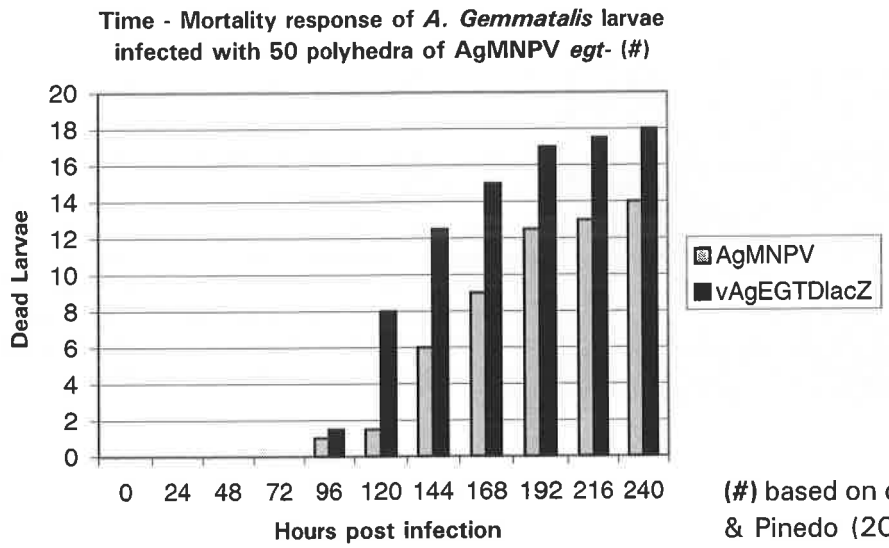
The deletion of two *enhancin* genes from the LdMNPV genome reduces viral potency by 12-fold. On the other hand, the insertion of TnGV *vef* in AcMNPV under the control of the very late p10 promoter reduces the LC_{50} to less than 50% of the wt AcMNPV, and the LT_{50} by 22%.

Another approach to improve the insecticidal activity of baculoviruses is to perforate or eliminate the basement membranes - a physical barrier to systemic infection. In this direction Harrison & Bonning (2001) constructed recombinant AcMNPVs that expressed different proteases and found that AcMLF9.ScathL (AcMNPV expressing *Sarcophaga peregrina* cathepsin L) killed *Heliothis virescens* significantly faster than a virus expressing an insect-selective scorpion neurotoxin from the p6.9 promoter, induced premature melanization, and led to a 26-fold decrease in lettuce consumption compared to the wt virus infected larvae.

Alteration of hormonal balance and insect development

Most baculoviruses express an *egt* gene which codes for an ecdysteroid-UDP-glycosyl-transferase, an enzyme that inactivates ecdysone, the molting hormone. Therefore, the infection with a *wt* baculovirus delays molting and the larva continues feeding, as a genetically-determined strategy to increase virus progeny.

The deletion of the *egt* gene in AcMNPV, therefore, leads to an earlier molting and detention of feeding, resulting in 20 and 40 % reductions in LT_{50} and food consumption, respectively (O'Reilly & Miller, 1991). Similar results have been obtained with other NPVs (LdMNPV, SpliMNPV, AgMNPV) and with CpGV.



(#) based on data published by Bergman & Pinedo (2001)

Insertion of juvenile hormone esterase (JHE) in AcMNPV had no effect on LT_{50} and LD_{50} on *T. ni* larvae as compared to the *wt* AcMNPV, but there was a decrease in food consumption. Probably, the homeostatic mechanisms of the larvae eliminate the excess of JHE via ubiquitinylation and proteolysis. The expression of a modified degradation-resistant JHE yielded a significant reduction of TL50 as well as 36-50% decrease of damage (Bonning & Hammock, 1996).

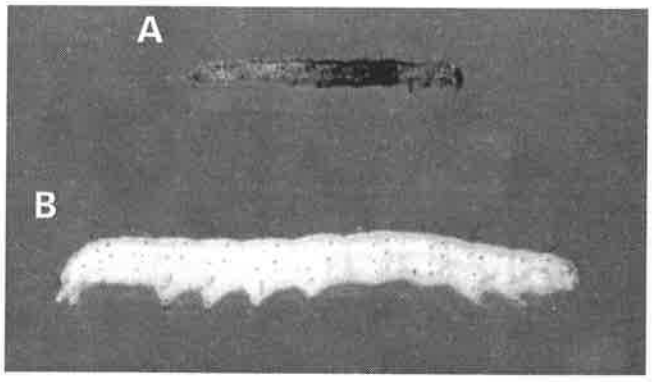
Most of the recombinants using different insect hormone genes did not live up to the expectations, probably due to a system that maintains homeostasis that counteracts any alteration caused by an overexpression by the baculovirus.

Insect-specific toxins

Expression of toxins would not lead to the problems related to compensation by the insect. However, if the expressed toxin kills the cell, it will compromise the viral replication and the larva will probably survive. Therefore, the expression of toxins that are secreted and have a tissue-specific mode of action would solve this problem. The first toxins used to construct AcMNPV recombinants were those from the scorpion *Butus eupeus* and the bacterium *Bacillus thuringiensis*. Both attempts were a failure; in the case of the *Bt* toxin, its target is the intestinal epithelium, which is hardly reached from the infected tissues, and the *Butus* toxin lacked a signal sequence.

The most successful modifications of baculovirus insecticidal properties achieved in the early 90's were based on the insertion of insect-specific neurotoxins isolated from the scorpion *Androctonus australis* (AaIT) and the mite *Pyemotes tritici* (TxP-1). None of them have any effect on mice when injected intracerebrally at high concentrations, but infection with a recombinant baculovirus reduces significantly the damage caused by the pest. The larvae stop feeding due to a paralysis caused by the toxin. Adjustment of the codon usage and secretion signal improved the results (Stewart *et al.*, 1991; Inceoglu *et al.*, 2001). The AaIT expressing AcMNPV AcST3 was the first recombinant baculovirus tested in a controlled field experiment (Cory *et al.*, 1994).

The expression of a range of neurotoxins from sea anemonae, spiders and scorpions have led to varying degrees of insecticidal efficacy improvement, depending on the virus-host system. The most promising of these is probably the Lgh1T2 toxin from the desert scorpion *Leiurus quinquestratus hebraeus* toxin (Gershburg *et al.*, 1998).



A. *Anticarsia gemmatalis* killed by infection with the recombinant AgMNPVM/*tox*$^+$, that expresses TxP-1.

B. *Anticarsia gemmatalis* killed by infection with wt AgMNPV.

The size difference is a reflection of the fact that the toxin expressing virus causes early cessation of feeding due to paralysis of the larva. The larvae infected with the wt virus continue to feed and large amounts of polyhedra accumulate, giving the whitish appearance to the cadaver (Arana *et al.*, 2001).

Combined genetic modifications

The attempts of improving the insecticidal properties of baculoviruses by introducing more than one genetic modification aimed at altering the hormonal balance during infection did not yield the expected results. The combination of Degt with JHE in AcMNPV did not alter the LT_{50} or the larval foliage consumption (Elridge *et al.,* 1992), but D*egt* recombinants expressing PTTH reduced the time to kill 100% of *S. frugiperda* L3 larvae to 6 days compared with the 8 or more days after infection with either D*egt* or PTTH single recombinant.

A combined D*egt* and scorpion toxin expressing recombinant HaSNPV was reported to kill the target insect 27.5% faster than the wt virus. Since it has been observed that injection of two different scorpion toxins causes a cooperative effect increasing their insecticidal by 10-fold, double recombinant baculoviruses have been constructed; however the observed cooperative effect was more modest (Prikhod'ko *et al.,* 1998, Inceoglu *et al.*, 2001).

Host range expansion

With the exception of few members, the Baculoviridae are endowed with a narrow host range and they can be used as "magic bullets" that affect almost exclusively the target insect, leaving the beneficial and other non-target species untouched. This characteristic of the baculoviruses is both, their major advantage over the broad-spectrum chemical insecticides and a disadvantage from the commercial perspective, when more than one pest affects the same crop.

Although, the host range determinants have been the object of many studies, there are no general conclusions that could be used for different virus-host systems. The idea that host range regulation could be at the level of virus entry has been discarded in view of the fact that not only insect cells but also cells from vertebrates can support the immediate early events of infection. Several gene products that function downstream in the infectious cycle have been identified as modulators of the host range using permissive and non-permissive host cells.

Helicase (p143). The wt AcMNPV replicates well in *Spodoptera frugiperda* cells (Sf9), but is not capable of replicating in *Bombyx mori* cells (Bm5). However, recombinant AcMNPV that had their helicase gene replaced with that of BmNPV, were capable of replicating in both cell lines. Moerover, the change in host range was mapped to one or two amino acids (Kamita & Maeda, 1997; Argaud et al., 1998). These results suggested that the block for AcMNPV replication in *Bm5* cells was at the level of DNA replication. However, the conclusions could not be extrapolated since the symmetric experiment, *i.e.,* the replacement for AcMNPV helicase in BmNPV genome did not allow this virus to replicate in the non-permissive *Sf9* cells.

Hrf-1. Thiem *et al.* (1996) have demonstrated that insertion of an LdMNPV DNA fragment containing a gene designated *host range factor 1* (*hrf-1*) enabled AcMNPV to undergo a complete infectious cycle in *Lymantria dispar*, larvae and cells, normally non-permissive for wt AcMNPV.

Hcf-1. The host cell factor 1 is expressed in AcMNPV and allows the replication in some host cells (*TN-368*), but is dispensable for replication in others (*Sf21*).

P35. The product of gene *p35* is a caspase inhibitor and is required for the replication of AcMNPV in *Sf21* and *Sf9* cells but is not required for replication in *Trichoplusia ni* cells. In this context, the antiapoptotic gene product P35 is a host range determinant.

The host range is a result of a block of viral replication at different points established in different insect hosts and the acquisition by the ancestral viruses of a series of genes necessary

to replicate on a particular host during evolution (López-Ferber & Del Rincón Castro, 2001). As a consequence, in order to expand the host specificity of one virus to a second host one could start from comparing the genomes of the viruses that infect each host. Theoretically, it is possible to speculate that the set of genes exclusive for each virus should include the host range determinants. However, the problem is more complex, and, as exemplified by the effect of helicase sequences on the expansion of the AcMNPV host spectrum, some conserved genes may also play a role in virus-host specificity.

Perspectives
Strategies for the generation of improved recombinant baculovirus insecticides

The classic example of successful application of wt baculoviruses in Brazil is a platform to further expand their use by improving their insecticidal characteristics by genetic engineering.

The strategies should aim at: 1) disorganizing or eliminating the physical barriers to the primary infection and/or the spread of systemic infection, 2) the deletion of viral genes that cause a more rapid release of virions form OBs in the midgut of the insect larva, 3) the insertion of insect-specific toxic genes and 4) other alternatives leading to disruption of normal insect physiology. New inhibitory peptides and proteins that do not carry the scary "neurotoxin" label will be an interesting addition to the repertoire.

Fundamental biochemistry, molecular and cell biology and genomics will give us more tools to design better ways to improve the properties of baculoviruses as biopesticides. In this framework, the consideration of the site of delivery of the toxic gene product is crucial to achieve the desired effect (e.g. *vef*, *cry, proteases, neurotoxins*); the type of promoter used to drive its transcription has an influence on the timing and level of expression and may determine the success or failure of the approach; changes in codon usage and protein engineering approaches could be instrumental in augmenting the effect of the foreign gene product, etc.

Regarding the commercial production of recombinant viruses that possess a an enhanced killing capacity, the option using of larvae for bioinsecticide production *in vivo* will be most likely excluded and bioreactors will be the choice.

Safety concerns and public acceptance

Studies on the persistence of the recombinant baculoviruses in the greenhouse microcosm and the field indicate that they show a reduced fitness and are outgrown by the wt virus (Burden *et al.*, 2000, Cory, 2000). To further diminish the potential risks of recombinant baculoviruses in the field until a considerable body of evidence builds up, genetically crippled viruses can be produced incapable of persisting outside the infected insect. To this end OBs of occlusion deficient baculoviruses can be produced in packaging cell lines that complement the lesions in the viral genome.

In the end, the wider use of insect viruses, in general, and recombinant baculoviruses, in particular, to control insect pests will depend upon their competitive characteristics compared to chemical pesticides, as well as the acceptance of the general public. Finally, effective pest management requires flexibility and a variety of tools, and more potent baculovirus pesticides offer clear benefits in terms of environmentally safe pest control.

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Symposium (Fungi 1)
Toward the Integration of Fungal Entomopathogens with Other Biological Control Agents

Interactions between entomopathogenic fungi and predators

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Microbial and arthropod natural enemies can contribute to the suppression of pest populations either as individual species or as species complexes. A pest population will be reduced to a greater extent by complexes than single natural enemies if interference between the natural enemies is minimal or if the natural enemies work in an additive or synergistic way. However, multiple species of natural enemies could act as intra-guild predators/ competitors and such interactions could result in an increase in the pest population. Therefore, assessment of interactions within complexes of natural enemies is essential if they are to be exploited effectively in pest management. Here we describe interactions between two groups of natural enemies - predators and entomopathogenic fungi.

Studies on the direct susceptibility of predators to entomopathogenic fungi have largely been carried out under laboratory conditions which favour the fungus. Under these conditions some species/ isolates are pathogenic to predators (e.g. Magalhaes *et al.*, 1988; James and Lighthart, 1994; Cook *et al.*, 1996; Poprawski *et al.*, 1998; Yeo, 2000). However, under real field conditions, predators are far less likely to be at risk unless there are prolonged periods of high humidity or the insects are stressed (Donegan and Lighthart, 1989; Pell and Vandenberg, 2002). For example, James *et al.*, 1995 demonstrated that the convergent ladybird, *Hippodamia convergens* was susceptible to infection by *Beauveria bassiana* in the laboratory but that in the field pathogenicity varied with weather conditions. Early in the season the incidence of the predator was reduced by 75 - 93% even at low concentrations of the fungus but the predator was not affected later in the season when it was hotter and drier. With appropriate management the fungus could, therefore, be applied when the risk to co-occurring predators was minimal. In laboratory studies by Pell and Vandenberg (2002) *H. convergens* was sprayed with field rates of *Paecilomyces fumosoroseus* and only became infected when periods of high humidity were prolonged or when the beetles had previously been stressed. However, beetles foraging in the environment of *P. fumosoroseus*-infected, sporulating aphid cadavers were at risk of infection under laboratory conditions demonstrating that the route by which inoculum was received may also be important. If secondary cycling after spray application of a mycoinsecticide is unlikely or unnecessary for the strategy to be successful this potential risk is reduced.

Laboratory studies cannot provide complete information concerning the potential risk associated with the application of an entomopathogen, however they can provide clear indications of where there is unlikely to be any risk and where there is potential for risk which requires further research under more realistic conditions. If a fungus does not infect a predator under optimal conditions in the laboratory then it is extremely unlikely that the predator would be at

risk from an application of that fungus in the field (Yeo, 2000). If laboratory studies indicate potential predator infection then further assessments must be made under realistic field conditions and using appropriate application strategies to determine whether the applied fungus is actually likely to have a negative impact on the biodiversity and/ or fitness of predators in the long term. For example, increased use of mycoinsecticides, such as *B. bassiana*, may increase the level of overwintering mortality of hibernating predators as *B. bassiana* is known to be an important mortality factor amongst overwintering predators (Flexner *et al.*, 1986).

Many species/ isolates of fungi have extremely narrow host ranges and do not infect non-host insects even under conditions that favour infection. *Erynia neoaphidis* is an aphid specific pathogen and does not parasitise other aphid natural enemies and so is not an intraguild predator and would not directly infect predator populations. However, it does interact with predators in other ways. If predators consumed infected aphids this could result in a reduction of the pathogen population density. Studies on the predation of *E. neoaphidis* infected aphids by four aphid predators (*Coccinella septempunctata* (ladybird), *Chrysoperla carnea* (lacewing), *Episyrphus balteatus* (hoverfly) and *Pterostichus madidus* (carabid)) demonstrated that only late instar ladybird larvae, ladybird adults and carabids consumed infected aphids (Pell *et al.*, 1997; Roy *et al.*, 1998). Carabids consumed entire infected aphids whereas ladybirds often only partially consumed them and always preferred uninfected prey. Carabids are ground predators and so their impact on fungal density would be minimal. Foraging ladybirds do reduce the number of conidia produced by aphids infected by *E. neoaphidis*, however, transmission of the fungus within an aphid population was not reduced when the source of inoculum was a cadaver damaged by predator feeding as opposed to an intact cadaver (Roy, *et al.*, 1998). Furthermore, the presence of a foraging ladybird significantly increased the transmission of the fungus from both intact and damaged cadavers. In addition, ladybirds foraging in the proximity of sporulating *E. neoaphidis* cadavers became contaminated with fungal conidia which they vectored to uninfected aphids spreading infection (Pell *et al.*, 1997; Roy *et al.*, 2001). This suggests that even though predators can consume a proportion of infected prey this is likely to be of limited impact on the fungus population. Indeed the presence of the predator enhances transmission and dispersal, favouring the development of epizootics. Although these studies were made in both the laboratory and the field they were on a small spatial scale and it is now important to determine whether they are relevant on a farm scale and whether they can be manipulated/ managed to improve pest managment. The relevance of these studies to other predator/ fungus associations also requires investigation.

The safety of fungal control agents is directly linked to their physiological and ecological host range (Goettel, 1994). The physiological host range is defined under laboratory conditions which favour the fungus and represent a worst case scenario. Fungi with narrow host ranges under laboratory conditions are safe to use in the field. The ecological host range represents the hosts actually infected in the field (Hajek and Butler, 2000) and is influenced by environment (e.g. James *et al.*, 1995) and the behaviour of the potential hosts (Roy and Pell, 2000; Pell and Vandenberg, 2002). It is essential to consider both the physiological and ecological host range when assessing potential interactions between natural enemies. The potential for direct infection of predators by fungi is only one interaction, however, and the role of predators in transmission and dispersal of fungi is also be very important and open to manipulation.

Studies conducted so far indicate the generally positive nature of the interactions between insect natural enemies and insect pathogenic fungi with respect to control of pest populations. More long-term studies are required to further investigate the many ways in which complexes of beneficial organisms interact in the agroecosystem and how they can be optimally exploited for pest management.

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Interaction of entomopathogenic fungi, insect parasitoids and their hosts

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Epizootics caused by entomopathogenic fungi are usually restricted to times of the year when humidity levels are high and sufficient numbers of insect hosts are present. Other natural enemies, including predatory and parasitic insects, often provide a greater role in the suppression of pest insects when drier conditions prevail or when host density is lower. Temporal separation of entomopathogenic fungi and other natural enemies is only one mechanism that helps to minimize their negative interaction. A better understanding of the factors that minimize antagonistic interaction of insect pathogens and other natural enemies could improve their integrated utilization against pest insects.

Competition between microorganisms and multicellular animals for insect hosts is pervasive throughout nature (Hochberg and Lawton, 1990). Although insect parasitoids can become infected by generalist entomopathogenic fungi under optimal conditions for the fungus (Goettel et al., 1990; Poprawski et al., 1992; Lacey et al., 1997; Furlong and Pell, 1996), there are numerous examples where parasitoids and entomopathogenic fungi coexist within insect populations. Highly specific fungi, such as *Entomophaga maimaiga* and group specific fungi (*i.e.* those that attack only certain families of insects) such as *Aschersonia* spp. pose no direct threat to parasitoids and other nontarget organisms. Premature death of the host due to fungal infection is one of the main antagonistic interactions between entomopathogens and parasitoids (Brooks, 1993). However, there is also mounting evidence for behavioral and biochemical mechanisms that minimize the negative interactions between fungi and insect parasitoids. In this review we will focus on the positive interaction between insect parasitoids and entomopathogenic fungi. Several examples of positive interaction between parasitoids and entomopathogenic fungi are presented in Table 1. The various types of interactions between these two groups of natural enemies are presented below by category of interaction.

Avoidance of oviposition in fungal-infected insect hosts

Parasitic Hymenoptera use a complex set of semiochemical and physical cues to determine the acceptability of hosts. These include both external and internal host cues. Several examples are reported in the literature where parasitoids selectively oviposit in host insects that are free of fungal infection or are in the early stages of infection. Brobyn et al. (1988) observed that the frequency with which the braconid, *Aphidius rhopalosiphi*, attempted to oviposit in the aphid, *Metopolophium dirhodum*, diminished in aphids treated with *Pandora neoaphidis* 3 days previously. Fransen and van Lenteren (1993) reported probing of the greenhouse whitefly, *Trialeurodes vaporariorum*, recently infected with *Aschersonia aleyrodes* by the aphelinid, *Encarsia formosa*, but oviposition mainly occurred in non-infected hosts. Similarly, Mesquita and Lacey (2001) observed decreased oviposition by *Aphelinus asychis* in the Russian wheat aphid, *Diuraphis noxia*, correlated with the length of time since treatment with *Paecilomyces fumosoroseus*.

TABLE 1. Interaction between insect parasitoids and entomopathogenic fungi in which biochemical defenses or avoidance behavior enabled survival of the parasitoids in the presence of fungal infections.

Insect Host	Fungus	Parasitoid
Trialeurodes vaporariorum	*Aschersonia aleyrodis*	*Encarsia formosa*[1]
Diuraphis noxia	*P. fumosoroseus*	*Aphelinus asychis*[2]
	Zoophthora radicans	*A. asychis*[3]
Metopolophium dirhodum	*Z. radicans*	*Aphidius rhopalosiphi*[4]
	P. neoaphidis	*Aphidius rhopalosiphi*[5]
Therioaphis maculata	*Z. radicans*	*Trioxys complanatus*[6]
Hyperomyzus lactucae	*Pandora neoaphidis*	*Aphidius sonchi*[6]
Heliothis zea	*Nomuraea rileyi*	*Microplitis croceipes*[7]
Cydia pomonella L.	*Beauveria bassiana*	*Ascogaster quadridentatus*[8]
Pieris brassicae	*B. bassiana*	*Apanteles glomeratus*[9]
Pieris brassicae	*B. bassiana*	*Pimpla turionellae*[10]
Diatraea saccaralis	*Metarhizium anisopliae*	*Apanteles flavipes*[11]
		Metagonistylum minense[11]
		Paratheresia claripalpis[11]

[1]Fransen & van Lenteren, 1993, 1994; [2]Mesquita & Lacey, 2001; [3]Poprawski et al., 1992; [4]Brobyn et al., 1988; [5]Powell et al., 1986; [6]Milner et al., 1984; [7]King & Bell, 1978; [8]El-Sufty & Führer, 1985; [9]El-Sufty & Führer, 1981a; [10]Willers et al. 1982; [11]Folegatti and Alves, 1987.

Avoidance of host feeding in infected insects

Several species of parasitoids feed on the blood of host insects as a source of protein. The average number of *D. noxia* probed in preparation for feeding by *A. asychis* females was not significantly influenced by host infection with *Paecilomyces fumosoroseus* (Mesquita and Lacey, 2001). However, the duration of ovipositor insertion was influenced by the length of the time interval between exposure to *P. fumosoroseus* and subsequent exposure to the parasitoid. Parasitoid females spent considerably less time with their ovipositor inserted in aphids that recently died and in aphids that had been exposed to *P. fumosoroseus* 72 h prior to contact with the parasitoids.

Parasitoid protection of host from fungal infection

The premature death of insect hosts due to fungal infection is a major source of mortality of parasitoids that are present in patently infected hosts (Brooks, 1993). But in some hosts that are parasitized prior to treatment, fungal infection is reduced or prevented. Führer and El-Sufty (1979), El-Sufty and Führer (1981a), and Willers et al. (1982) reported the production of fungistatic substances by parasitoid larvae that prevented fungal infection in host Lepidoptera. Fransen and van Lenteren (1994) observed that when parasitized whitefly hosts are treated with *A. aleyrodes* spores four days after parasitization by *E. formosa* takes place, the fungal infection will not establish, and healthy females emerge from their hosts. Mesquita and Lacey (2000) reported that the number of mummies produced by two female *A. asychis*, during 24 h of exposure, varied from 20.3 to 23.0 and was not significantly different when the aphids were first exposed to the parasitoids, then treated with *P. fumosoroseus* 24, 48, 72 and 96 h after exposure. However, they observed a higher percentage of emergence of *A. asychis*, from *D. noxia* that were treated with *P. fumosoroseus* 48-96 hours after parasitization. When the fungus was applied to the aphids prior to exposure to the parasitoids, the development of mummies was significantly reduced as a function of the time between treatment with *P. fumosoroseus* and parasitoid oviposition. Several other cases of parasitoid survival in hosts

that have been treated with fungi are reported (King and Bell, 1978; El-Sufty and Führer, 1981a, 1981b; Milner et al., 1984; Powell et al., 1986; Folegatti and Alves, 1987; Poprawski et al., 1992;). In related studies on fungal-infected and parasitized hosts, fungal infection of parasitoid larvae within the host was either not observed (Powell, 1986) or only reported for a small percentage of individuals (Keller, 1975; Askary and Brodeur, 1999).

Transmission of fungal spores by parasitoids

A potential benefit to fungi of the interaction of parasitoids with fungus-infected hosts is the transmission of spores or hyphal bodies after probing by ovipositing insects. Limited transmission of *A. aleyrodis* from infected *T. vaporariorum* nymphs to other nymphs by *E. formosa* was observed by Fransen and van Lenteren (1993). Similar observations were made by Poprawski et al. (1992) for transmission of *Zoophthora radicans* spores by *A. asychis* to *D. noxia.* Although the parasitoid, *Diadegma semiclausum,* did not directly vector spores of *Z. radicans* to the diamondback moth, *Plutella xylostella,* the incidence of infection in the host was increased due to greater movement of larvae in proximity to the fungus due to disturbance of the host larvae caused by the foraging parasitoid (Furlong and Pell, 1996). Other examples are presented by Roy and Pell (2000).

Parasite facilitation of fungal infection

There are some observations that parasitized hosts are more susceptible to fungal infection, ostensibly due to suppression of the melanization response or weakening of the cuticle. El-Sufty and Führer (1981a) showed that parasitism by *A. glomeratus* promoted penetration of *B. bassiana* through the cuticle of *Pieris brassicae,* however fungistatic factors in the hemolymph of parasitized insects stopped further growth of the fungus. Similar observations for the interaction of the parasitoid *Microplitis croceipes,* the fungus, *Nomuraea rileyi* and the host, *Heliothis zea* were made by King and Bell (1978).

Integration of fungi and parasitoids for practical control of insect pests

Integration of natural enemies has been proposed in a variety of crops. One environment that has received the most attention in terms of integrating fungi and parasitoids is the greenhouse (glasshouse). Studying the complex interaction between *E. formosa*, *A. aleyrodis*, and *T. vaporariorum*, Fransen and Van Lenteren (1993; 1994) found that both natural enemies can act complementarily. Greenhouses that have been treated simultaneously with *E. formosa*, *A. aleyrodis* resulted in successful suppression of whitefly populations (Landa, 1984). Mesquita et al., (1997) reported complementary activity of *P. fumosoroseus* and *A. asychis* for control of *D. noxia*. Similarly, Folegatti and Alves (1987) observed better control of *Diatraea saccharalis* when parasitoids and *M. anisopliae* were combined.

Environmental conditions that favor hymenopteran parasitoids or fungi will influence the type of interaction and compatibility or antagonism of these two groups of biological control agents. Parasitoids are better at exploiting uninfected hosts because of their abilities of search, whereas most pathogens must wait for chance encounters and proper environmental conditions. Based on the models of Begon et al. (1999), coexistence and enhanced biological control are favored by complementarity between parasitoid and pathogens in terms of their extrinsic and intrinsic qualities.

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Interactions between fungi and other entomopathogens

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Abstract

Interactions of fungal and other insect pathogens in the environment are almost unavoidable. Such interactions can be antagonistic, additive or synergistic in nature, and all types have been recorded between pathogens. In addition, interactions between pathogens can change the behaviour of pathogens, such as the host preference among entomopathogenic nematodes, or other aspects of fungal epizootiology. Environment and distribution of hosts also greatly influences the interaction between fungi and other pathogens. Use of multiple pathogens to control insects is becoming more feasible and knowledge of interactions between pathogens necessary for implementation of multiple pathogen systems.

Introduction

Entomopathogenic fungi are ubiquitous in nature, attacking most insect groups. However, the fungi are not the only group of pathogens and often more than one pathogen will be active against a single insect species. While the occurrence of multiple pathogens from a single host has regularly been recorded, few studies have tried to elucidate the complex interactions between microbial pathogens which attack the same host. The effect of two pathogens attacking the same host at the same time could, theoretically, be antagonistic, additive (neutral) or synergistic. Care needs to be taken in interpreting results as, for example, true synergism is a significant increase above the level of mortality expected from both pathogens added, not just a level above the highest kill recorded for one pathogen.

Interactions between entomopathogenic fungi

Several studies have examined the effect of two pathogens attacking a single host. For example, Inglis *et al.* (1997; 1999) examined the use of *Beauveria bassiana* and *Metarhizium flavovirde* against the migratory grasshopper, *Melanoplus sanguinipes*. They found that less mortality occurred for *B. bassiana* at high temperatures than *M. flavoviride* and the reverse was observed at lower temperatures. Co-application of both fungi could result in increased mortality at temperature extremes (Inglis *et al.* 1997) but did not increase mortality under an oscillating temperature regime (Inglis *et al.* 1999). Generally, use of fungal combinations, such as *Metarhizium* and *Beauveria,* results in less than additive mortality, suggesting competition rather than any synergism (e.g. Garcia *et al.* 1990).

Double infections of cadavers due to Entomophthorales has been recorded, but when *Entomophthora muscae* and *E. schizophora*e were released simultaneously in populations of houseflies, less than 10% of cadavers had symptoms of double infections (Jensen *et al.* 2001). Usually in competitive situations, one fungus is more virulent than the other, whether because of environmental factors or because of reduced time to kill. With *E. muscae* and *E. schizophorae*, the latter species was generally more common in houseflies, even when release of *E. muscae* was one day earlier than *E. schizophorae.*

Interactions between entomopathogens are not restricted to between different species of pathogen. For example, Glare *et al.* (1989) found that inoculating spotted alfalfa aphids with two strains of the entomophthoralean fungus, *Zoophthora radicans* resulted in increased resting

spore production. This was attributed to an underlying genetic elements to resting spore production, but does indicate that within species interactions can alter the biology and/or ecology of fungal pathogens.

Entomopathogenic fungi and viruses

Entomopathogenic viruses infect an lot of insect species and it is highly probable that insects will be attacked by fungi even when already infected with virus. There have been a few reports concentrating on the interaction between virus and fungi, mainly reporting on the gypsy moth (*Lymantria dispar*) pathogens, gypsy moth nucleopolyhedrovirus (LdMNPV) and the fungus *Entomophaga maimaiga.* Both the virus and *E. maimaiga* are major causes of mortality in gypsy moth populations in North America often in the same population. However, the virus was not influenced by artificial rain, which was used to increase the levels of *E. maimaiga* (Malakar *et al.* 1999a). A model developed predicted that *E. maimaiga* will have little impact on LdMNPV-induced mortality in *L. dispar*, because *E. maimaiga*-induced mortality was highest only in older instars (Malakar *et al.* 1999a). Within the host, the virus and fungus may act synergistically, as more virus-containing cadavers were found in dual inoculation experiments than at low doses of the virus alone (Malakar *et al.* 1999b). Most larvae of gypsy moth inoculated with both pathogens actually died from *E. maimaiga* infection in these experiments.

Entomopathogenic fungi and nematodes

Some entomopathogenic nematodes have similar penetrative abilities as fungi, but the mode of action once inside a host is through the release of symbiotic bacteria such as *Photorhabdus* and *Xenorhabdus*. An example of the effect of interaction between fungi and nematode pathogens of insects was reported by Barbercheck and Kaya (1990; 1991a; b). Nematodes and *B. bassiana* together killed caterpillars more quickly than either pathogen alone, but rarely co-produced on hosts (Barbercheck and Kaya 1990). More caterpillars of *Spodoptera exigua* previously exposed to *B. bassiana* died when exposed to the nematode *Heterorhabditis bacteriophora* than with the fungus or nematode alone, while the two treatments were less than additive when *Steinernema carpocapsae* and *B. bassiana* were used (Barbercheck and Kaya 1991a). When the order of application was reversed, mortality of *S. exigua* from *S. carpocapsae* and *H. bacteriophora* was reduced relative to the treatments with nematodes alone. However, these nematodes were more likely to occur in hosts not infected with *B. bassiana* in choice tests in soil, suggesting a behaviour which minimises antagonistic interactions between entomophilic nematodes and *B. bassiana* in soil (Barbercheck and Kaya 1991b).

Entomopathogenic fungi and bacteria

The most widely used biopesticides are all based on the bacterium *Bacillus thuringiensis* (*Bt*) and it is not surprising that several studies have reported on the effects of entomopathogenic fungi with *Bt*. Both *Beauveria* and *Metarhizium* have been used in conjunction with *Bt*. In the laboratory against *Ostrina nubalis,* the actions of *Bt kurstaki* and *B. bassiana* were "independent" (additive?) (Lewis and Bing 1991), whereas El-Maghraby *et al.* (1988) found the same combination antagonistic on *Spodoptera littoralis* larvae. Costa *et al.* (2001) found sublethal exposure of the Colorado potato beetle to delta-endotoxin from *Bt* did not increase the susceptibility to *B. bassiana*, despite obvious negative impacts on beetle development. However, combinations of the *Bt* products Bitoxibacillin [*Bt thuringiensis*] and Novodor [*Bt tenebrionis*] with Boverin (*B. bassiana*) against Colorado potato beetle larvae suppressed larval feeding to a greater extent than any preparation alone (Lesovoj and Goral 1996). Mixtures of Boverin and

Ecotec Bio (*Bt kurstaki*) were ineffective with *Bt* suppressing the fungal product (Lesovoj and Goral 1996). In a study using only the destruxin toxins from *M. anisopliae*, Brouseau *et al.* (1998) found some evidence of synergism with *Bt kurstaki* at low doses. The use of the fungus *Nomuraea rileyi* in combination with *Bt kurstaki* against 1-2 day old larvae of *Trichoplusia ni* was less than additive (Ignoffo *et al.* 1980b).

Another bacterial pathogen, *Serratia entomophila* (Enterobacteriaceae), causes amber disease in the New Zealand grass grub, *Costelytra zealandica*. Application of *S. entomophila*, which causes a chronic disease in the larvae, with *M. anisopliae* resulted in a synergistic interaction against 2nd instar larvae (greater than additive inside the 41 days of the experiment) (Glare 1994). Treatment of 3rd instar larvae with both pathogens did not increase mortality compared with the fungus alone. While this combination suggested some synergism occurs between the pathogens, it probably has little impact in the field as all larvae infected with the bacteria would have died anyway. Similar results have been found using *B. bassiana* and *S. entomophila* against the *C. zealandica* (Glare, unpubl. data)

Outside the Deuteromycetes, Eilenberg *et al.* (2000) reported on studies of the natural coprevalence of *Strongwellsea castrans* (Entomophthorales), *Cystosporogenes deliaradicae* (Microspora) and *Bt* on adults of *Delia radicum* in Denmark. They found a significantly higher risk of natural infection with *C. deliaradicae* in *D. radicum* which were also infected with *S. castrans*, than in uninfected *D. radicum,* while only two flies were found infected with *Bt* and *S. castrans.*

Entomopathogenic fungi and protozoa

Protozoa and fungal interactions are rarely reported, however Fuxa (1978) found that on the caterpillar *Heliothis zea,* the interaction between the protozoan *Vairimorpha necatrix* and the fungus *Nomuraea rileyi* was at least additive, but variable depending on the different proportions of the pathogens applied. The study of Eilenberg *et al.* (2000) reported above also recorded a higher risk of fungal infection following microspore infection.

Epizootiology

While laboratory studies can demonstrate direct interactions between hosts and the effects of some variables, such as temperature, timing of infection and host stage, the interaction between pathogens in the field will be dependent upon a wide range of variables. Few insects suffer from only a single disease and interactions between various pathogens will be extensive. For example, the New Zealand grass grub, *C. zealandica,* has over 40 described pathogens (Glare *et al.* 1993) and the interaction between them is poorly understood. Different pathogens predominant in different parts of the country, generally due to temperature differences (e.g. Popay 1992). In the laboratory, Barbercheck and Kaya (1990) demonstrated temperature influenced the outcome of development in *S. exigua* larvae infected with nematodes and *B. bassiana*. In early sequential treatments *B. bassiana* was more likely to develop to the exclusion of nematodes at 15°C, while nematodes were more likely to develop in these treatments at 22 and 30°C. Similarly, temperature was the influential factor in competition between *B. bassiana* and *M. flavoviride* in the experiments of Inglis *et al.* (1997; 1999). Obviously, the spatial distribution of hosts influences interactions between pathogens, altering the amount of inoculum and timing of multiple pathogen infections. There is still a great deal more to understand about how pathogens interact and pathogen combinations may yet be a method to overcome the limitations of using single pathogens (i.e. as biopesticides) in pest control.

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Interactions between entomopathogenic fungi and chemicals pesticides

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Interactions between entomopathogenic fungi and chemical pesticides may be of two basic types. Interactions are positive (synergistic) when there are improvements in the control efficiency of the pathogen, or negative (antagonistic) when this efficiency is reduced (e.g., due to inhibition of pathogen germination or reduction in pathogen inoculum). Control agents may also exhibit no significant positive or negative interaction, in which case the combined independent effects are generally referred to as additive.

Negative interactions and selective effects that enhance compatibility between insect pathogenic fungi and chemical pesticides (by precluding negative interactions) have been more extensively studied than positive interactions and are important factors in successful Integrated Pest Management (IPM) programs. Making an analogy with parasitic insects, we may say that chemical selectivity with respect to these organisms, investigated in many IPM programs, has contributed to the use of more selective products, preserving this important natural control resource. However, with regard to studies of selectivity/compatibility of chemicals used in combination with entomopathogenic fungi, there are no standard methodologies (protocols). This subject is important, but beyond the scope of this paper, which will focus on positive interactions.

Positive interactions between chemicals and entomophatogenic fungi were first investigated for *Beauveria bassiana* and DDT combinations applied against Colorado potato beetle (CPB) *Leptinotarsa decemlineata*; however, no synergism was observed (Fargues, 1973). *B. bassiana* combined with carbaryl and azinphos-ethyl also did not show synergistic effects in CPB control (Fargues, 1975), and similar results were observed by Anderson et al. (1989) who tested combinations of *B. bassiana* and carbaryl, fenvarelate, abamectin, triflumuron, and thuringiensin.

Studies by Hassan and Charnley (1989) demonstrated that the cuticle of *Manduca sexta* larvae treated with the benzoylurea insecticide diflubenzuron (Dimilin) was less resistance to penetrant hyphae of *Metarhizium anisopliae* than untreated cuticle; however, larval mortality enhancement was low. Laboratory assays also revealed that a mixture of diflubenzuron and *B. bassiana* could increase the rate of mortality among grasshoppers (Reuter *et al.*, 1996). Field applications in the United States demonstrated that a combination of *B. bassiana* and diflubenzuron was more effective than other *B. bassiana*/chemical pesticide combinations, but in low effective rates (Foster *et al.*, 1996 cited by Delgado *et al.,* 1999)

Another study, on control of the grasshopper complex in Mali, showed only an additive effect of *B. bassiana* and diflubenzuron. On plots treated only with *B. bassiana* or diflubenzuron, grasshopper populations decreased 38.1% and 29.4%, respectively, within 14 days after application, whereas on plots treated with *B. bassiana* plus diflubenzuron, populations decreased 55.6% (Delgado *et al.*, 1999).

Another pesticide that has shown synergic effects when used in combination with entomopathogenic fungi is the chloronicotinyl insecticide imidacloprid. This effect has been

observed with termites, soil inhabiting Curculionidae larvae and cockroaches. First observations were made in subterranean termites. When nitroguanidine imidacloprid was applied in baits, collected termites rapidly succumbed to infections by the soil-borne pathogens *Conidiobulus coronatus* and *M. anisopliae* (Zeck, 1992). Following these observations, Boucias *et al.* (1996) investigated the effects of imidacloprid and *B. bassiana* on the termite *Reticulitermes flavipes*. These authors observed that after treatment with imidacloprid, termites showed hypersensitivity to artificially introduced fungi such *B. bassiana* and to indigenous pathogens such *Conidiobulus coronatus* and concluded that the imidacloprid action involved an immediate disruption of social behavior. Grooming, trophalaxis and tunnel construction were suppressed within 1 to 2 hrs after exposure to imidacloprid.

In field studies for control of *Heterotermes tenuis*, a low level of synergism was observed when *B. bassiana* was applied in combination with imidacloprid in baits (Almeida and Alves, 1996). Almeida *et al.* (1998) also studied *H. tenuis* control in sugarcane using Termitrap baits impregnated with low concentrations of insecticides and associated or not with *B. bassiana*. The treatments with imidacloprid and fipronil associated with *B. bassiana* were the most efficient for termite control. In other studies during the dry season when Termitrap baits were impregnated with triflumuron 0.2%, triflumuron 0.1% + *B. bassiana* and *B. bassiana* alone there was total elimination of termite populations in the focus 15 days after application. However, when triflumuron was applied alone, at a rate of 0,1 and 0,15%, the decrease in population was smaller (Almeida and Alves, 1999). Also, the insecticide fipronil in sublethal doses associated with *B. bassiana* in Termitrap baits was highly effective against *Cornitermes cumulans* and *H. tenuis* in sugarcane (Almeida *et al.*, 2000). Moino Jr. and Alves (1998) observed changes in grooming behavior of *H. tenuis* as a result of imidacloprid intoxication. It was revealed by scanning electron microscopy that *B. bassiana* and *M. anisopliae* conidia were removed from workers 6 hrs after application of the fungi alone, and that, in the same time, imidacloprid treated insects did not remove conidia. These observations are in agreement with those of Boucias *et al.* (1996).

Neves & Alves (1999) found that by combining imidacloprid and *B. bassiana* or *M. anisopliae*, it was possible to reduce the recommended dosages of imidacloprid (Gaucho 70 PM) by more than 99% and of conidial concentrations by 75% and still achieve e"80% control of large nests of the mound termite *Cornitermes cumulans*. The recommended combination for control of large nests is 6 g of the inert ingredient calcene, 1.9 mg of Gaucho 70 PM and 500 mg of *B. bassiana* conidia. Other studies were carried out to verify inhibition in *C. cumulans* grooming behavior when treated with sublethal rates of imidacloprid and entomopathogenic fungi (Neves and Alves, 2000). Scanning electron microscopy revealed that grooming by termite workers was highly efficient, removing all conidia from the cuticle of soldiers and other workers in the first hours after fungal application. The insecticide imidacloprid, when used in sublethal concentrations, inhibited grooming behavior and allowed conidia to germinate and penetrate the insect cuticle, causing infection.

Sublethal doses of imidacloprid, applied as a contact or oral treatment, increased synergistically the pathogenicity of *M. anisopliae* and *B. bassiana* against first-instar larvae of *Diaprepes abbreviatus*, a soil inhabiting insect (Quintela and McCoy, 1997). Synergistic effects were also observed when sublethal doses of imidacloprid were combined with conidia of *M. anisopliae* or *B. bassiana* applied to the soil (Quintela and McCoy, 1998). These authors concluded that a retarded locomotory response of first-instar *D. abbreviatus* treated with sublethal rates of imidacloprid was an important factor in the synergism. The loss of mobility prevented larvae from removing conidia upon contact with the soil substrate. Quintela (1996), cited by Quintela and McCoy (1998), observed that conidia were sheared from cuticle by the active movement

of larvae through soil. They supported this hypothesis by the fact that in all bioassays, larval mortality and mycosis were highly correlated with larval mobility in soil.

Two *Blatella germanica* (German cockroach) strains, one susceptible and the other resistant, to imidacloprid, were killed significantly faster when the insects fed on imidacloprid after a topical application of *M. anisopliae* conidia. This indicated a possible synergistic interaction between imidacloprid and *Metarhizium* (Kaakeh *et al*, 1997). Pachamuthu and Kamble (2000), testing the combined toxicity of *M. anisopliae* with sublethal doses of chlorpyrifos, propetamphos and cyfluthrin observed that the percentage of *Blattella germanica* mortality was significant higher than the insecticide alone and in certain combinations higher than *M. anisopliae* alone. The interactions indicated an additive effect for chlorpyrifos and cyfluthrin, and a synergistic effect for propetamphos.

As we may observe, the most recent and numerous studies demonstrating positive interactions between chemical pesticides and fungal entomopathogens have focused on combinations of *M. anisopliae* or *B. bassiana* and sublethal rates of imidacloprid. Imidadcloprid appears to act as a stressor primarily by affecting insect behavior, especially with regard to locomotion and grooming (Boucias *et al.*, 1996; Quintela and McCoy, 1997 and Neves and Alves, 2000). Boucias *et al.*, (1996) observed that imidacloprid in non-lethal doses did not cause alterations either in the insect cuticle or in the immune system. However, for diflubenzuron synergism there was a physiological disruption in the insect cuticle (chitin lamellae) that facilitated penetration by the fungus (Hassan and Charnley, 1989). Other products may have the capacity to enhance fungal efficacy via different mechanisms (e.g., by causing a direct or indirect alteration in the insect immune system).

Although, some studies have been made on positive interactions of entomopathogenic fungi with chemicals, this is a subject that warrants considerable additional study, especially with respect to new-generation insecticides such as neonicotinoids. These products show a high compatibility with *B. bassiana*, *M. anisopliae* and *Paecilomyces* sp. *in vitro* (Neves *et al.*, 2000). The exploitation of positive interactions among entomopathogenic fungi and chemicals in pest control has great potential to increase the use of microbial pesticides and enable greater realization of their ecological benefits. Additional study should be directed as well toward achieving a better understanding of the negative interactions among chemicals and fungal pathogens, with the objective of preserving these important biological control agents in IPM strategies.

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Symposium (Viruses 1) Arthropod-borne Virus

Contributions of invertebrate pathology to vector control

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Control of the invertebrate host is an integral part of any integrated program to prevent the spread of vector borne diseases of man and animals. This includes important mosquito borne arboviruses such as yellow fever, dengue and the various types of encephalitis including West Nile Virus, St. Louis Encephalitis, Eastern Equine Encephalitis and Venezuelan Equine Encephalitis among many others. Efforts to control mosquito vectors have relied primarily on chemical adulticiding and larviciding, source reduction and biological control. Investigations on biological control, specifically microbial control, have resulted in many important contributions to both applied and basic research on pathogens of mosquitoes and other Diptera. Invertebrate pathologists worldwide have discovered important diseases of mosquitoes including bacteria, fungi, protozoa, microsporidia, nematodes and viruses some of which are currently widely utilized in vector control programs.

The microbial insecticide, *Bacillus thuringiensis* var. *israelensis* (*Bti*) was first discovered in 1976 (Goldberg and Margalit, 1977) and since that time has proven to be highly pathogenic for many aquatic Diptera mainly the Culicidae, Simuliidae, Dixiidae, Chironomidae, and some Ceratopogonidae (Lacey, 1997). *Bti* was registered by the US EPA in 1983 and has been successfully utilized for control of mosquitoes and black flies worldwide with more than 25 products registered for mosquito and black fly control. *Bti* has an excellent safety record and resistance has not been found probably due to its complex mode of action involving synergistic interaction between up to four proteins (Becker and Margalit, 1993). *Bacillus sphaericus* (*Bs*) is another successful microbial insecticide isolated from mosquitoes in 1965 (Kellen et al., 1965; Lacey, 1997). It was registered in 1991 and there are currently two registered products for mosquitoes with good activity for *Culex* and some *Anopheles* mosquitoes but less effective for *Aedes* spp. *Bs* also has an excellent safety record but there have been numerous reports of resistance development (Rao et al., 1995).

Fungi belonging to the genera *Lagenidium, Culicinomyces and Coelomomyces* have been studied as microbial control agents for mosquitoes (Lacey and Undeen, 1986). *Lagenidium giganteum* has been the most extensively developed (Brey and Remaudiere, 1985; Kerwin and Petersen, 1997) and is registered with the U.S. Environmental Protection Agency and several states, including California and Florida, for operational control of larval mosquitoes. *L. giganteum* is specific for mosquitoes and has shown good recycling capabilities for weeks, months, or even years in certain breeding habitats after a single application (Kerwin et, 1994).

Other groups of mosquito pathogens have been extensively investigated including the ciliate *Lambronella clarki* (Washburn and Anderson, 1991), microsporidia such as *Branciola (=Nosema) algerae* (Anthony et.al, 1978), *Edhazardia aedis* (Becnel et al.,1989) and *Amblyospora connecticus* (Andreadis, 1988; 1990) and the mermithid nematodes *Romanomermis culicivorax*

(Petersen, 1985) and *Strelkovimermis spiculatus* (Camino and Garcia, 1990; Garcia and Camino, 1991). While these are important natural control agents for mosquitoes, their use for operational mosquito control is limited because of requirements for in vivo production and a short shelf life. However, some of these have demonstrated effectiveness as introduced agents and may play a role in classical biological control projects such as *E. aedis* for *Aedes aegypti* (Becnel and Johnson, 2000) and *S. spiculatus* for *Culex* mosquitoes (Becnel and Johnson, 1998).

Mosquito pathogenic viruses such as baculoviruses and iridescent viruses have been known since 1963 but none have shown any potential due to the inability to transmit these pathogens to the host. Recently, a new baculovirus for *Culex* mosquitoes (CuniNPV) has been isolated where transmission has been determined to be mediated by divalent cations: magnesium is required for transmission whereas calcium inhibits transmission (Becnel et al., 2001). The ability to transmit CuniNPV has, for the first time, allows the evaluation of these viruses as control agents for mosquitoes and also to conduct molecular studies to investigate genes that determine transmission, host range, virulence and other key biological features required to infect and kill mosquitoes (Afonso et al., 2001; Moser et al., 2001).

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Yellow fever in South America

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Yellow fever, the original viral hemorrhagic fever, is endemic in tropical regions of Africa and South America where the virus is maintained as a zoonotic infection, within complex cycles involving jungle mosquitoes and monkeys (Monath, 2001; Robertson et al., 1996; Vasconcelos et al., 1999)., It is estimated that approximately 200,000 yellow fever infections occur annually, with several thousand deaths. But, the disease is grossly underreported and only about 5000 and 300 cases reported annually in Africa and South America, respectively (Monath, 2001).

Yellow fever virus is the prototype of the genus *Flavivirus* of the family *Flaviviridae*; it is a single-stranded RNA virus positive sense (Westaway et al., 1985). The virus genome contains a single open-reading frame with 10, 233 nucleotides that encodes three structural (prM, C, and E) and seven non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins, and two short non-coding regions (3′ and 5′ NCRs) (Rice et al., 1985; Hahn et al., 1987). Presently, seven yellow fever virus genotypes (five in Africa and two in south America) are recognized within a single serotype (Wang et al., 1996; Mutebi et al., 2001).

The diagnosis of yellow fever, is based on the clinical symptoms and plus at least detection of specific IgM antibodies in unvaccinated persons (Kuno et al., 1987), virus isolation in animals or cell cultures (Tesh, 1979; Tesh et al., 2001), detection of antigens by immunohistochemistry (Hall et al., 1991), and/or genome recovery by RT-PCR (Lanciotti et al., 1992).

Clinically, yellow fever occurring during an epidemic or in a patient having the classical signs of severe disease is relatively easy to diagnose. The problem is that only 10-20% of people developing yellow fever virus infection shows the classical signs of yellow fever. Abortive infections are difficult to recognize, except during epidemics when multiple cases alert physicians to its presence. In classical yellow fever, patients show a severe systemic illness with fever, headache, myalgia, Faget' sign, conjunctival congestion, flushing of neck and face, and other non specific symptoms/signs that can not be easily differentiated from other febrile illnesses.

The early phase of more severe forms shows similar symptoms/signs and is called the "period of infection". This phase lasts about three days. During this period, yellow fever virus can be detected in blood; therefore patients in this stage are infectious for mosquitoes. After this period, patients with abortive forms show rapid recovery, while those with severe forms have apparent improvement of symptoms that lasts several hours to one or two days and is known as "period of remission". This brief period is immediately followed by the "period of intoxication" that is characterized by low viremia or absence of it, return of high fever, and presence of jaundice, vomiting, epigastric pain, dehydration, hemorrhagic diathesis, prostration and renal failure. Other symptoms/signs commonly present at this stage include thrombocytopenia, albuminuria, tachycardia, hypotension, encephalopathic signs, and azotemia. The picture is easily recognized when, coffee-ground hematemesis, oligo-anuria and jaundice are evident. About 50% of these patients have a fatal outcome. Other intermediate more or less severe forms have also been described (Serié et al., 1968; Monath, 1988; Vasconcelos et al., 1997; Monath, 2001; Vasconcelos, 2000).

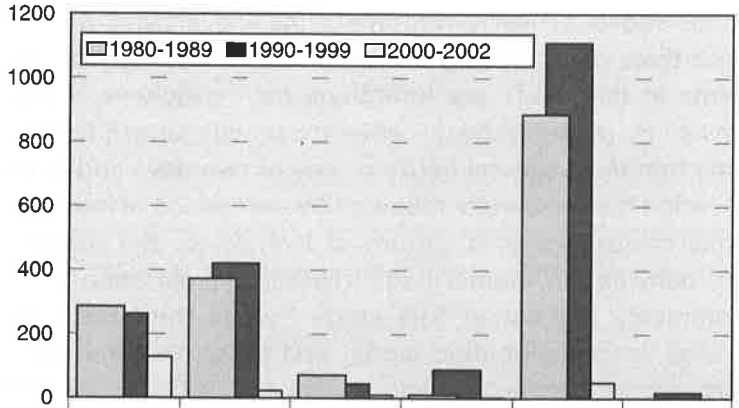
Yellow fever virus is transmitted in different cycles in Africa and South America. In Africa, a third or intermediate cycle has been recognized in addition to the urban and jungle cycles (Digoutte *et al.*, 1995). In South America, only two cycles (urban and jungle) has been recognized until now. In Africa, the vectors are mosquito species of the genus *Aedes*; in South America, *Haemagogus* and *Sabethes* species are responsible for the jungle cycle transmission (Monath, 1988).

The recent increases in distribution and density of *Aedes aegypti* in urban areas of the Americas with resulting epidemics of dengue fever and dengue hemorrhagic fever, brings again the concern of the re-urbanization of yellow fever (Mondet et al., 1996; Vasconcelos et al., 1999; Travassos da Rosa et al., 2000). It is worthy of note that the last urban epidemic in the Americas occurred in Rio de Janeiro in 1929-30, and the last case was reported in Port-of-Spain (Caribbean region) in 1954 (Nobre et al., 1994; Dégallier et al., 1996; Vasconcelos et al., 1999).

In South America, in the last two decades, yellow fever has been reported in Bolivia, Brazil, Colombia, Ecuador, French Guyana (a single fatal case reported in 1998), Peru and Venezuela (Figure 1), and excepting for a few urban cases reported in Bolivia (Van der Stuyft et al., 1999), all of them were of jungle yellow fever. However, more than 93%, of the total have been reported by three countries: Bolivia, Brazil and Peru. While Bolivia reported about 20% and Brazil almost 18%, Peru reported more than 50% of all cases on the continent (Figure 2). The case-fatality rate was high, reaching 49.6%, and clearly represents an underestimate, since almost all reported cases were due to classical yellow fever, and in general was diagnosed after the patient's hospitalization. Only exceptionally is the case-fatality rate in sylvan cases under 30%, which was observed in Brazil in 1993 and 1994 (Figure 3) (Vasconcelos et al., 1997; Vasconcelos et al., 1999). The overall case-fatality rate ranges from 33.3% in Venezuela through 79% in Colombia (Figure 4).

Yellow fever, in South America, is transmitted mainly in Amazon, Araguaia-Tocantins and Orinoco basins, especially by *Haemagogus janthinomys*; but other *Haemagogus* and *Sabethes* species have also been recognized to be potential vectors of yellow fever virus in the region (Monath, 1988; Monath, 2001; Vasconcelos et al., 2001b).

The coastal zone of South America, the most populated area, is in general yellow fever free, but the presence of *Aedes aegypti*, sometimes in increased indexes is noteworthy. The



	Brazil	Bolivia	Colombia	Ecuador	Peru	Venezuela
1980-1989	286	373	72	11	887	0
1990-1999	263	421	45	90	1111	18
2000-2002	129	23	10	2	49	0

FIG. 1. Yellow fever reported cases in South America, 1980-2002 (figures for March).
Source: PAHO/WHO

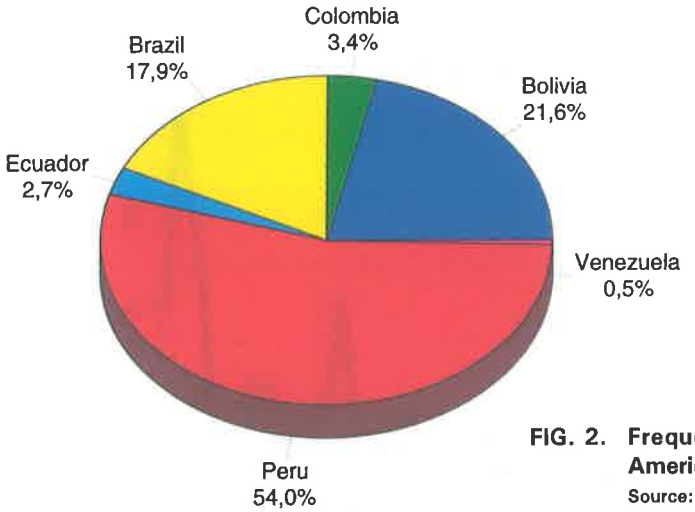


FIG. 2. Frequency of yellow fever by country in South America, 1980-2002 (figures for March).
Source: PAHO/WHO.

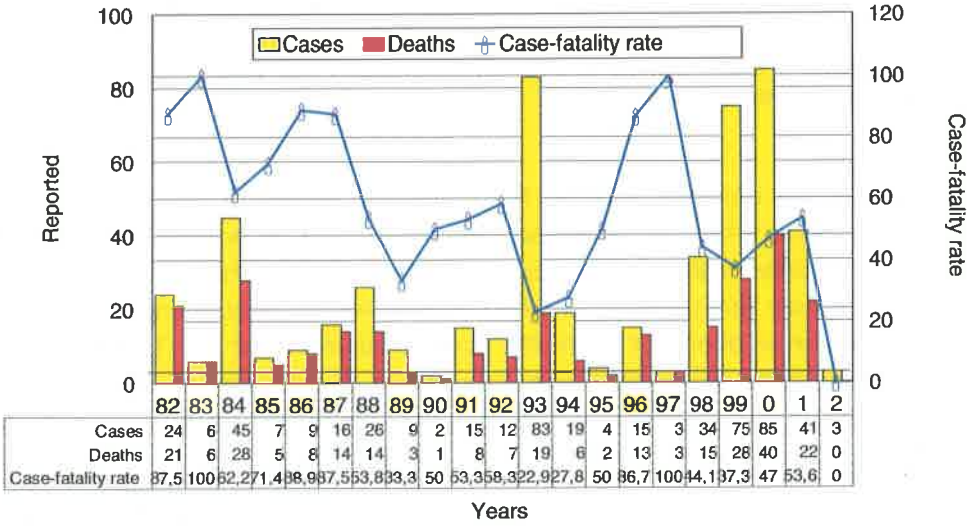


FIG. 3. Yellow fever reported cases, deaths, and case-fatality rate in Brazil, 1982-2002 (figures for March).
Source: CENEPI/FUNASA/MS

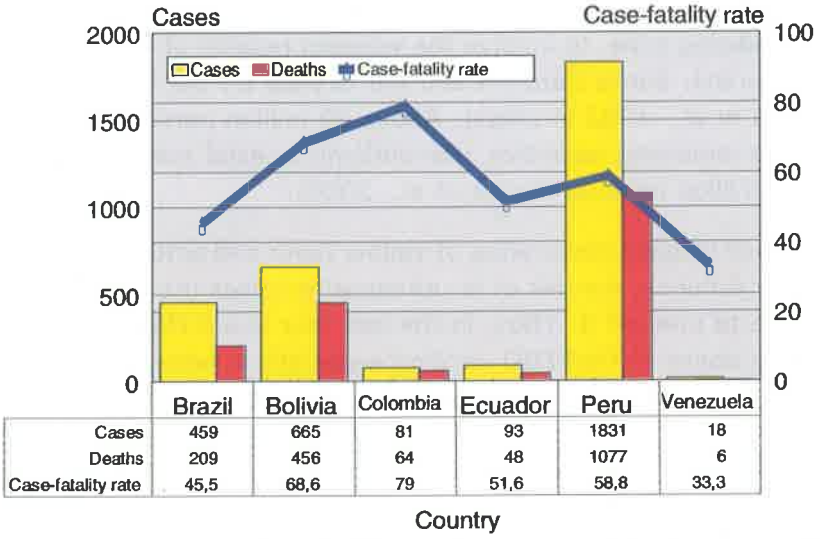


FIG. 4. Yellow fever cases, deaths and case-fatality rate by country in South America, 1985-2002 (figures for March).
Source: PAHO/WHO.

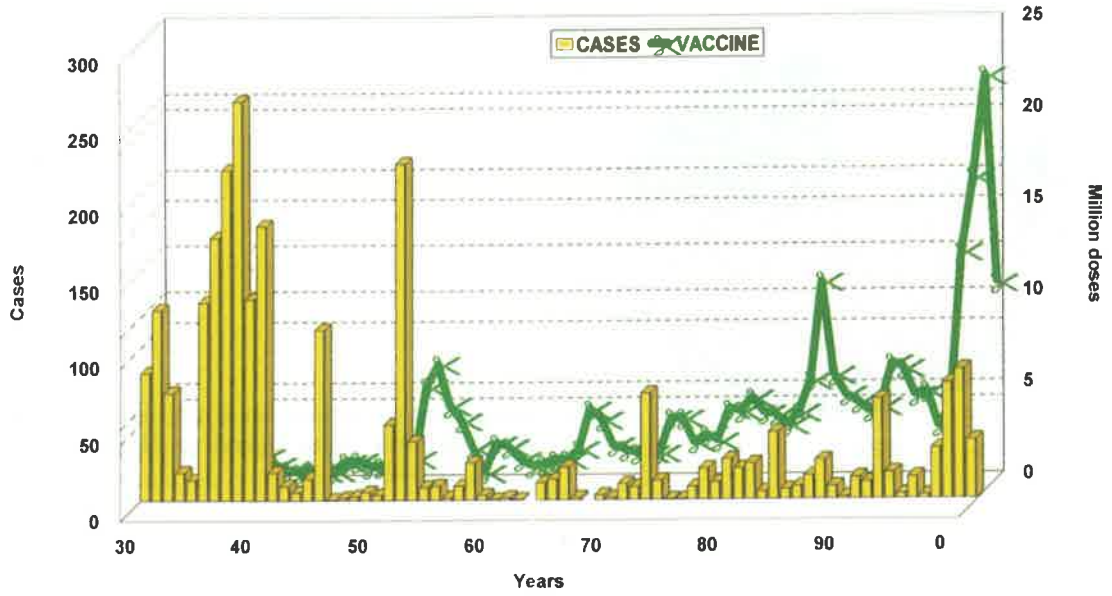


FIG. 5. Yellow fever in Brazil: number of reported cases and 17DD vaccine doses used, 1930-2001.
Source:CGVEP/CGPNI/CENEPI/FUNASA/MS

reintroduction of this mosquito species has increased the risk of urban yellow fever, especially in the last five years when an increased reemergence of virus transmission was observed in the continent, mainly in Brazil. This country, like several others in South America, has an extensive Amazonian forest region in which jungle yellow fever is endemic and a coastal zone in which yellow fever does not currently occur (Robertson et al., 1996; Vasconcelos et al., 1997). The boundary between these two zones designated as pre-Amazon forest, and intermediate savanna and gallery forests may be affected by periodic expansions in epizootic activity (Mondet et al., 1996; Vasconcelos et al., 2001b).

In Brazil, the endemic area includes 12 states in the western two-thirds of the country inhabited by 29.3 million people. Beginning in the first quarter of 1998 and continuing to 2001, one of the largest epizootics in history has occurred, leading to the occurrence of 236 registered human cases of jungle yellow fever ;105 of them or 44.5% had a fatal outcome (Vasconcelos et al., 2001a; Vasconcelos et al., 2001b). The epizootic has expanded beyond traditional boundaries of the endemic zone, to involve the western regions of the states of Minas Gerais, São Paulo, Bahia, Paraná, Santa Catarina and Rio Grande do Sul States (Vasconcelos et al., 2001b; Vasconcelos et al., 2002-in press). About 20 million persons inhabit this transitional epizootic region. The remaining receptive, non-endemic coastal zone includes 15 states and is inhabited by 126.3 million persons (Costa et al., 2002).

Considering the annual disease occurrence of yellow fever and actual figures of *Aedes aegypti* indexes in the South America, the risk of re-urbanization raises the question of massive use of yellow fever vaccine to prevent it. Thus, in the last four years (1988-2001), Brazil has used more than 60 million doses of the17DD vaccine especially in endemic and transitional yellow fever zones (Figure 5). This massive use, resulted in the appearance of several serious adverse events, including deaths due the vaccine (Vasconcelos et al., 2001c; Galler et al., 2001). In consequence, Brazilian authorities reviewed the policy for vaccine utilization and yellow fever vaccination is now only recommended to people living in the high risk areas (endemic and transitional zones), and for those that are travelling or will travel to these areas, focusing on tourists, agricultural workers, and migrants, the groups most affected by the disease (Vasconcelos et al., 2001b).

To conclude, we suggest that other South American countries establish national policies for the use of yellow fever vaccine. But, it is important to emphasize, that the most important step is trying to control the *Aedes aegypti* levels. This is the best way to avoid re-urbanization of yellow fever and to prevent the lost of many human lives.

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Dengue transmission and *Aedes aegypti* control in Brazil

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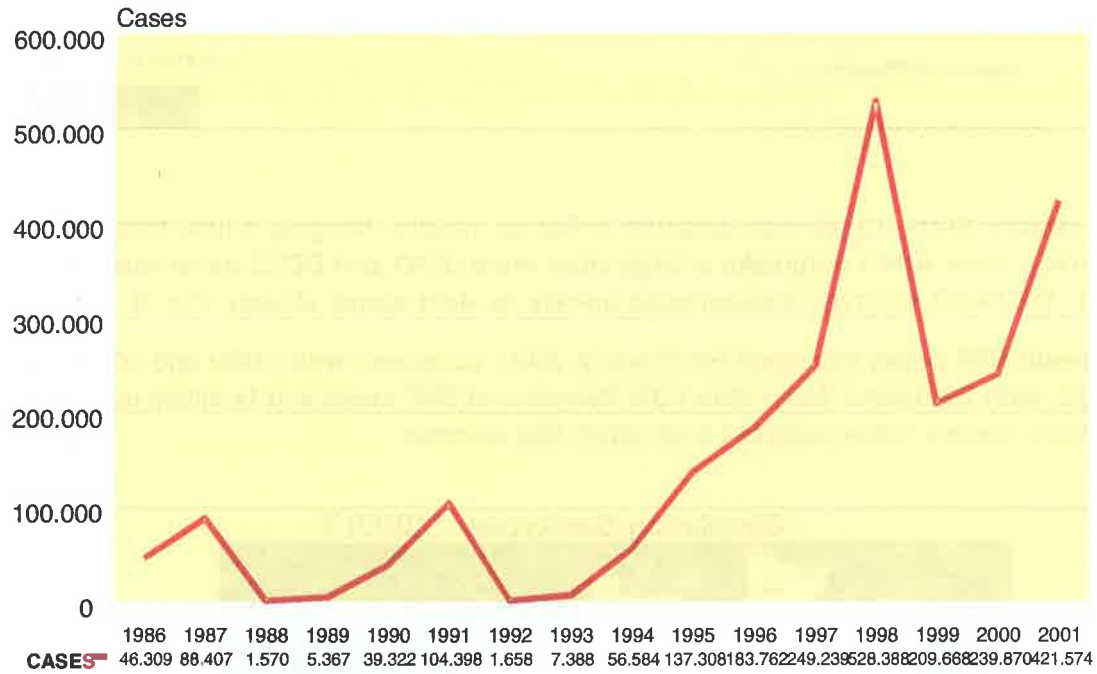
Last time *Aedes aegypti* was considered eradicated in Brasil was in 1972. Up to the 70s, surveillance activity was carried out, mainly in coastal and border areas. Such activity was not enough to prevent the reintroduction of the vector leading to first dengue outbreak in Roraima, most northern part of the country, by 1982. During the 80s and 90s, in spite of all efforts to control the vector by health authorities, the vector spread through all regions of Brasil, achieving 3650 of the existing 5650 counties. Dengue records developed a stepwise line, with a faster increase in dengue transmission after 1994, reaching 530,000 notified cases in 1998 (Fig 1).

Notified Dengue Cases - BRAZIL - 1986 a 2001 (*)

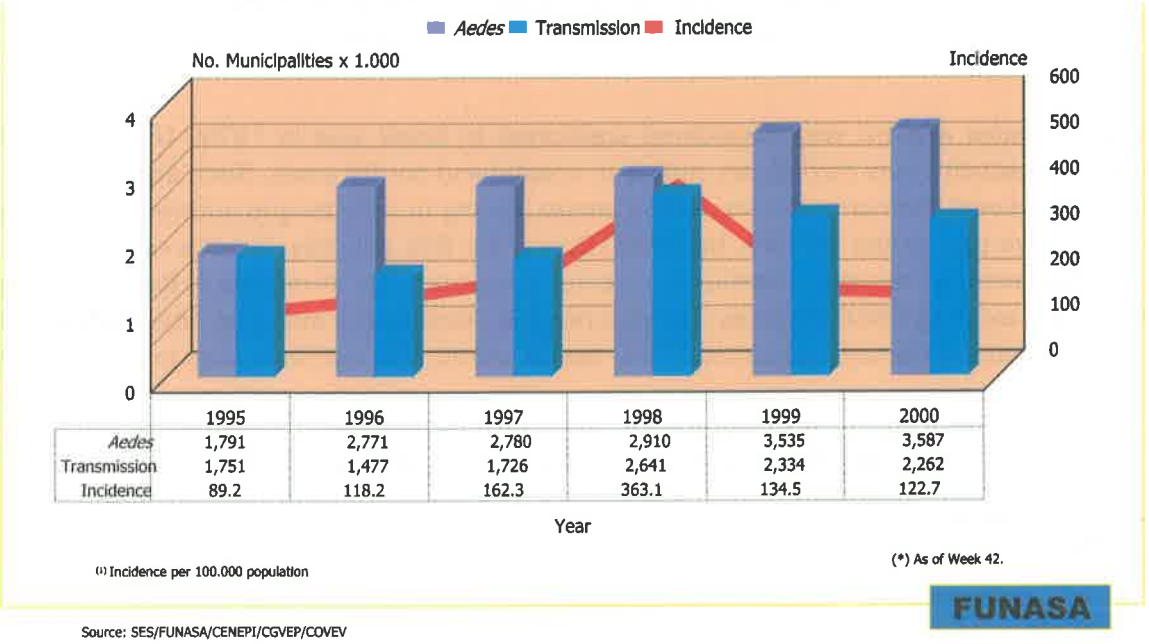


(*) Up to week 52.

The health authorities decided to implement a national program to eradicate *Aedes aegypti*, which was designed in 1996 and established in 1997. The program was planned to act in vector control, health sanitation, community participation, legislation and health education. From 1997 to 1999, 524 million US$ were invested, through contracts of Ministry of Health with states and municipalities, in which the money was transferred to buy equipment in health states secretaries and hire field personnel in municipalities. After four years, the program achieved 3900 from the 5507 counties nationwide (Fig 2).

In the year 2000, the vector was detected in 3587 counties, and 230,000 dengue cases from 2262 counties were detected, meaning that the vector eradication was unfeasible.Of 27 states, 25 have dengue transmission, with serotypes DEN1 and DEN2 circulating widely.

Yearly comparison of municipalities with dengue transmission, Dengue incidence and infestations of *Aedes aegypti*. BRAZIL - 1995-2000 (*)



	1995	1996	1997	1998	1999	2000
Aedes	1,791	2,771	2,780	2,910	3,535	3,587
Transmission	1,751	1,477	1,726	2,641	2,334	2,262
Incidence	89.2	118.2	162.3	363.1	134.5	122.7

(1) Incidence per 100.000 population

Source: SES/FUNASA/CENEPI/CGVEP/COVEV

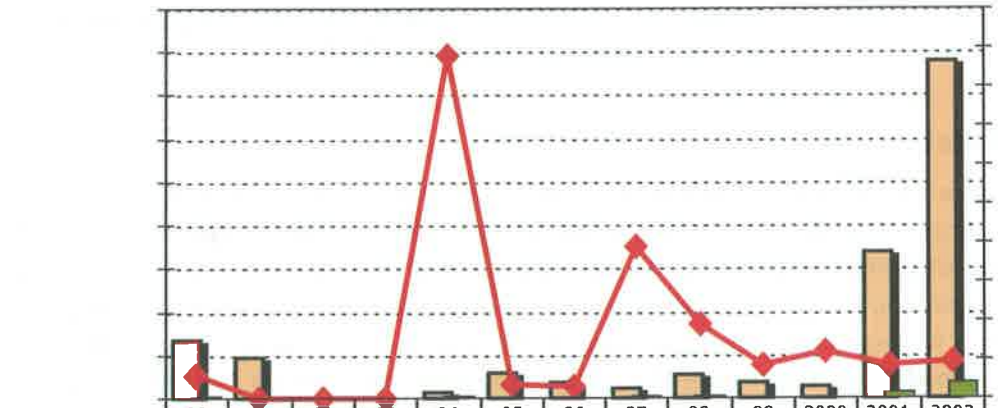
Since january 2001, DEN3 was detected in Rio de Janeiro, bringing a new threat of dengue hemorragic fever (DHF) outbreaks in large cities were DEN1 and DEN2 transmission occurred. In fact, the DEN3 serotype disseminated quickly to eight states already (Fig 3).

As a result DHF cases increased ten times in 2001 compared with 2000 and achieved 1559 in 2002, with 69 deaths. More than 50% dengue and DHF cases and fatalities occurred in Rio de Janeiro were a major outbreak took place this summer.

Circulating Serotypes - 2002(*)

Dengue Hemorragic Fever - Reported cases, deaths and mortality rates

Brasil, 1990 to 2002



	90	91	92	93	94	95	96	97	98	99	2000	2001	2002
Casos	274	188	0	0	25	114	69	46	105	72	51	675	1559
Óbitos	8	0	0	0	11	2	1	9	10	3	3	28	69
Letalidade	2,92	0,00	0,00	0,00	44,00	1,75	1,45	19,57	9,52	4,17	5,88	4,15	4,78

(*) Dados até a s.e 19.

FUNASA

Fonte: SES/FUNASA/CENEPI/CGVEP/COVEV

To fight the rise in dengue and dengue haemorragic epidemics, the Health Ministry launched national plan in September 2001, to strength epidemiological surveillance and vector control in 657 counties, which account for 72% of dengue cases. The new plan was designed to strength virus surveillance for early detection of outbreaks, and vector control interventions associated with community participation. Counties have received monthly financial transfers, based upon population, area size and epidemiological situation. The money is aimed to support expenses with field staff and routine supplies. National Health Foundation (FUNASA) provides technical guidelines, training and strategic supplies such as insecticides and diagnostic kits. Field operations are designed to provide house inspections every two months to all premises in infested cities. Risk areas are mapped to receive one visit each two weeks and insecticide residual spray once a month. Areas with dengue transmission must be submitted to space spray (ULV), coupled with community mobilization to collect and eliminate breeding sites. Resistance to the larvicide temephos is widespread in the country, where solid formulations of *Bacillus thuringiensis israelensis* have been used as alternative for focal treatment. *Aedes aegypti* populations resistant to piretroids also have been detected in Sao Paulo state and other areas, leading to substitution of piretroids by malathion and fenitrothion as adulticides. The full implementation of the dengue fighting programs and the success of dengue prevention is largely dependent of local governmental policies for inter-sectorial actions and community participation. These are the big challenges to fight dengue transmission and consequently DHF outbreaks in large cities.

West Nile virus: an exotic emerging pathogen in North America

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Introduction

In the summer of 1999, West Nile virus (WNV), an mosquito-borne flavivirus native to Africa, Asia and Europe, was discovered in the western hemisphere for the first time when it caused an epidemic of human encephalitis in the metropolitan area of New York City.[1,2] Concurrent with this human epidemic were the deaths of thousands of birds in northern New Jersey and southeastern Connecticut[3,4] and a number of horses on Long Island. In three short years the virus has spread at an unprecedented rate throughout the eastern half of the United States and has emerged as a major public health concern. In this symposium, I will review our current knowledge of WNV and discuss recent developments in the ecology and epidemiology of this exotic virus in the United States.

History and geographic distribution

West Nile virus is an Old World arbovirus with a wide distribution that includes all of Africa, southern Europe, southwestern and south central Asia, and Australia (Fig. 1). It was first isolated from the blood of a febrile woman in the West Nile district of Uganda in 1937 and was among the earliest arthropod-borne viruses to be discovered.[5] The virus became recognized as a cause of severe human illness (meningoencephalitis) in elderly patients during an outbreak in Israel in 1957. Equine cases were first noted in Egypt and France in the early 1960's. Recent outbreaks in humans involving hundreds of cases of severe neurological disease and fatal infections have occurred in Algeria (1994), Romania (1996-97), the Czech Republic (1997), the Democratic Republic of the Congo (1998), Russia (1999-2000), and Israel (2000-01). The occurrence of three major epidemics, in southern Romania, the Volga delta in southern Russia and the northeastern United States were the first epidemics reported in large urban populations and were totally unexpected. Epizootics of disease in horses have occurred in Morocco (1996), Italy (1998), and France (2000).[6,7]

FIG. 1. West Nile Virus distribution pre-1999.

Virology

West Nile virus is a member of the Japanese encephalitis antigenic complex of the genus *Flavivirus,* family Flaviviridae that includes a number of important human pathogens including Japanese encephalitis in Asia; St. Louis encephalitis in North and South America; and Kunjin (a subtype of WNV) and Murry Valley encephalitis viruses in Australia.[8] The WNV genome is positive-sense, single-stranded RNA (approximately 10,000 – 11,000 bases) that encodes 3 structural proteins (capsid, premembrane, and envelope) and 7 nonstructural proteins.[9,10] The mature virion (Fig. 2) (approximately 40 nm in diameter) is a nucleocapsid enveloped in a lipid bilayer with projecting envelope proteins that mediate cellular attachment and membrane fusion and appear to be important virulence factors.[9,10] Sequence analysis of WNV suggests two evolutionary lineages.[2] Lineage I includes isolates from Europe, the Middle East, Africa, India, Australia (Kunjin) and the recent isolates from North America. Lineage II includes isolates from central and south Africa. Epidemics are most commonly associated with lineage I, but lineage II viruses have caused sporadic human cases in South Africa.

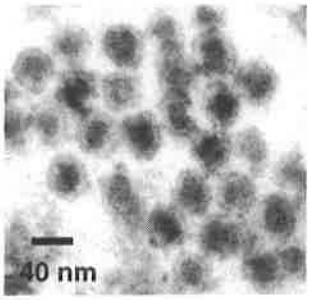

FIG. 2. West Nile virus

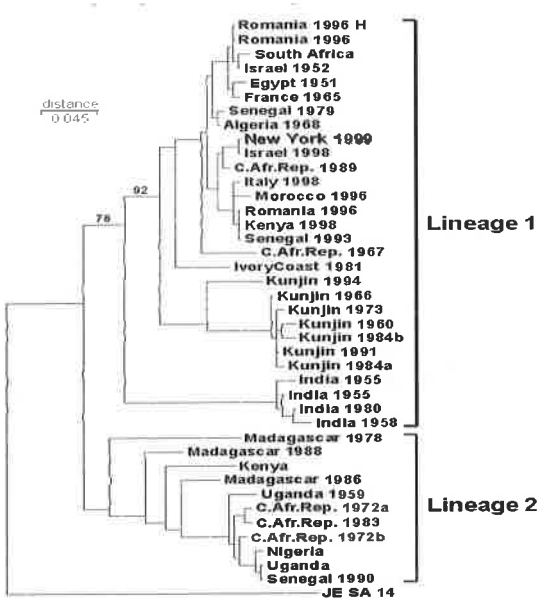

FIG. 3. Molecular phylogeny of West Nile virus[2]

Human Infection

The severity of human disease varies considerably and is partially age-dependent, being mild in the young and increasing in severity with age, with encephalitis generally confined to the aged. The viral incubation period ranges from 3-15 days. Most infections are clinically inapparent, but febrile illness, ranging from nonspecific viral syndrome to fatal encephalitis, are not uncommon. The ratio of symptomatic to asymptomatic infection varies between 1:140 and 1:300. Mild illness includes 3-6 days of fever, headache, backache, myalgia, and muscle weakness. A roseolar or maculopapular rash may be present, but this occurs more frequently in children. Severe neurological syndromes leading to coma and death occur principally in the elderly, but have also been reported in children.[7,11] In recent outbreaks, the case fatality rate was 4% in Romania, 6% in Russia, and 11% in New York.[7] Recovery is usually complete and rapid, but the potential for clinical relapse occurring weeks to months after recovery remains a question. Therapy for WNV encephalitis is supportive – respiratory support, management of cerebral swelling, and prevention of secondary bacterial infection. Currently there is no known effective antiviral therapy or vaccine. Ribavirin has been shown to inhibit the virus in nueral cell culture[12] and has been administered to a small number of patients, but its effectiveness is still unknown.

Transmission Cycle

The enzootic WNV transmission cycle is predominately maintained by virus transmission between ornithophilic mosquitoes of the genus *Culex* and wild birds.[5,6,13] The virus is amplified during periods of adult mosquito blood-feeding (summer and early autumn) by continuous transmission between mosquito vectors and avian reservoir hosts. Infectious mosquitoes carry virus particles in the salivary glands and infect susceptible bird species during blood feeding. Competent bird reservoirs will sustain an infectious viremia for 1 to 4 days, after which they either die or develop life-long immunity. A sufficient number of vectors must feed on an infectious host to ensure that some survive the extrinsic incubation period (approximately 2 weeks, depending on temperature) to feed again on a susceptible host. Humans, horses and most other mammals generally do not develop sufficient viremias in the blood to affect transmission and are thus considered "dead-end" of "incidental hosts". Direct bird to bird transmission of WNV has been experimentally demonstrated in crows sharing common cages, but it is not known if this occurs in nature. West Nile virus has also been detected in bats, chipmunks, raccoons, squirrels, and domestic rabbits but their role in the transmission cycle is uncertain. Serological evidence of WNV infection in domestic dogs and cats has also been demonstrated.

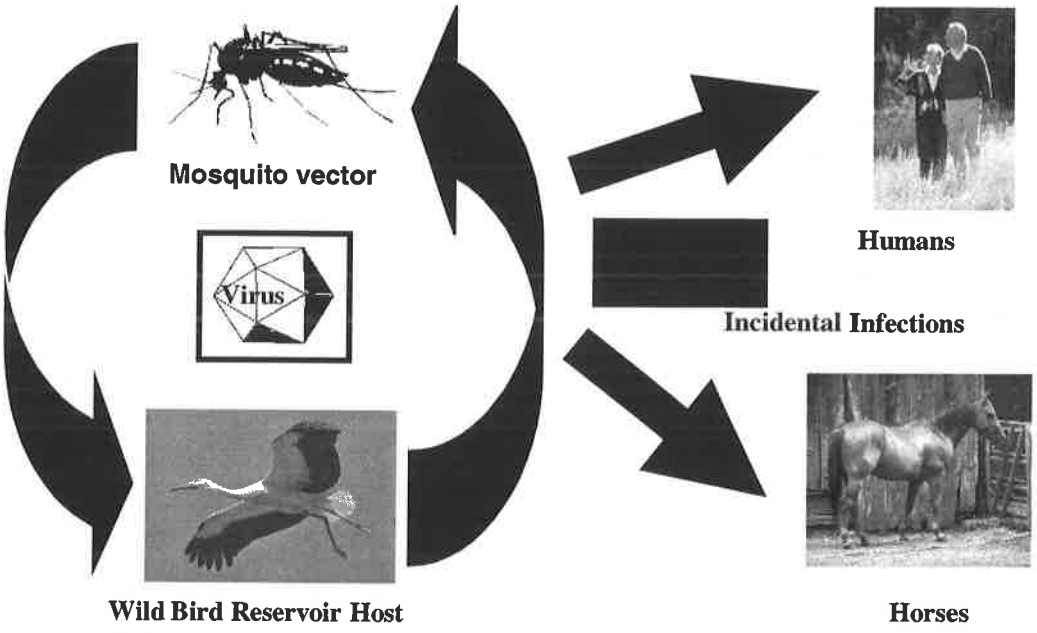

FIG. 4. West Nile virus transmission cycle

Ecology and Epidemiology

Bird reservoirs

Wild birds are the principal reservoir hosts worldwide and the virus has been isolated from a number of wetland and terrestrial species.[5,6] High bird morbidity and mortality observed in the United States is unique to the western hemisphere where at least 82 species have been found infected. American crows, *Corvus brachyrhynchos* have been the most commonly infected bird, suggesting that crows are most susceptible species to the virus. The cause of this increased susceptibility is unknown but since 1999, dead crows have proven to be the most sensitive indicator of local virus activity. Blue jays, *Cyanocitta cristata* a closely related corvid, are also highly susceptible. There is a strong association between high dead crow density and subsequent human infection[14] and dead crow surveillance is widely used in the United States

to monitor WNV activity. The house sparrows, *Passer domesticus,* cardinals, *Cardinalis cardinalis* and rock doves, *Columba livia* have been implicated as important reservoir hosts for amplification of the virus in urban areas[15,16]. WNV-infected migrant birds have also been hypothesized as a potential mechanism for the spread of WNV.[17]

Mosquito vectors

Mosquitoes, largely bird-feeding species, are the principal vectors of WNV. The virus has been isolated from 43 mosquito species, predominately of the genus *Culex* in the Old World[6] and from 26 species in the United States and Canada[4,18-20] (Table 1).

TABLE 1. Mosquito species from which WNV has been isolated in the United States[a]

Culex nigripalpus	*Ochlerotatus atlanticus*	*Aedes albopictus*
Cx. pipiens	*Oc. atropalpus*	*Ae. cinereus*
Cx. quinquefasciatus	*Oc. canadensis*	**Ae. vexans**
Cx. restuans	*Oc. cantator*	
Cx. salinarius	**Oc. japonicus**	Anopheles barberi
	Oc. sollicitans	*An. punctipennis*
Culiseta melanura	***Oc. taeniorhynchus***	*An. quadrimaculatus*
	Oc. triseriatus	
Coquillettidia perturbans	*Oc. trivittatus*	*Psorophora columbiae*
		Ps. ferox
Orthopodomyia signifera	*Uranotaenia sapphirina*	

[a] Species in **bold** have been shown to be vector competent in laboratory bioassays.[21-23]

Based on the number of isolations made from *Culex pipiens* and *Culex restuans,* these two species appear to be the most important vectors for enzootic maintenance and amplification of the virus among wild birds in the northeastern United States.[18-20] Both species are strongly ornithophilic, are widely distributed throughout the region, and occur in both urban and rural environs. Recently completed studies have further demonstrated that both are competent vectors for WNV in the laboratory. *Culex restuans*, which is most abundant in June and July, appears to be important in initiation of WNV transmission among birds in early summer while *Cx.* pipiens, which is more prevalent in August and September, appears to play a greater role in amplification of the virus later in the season. Isolations from *Culiseta melanura* collected in more rural environs further suggest this almost exclusive avian feeder may be important in circulation of the virus among birds in sylvan environments. *Culex salinarius* is a strong suspect bridge vector of WNV to humans and horses. This species is a well-recognized general feeder that feeds indiscriminately on both birds and mammals and will readily bite humans. It has been demonstrated to be a competent vector in the laboratory. In both 2000 and 2001 an increased number of isolations from this species were correspondently found in locales where both human and horse cases were reported. Several species of *Aedes* and *Ochlerotatus* which readily feed on both birds and mammals must also receive strong consideration as potential "bridge vectors" to humans and horses.

Transovarial transmission of WNV has been experimentally demonstrated in *Cx. pipiens, Culex tritaeniorhynchus, Aedes aegypti* and *Aedes albopictus* indicating a potential for vertical transmission of the virus in nature [24]. The virus was also isolated from hibernating *Cx. pipiens*

near the "epicenter" in New York City following the 1999 outbreak [25]. Survival in over wintering adult mosquitoes has been hypothesized as a possible mechanism for local virus persistence.

West Nile virus isolations from bird-feeding argasid (soft-bodied) and argasid (hard-bodied) ticks have occasionally been reported from Africa and Europe but they do not appear play a major role in epizootic transmission.[6]

West Nile virus in the United States

In three short years the WNV has spread at an unprecedented rate throughout the eastern half of the United States and southern Ontario, Canada extending from Maine to Florida and west to Iowa and Louisiana (Fig. 5). One hundred and forty nine human cases with 18 fatalities have been reported in 10 states through December 2001. Over this same time, over 800 hundred horses have been afflicted, a third of which have died or been euthanized. Tens of thousands of wild birds, representing 82 species, have been killed, and the virus has been found in 6 species of mammals and 26 species of mosquitoes. In 2001, there were two major transmission foci in the United States, one in the northeast, and the other in the southeast. The human cases were sporadic despite the intense epizootic in wild birds and horses.

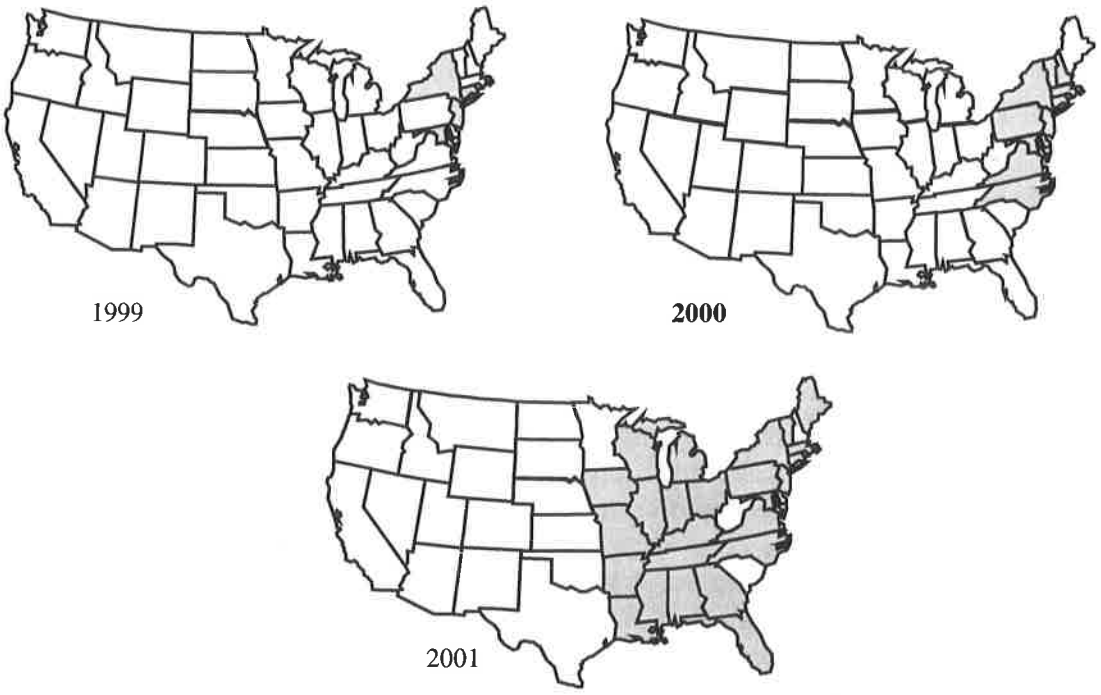

FIG. 5. Distribution of West Nile virus in the United States 1999-2001

Intensive regional studies conducted by in Connecticut, USA over the last three years have provided some important information concerning the epidemiology/epizootiology of the WNV and the risk of human infection.[3,18,26] The majority of the WNV isolations from mosquitoes have mostly been made from females collected in densely populated residential locales where the highest rates of dead crow sightings have been observed and where WNV activity was detected in the previous year. Furthermore, the detection of WNV activity in mosquitoes (i.e. number of isolations and species) has been consistent in time and space with the incidence of human cases (Fig. 6). In 2000 and 2001, WNV-infected mosquitoes were detected five weeks prior to the onset of symptoms of the first human case, reinforcing the sensitivity and efficacy

of a mosquito surveillance program. Overall, the detection of WNV-infected mosquito pools has proven to be a sensitive indicator of epizootic activity associated with subsequent human disease.

From our investigations we conclude the following

1. West Nile virus appears to over winter locally, probably in hibernating *Culex* mosquitoes, and reemerge in the same area.
2. *Culex* mosquitoes are the most important vectors for maintenance and amplification of the virus in wild birds.
3. Long range dispersal of the virus is likely facilitated by wild bird migration.
4. Crow mortality is a sensitive indicator of virus activity but does not indicate immediate risk for human illness.
5. The detection of WNV-infected mosquitoes is a sensitive indicator of epizootic activity associated with subsequent human disease.
6. Horse cases occur independently are not a sensitive sentinel for prediction of human infection.
7. High local dead bird density and multiple isolations of the virus from mosquitoes are high predictors for human risk of infection.
8. Highest virus activity and greatest risk for human infection occurs I mid-August through the end of September.

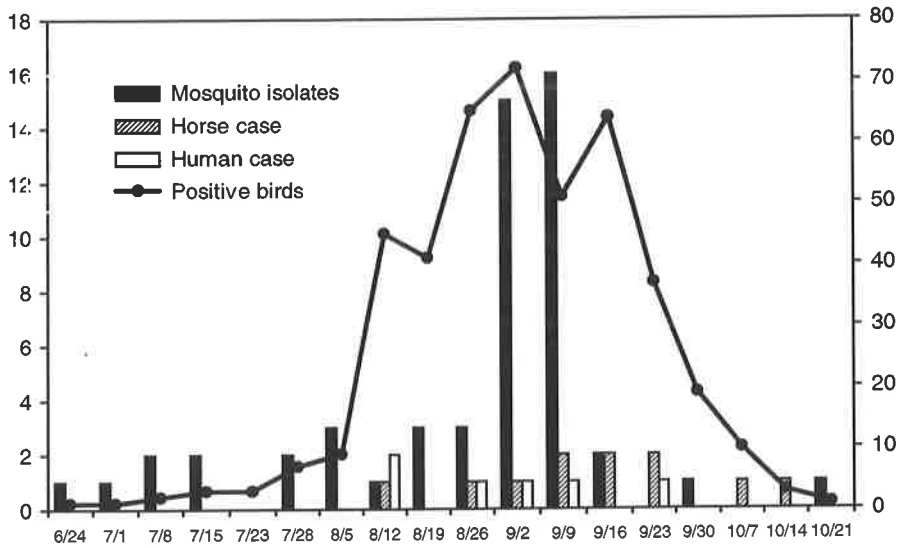

FIG. 5. West Nile virus activity in Connecticut, USA in 2001

Summary

West Nile virus is now endemic in the United States and is expected to be a major public health problem in the next decade. Given its rapid expansion during the last three years, it is likely to spread across the country and reach into Central and South America in the coming years. The involvement of new species of vector mosquitoes and reservoir hosts will alter its ecology and epizootiology. West Nile virus will continue to kill large numbers and varieties of

birds and its long-term impact is unknown. West Nile virus will also become a significant disease factor for the horse industry. A vaccine (formalin inactivated virus) for horses is now available and has been granted a conditional license by APHIS. Its effectiveness remains to be evaluated. A human vaccine is currently under development.[27]

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Symposium (Fungi 2) Microecology of Entomopathogenic Fungi

Ecology of entomopathogenic fungi in field soils

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What are soils?

Soils vary markedly in response to geology, climate, altitude, topography and salinity and can be derived from various parent rock (eg. limestone, granite, basalt), or can be the complex alluvial soils associated with river flats or flood plains, or can be organic peats (Davies, 1988). Most soil types can be differentiated into several horizons (eg. A, B, C, R) from the litter layer (A_{00} horizon) through the remaining A and B horizons to the bed rock (R). They can be further classified by the size of their component mineral particles and by their texture into sandy clay loam, slit loam, medium clay, etc (Yanagita, 1990). The permeability of water, soil aeration, adaptability for plant growth and other characteristics of soil can be deduced from the soil texture classification.

Soil structure is composed of three phases – solid, liquid and gas. Usual soils contain in volume 45% inorganic and 5% organic soil phase, 25% water phase and 25% air phase. Plant roots and microorganisms live in pores in soil containing water and air phases (Yanagita, 1990). Clay minerals in soils strongly influence the physio-chemical properties of soil, the adhesion of individual microorganisms (including entomopathogenic fungi, eg., Ignoffo *et al.*, 1977) and the adsorption of nutrients and metabolic products, effecting the growth of microorganisms (Filip, 1988).

Abiotic interactions in soils

The soil environment can be seen as an extremely complex ecosystem enveloping and interfering with the primary interactions between the subterranean insect host and the fungal pathogen, or it can be seen as a simple and very stable ecosystem cocooning the host-pathogen interaction. There is no doubt that the latter view is true. Soils are generally very old, in any one location they do not change much within a year, or from year to year. The top 25-100 mm of uncultivated soil is buffered from the extreme daily fluctuations of temperature (Rath *et al.*, 1995) and is unaffected by the detrimental effects of UV irradiation (eg. Braga *et al.*, 2001; Moore *et al.*, 1993) and solarisation (Vänninen *et al.*, 2000). Soil moisture levels can fluctuate widely, but humidity is regarded as critical to fungal infection of insects (eg. Hallsworth & Magan, 1999) and soil humidity both in soil air spaces as well as around burrowing or tunnelling insects may be less limiting (Milner *et al.*, 1997).

There are many abiotic factors that affect fungal germination, growth and host-pathogen interaction in the soil, including soil texture, temperature, moisture, pH, conductivity, organic matter (Beyer *et al.*, 1997b; Hoitink & Boehm, 1999), nutrients (Ritz, 1995; Rosenzweig & Stotzky, 1980), fertilizers (Rosin *et al.*, 1996), agro-chemicals (Gardner & Storey, 1985; Pung

et al., 1993) and cultivation practices (Gaugler *et al.*, 1989; Vänninen 1996). Soil temperature affects the persistence of conidia and the mortality of subterranean insects exposed to the fungus. High temperatures are detrimental to conidial persistence (Lingg & Donaldson, 1981) and low temperatures (even freezing temperatures) enhance persistence (Vänninen *et al.*, 2000), however Rath *et al.* (1995) found that the LT_{50} of *Metarhizium anisopliae* to *Adoryphorus couloni* (Col.: Scarabaeidae) was greatly increased as soil temperatures declined from 15° to 5°C. Soil temperature can also render a pathogen non-pathogenic. Rath (2000) showed that an isolate of *M. anisopliae* with a germination temperature range of 2 – 33°C was highly virulent to subterranean termites at 25°C, however, nest temperatures often exceeded 35°C, rendering the fungus ineffective in controlling termite nests. This situation is not likely to be common in agricultural or forestry soils as it is probably restricted to social insects such as ants and termites. Soil moisture alone or in combination with soil temperature and soil type can affect the persistence of conidia (Beyer *et al.*, 1997; Li & Holdom, 1993), host mortality and the sporulation of entomogenous fungi from insect cadavers (Krueger *et al.*, 1991). Soil moisture is critical in the application of mycelial formulations of entomogenous fungi where conditions need to be optimised to encourage sporulation (Hartwig & Oehmig, 1992; Pereira *et al.*, 1993). Soil pH can affect the fungistatic nature of soils and Groden & Lockwood (1991) showed that fungistasis increased exponentially as soil pH increased from 5 to 7, however fungistasis may also be an important mechanism for conidial survival, inhibiting spore germination until there are hosts present (Clerk, 1969). Vänninen *et al.* (2000) and Lingg & Donaldson (1981) found little effect of pH on the persistence of *M. anisopliae* or *Beauveria bassiana*, and Rath *et al.*, (1992) found no differences in the rate of isolation of *M. anisopliae* from Tasmanian soils of differing pH's.

The vertical distribution of entomopathogenic fungi is important to the control of subterranean insect pests. If conidia accumulate at or near the soil surface then insects living deeper in the soil will not be affected, too much vertical movement and conidia may be removed from the required 'infective zone' or may contaminate ground water. Soil texture effects the vertical movement of conidia in laboratory studies using shifted field soils (eg. Ignoffo *et al.*, 1977; Storey & Gardner, 1987) but in the field there is considerably less vertical movement of conidia and no relationship to the sand, clay or slit content (Storey & Gardner, 1998). Vänninen *et al.* (2000) found that after 3 years exposure to rainfall and melting snow there was little difference in the vertical movement of *M. anisopliae* or *B. bassiana* isolates in relation to soil type with most of the fungi being retained in the layer of application. They did find that there was more movement in peat, but it was unclear as to whether this was caused by water percolation, mite activity or saprophytic growth. Vänninen (1996) has also shown that soil type did not affect the occurrence of *M. anisopliae*, *B. bassiana*, *Paecilomyces farinosus* or *P. fumosoroseus*.

Natural distribution and abundance of entomogenous fungi

Insect fungal pathogens such as *Metarhizium* spp. are widely distributed in the world's soils but different varieties, or clads, or pathotypes, etc may have a much more restricted distribution (Driver *et al.*, 2000). What factors are involved in the distribution and abundance of natural populations in the soil? Rath *et al.* (1992) took 419 soil samples from all major pastoral localities in Tasmania (Australia) and recovered 132 isolates of *Metarhizium* which they classified into 14 strains (Yip *et al.* 1992; reclassification has been undertaken by Driver *et al.*, 2000). They found that the distribution of the strains varied with soil-type and average annual rainfall, but not with soil pH, conductivity, temperature or altitude. They also showed that the density at which the isolates were recovered was not correlated with any of the environmental variables examined. Vänninen (1996) studied the distribution and abundance of *M. anisopliae*, *B. bassiana*,

Paecilomyces farinosus and *P. fumosoroseus* in Finland by taking 519 soil samples over a five year period. She showed that there were differences between the fungal species and that the distribution of *Metarhizium* was mostly strongly correlated to geographical location; the *Metarhizium* being mostly found in southern Finland. She concluded that both the mesophilic nature of the genus as a whole combined with the general reduction in insect species from south to north was the reason for this correlation. This is perhaps supported by both Roddam & Rath (1997) who only found 6 isolates out of 163 soil samples taken from subantarctic Macquarie Island where there are only 31 recorded species of insect and Milner (1992), who found 66 isolates out of 125 soil samples taken from warm, wet climatic zones of Australia. However, the relative isolation of Macquarie Island must also be a major contributing factor to the rarity of entomogenous pathogens there.

The importance of abiotic soil factors on the distribution and abundance of entomogenous fungi, which provides perhaps a 'sneak preview' of the possible persistence of strains introduced for biocontrol, is highlighted by Driver *et al.* (2000) in their taxomonic revision of *Metarhizium*. They conclude that the distribution of some clades (particularly of cold-active *Metarhizium* strains) is probably determined by climatic factors rather than host specialisation. Bidochka *et al.* (2001) also conclude that *M. anisopliae* may be an "assembly of cryptic species, each adapted to certain environmental conditions" and that "habitat preferences should be considered as a feature for selecting fungal strains used for insect biocontrol efforts". However, while Bidochka *et al.* (2001) bioassayed their isolates against four insect species, there is no reason to suggest that these insect species were the 'natural' host of any of the isolates. *M. anisopliae* is regarded as a common pathogen of insects with a wide host range, however, specific strains can be very host specific, only affecting one or a few closely related hosts, while other strains are not pathogenic to some insect species (Cook *et al.*, 1996). This may be particularly true for subterranean scarab species (Rath *et al.*, 1995). The influence of the host is discussed later.

Biotic factors

Soil fungistasis, microbial competition, mycophagous invertebrates and the plant community all affect the persistence and efficacy of entomogenous fungi in the soil. Soil fungistasis appears most likely to be caused by microbial inhibition. Lingg & Donaldson (1981) were able to correlate the isolation of *Penicillium urticae* (which produces a water-soluble inhibitor of *B. bassiana*) with a fungistatic effect. Fungistasis could be reduced by sterilizing the soils (Beyer *et al.*, 1997a; Clerk, 1969) and this is probably due to removal of either competitive or inhibitory microbes. Collembola can alter fungal populations and affect germination through grazing activities (Lartey *et al.*, 1994). Earthworms can disperse fungal inoculum to the surrounding soil (Doube *et al.*, 1994), as can infected hosts and other insects (Rath *et al.*, 1995; Shimazu *et al.* 2002; Zimmerman, 1992).

Influence of the host

While biotic and abiotic factors all play a part in the persistence and efficacy of entomogenous fungi in the soil ecosystem, it is probably the host species that plays the greatest role in relation to survival of the fungal pathogen at a given location. Rath *et al.* (1995) have shown that following application of 5.1×10^4 *M. anisopliae* spores/g of soil (2 cm below the soil surface using direct-drill seed applicators) the recovery of the fungus from the top 10 cm declined over a six month period, but when the target species (*A. couloni*) starting dying from fungal infection, the fungal load in the soil increased 10 fold and then stayed relatively constant for the next 18 months. A further assessment 7 years after inoculation showed that the

fungus was still present in high numbers and that the fungus was found in the untreated plots at similar levels. It is believed that the adults were responsible for the movement of the fungus from the treated pasture plots to the untreated plots (Rath, unpublished). Callot *et al.* (1996) also describe how adults of the cockchafer *Hoplochelus marginalis* (Col.: Scarabaeidae) can facilitate dissemination of *Beauveria brongniartti* 1-2 km on the island of Reunion. They also show how the fungus can grow from the infected cadaver through the soil and can extend 30-40 mm from mummified insects. The fungus can produce from 5×10^5 to 5×10^7 spores per 'pellet'.

The effect of the soil environment on the host insect is just as important as the effects on the fungi. Insects do not survive, or survive very poorly, in soils exposed to constant high or low temperatures, waterlogged soils, very dry soils, etc or in the absence of the host plants or other food source. Reduced efficacy in field soils may have more to do with the life strategy of the hosts than on that of the fungus. In the hot dry soils typical of Tasmanian pasture soils in late summer and early autumn, there can be virtually no mortality, even though the larvae of the scarab *A. couloni* are in the 'fungus zone' and the temperatures are conducive to mortality. Under these harsh conditions the larvae are not eating and hence not moving through the soil – they remain semi-dormant in 'cells' in the soil which probably protects them from desiccation. As the larvae are not moving through the soil they cannot encounter fungal spores and no control is possible (Rath, unpublished data).

Conclusions

Biotic and abiotic factors all impact on the host-pathogen relationship and can affect the progress of biological control. Soil factors affect fungus and host persistence and the interaction between the two. Successful control of soil insects will utilise fungal strains that are well suited to the environmental niche of the host, but factors such as the distribution and abundance of the host, the delivery of the fungus to the ecosystem and the virulence of the fungal isolate are more critical factors.

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Phyllosphere ecology of terrestrial entomopathogenic fungi

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Introduction

Terrestrial plant leaf surfaces (phylloplanes) and microhabitat zones surrounding plant leaves (phyllospheres) comprise the largest biosphere-atmosphere interface on earth, a boundary zone with an area greater than all continents combined (Riederer and Schreiber, 1995). It is a habitat so exceedingly variable as to nearly defy characterization and one usually described as harsh or extreme by microbial ecologists. This view is generally shared by many insect pathologists and microbial control specialists. This paper will briefly describe the major biotic and abiotic attributes of the phyllosphere and outline some of the means by which entomopathogenic fungi (EPF) exploit and tolerate this microhabitat.

The phyllosphere environment

Wind. Viscosity of an air stream moving parallel to a surface increases as the distance from the surface decreases. This phenomenon generates a boundary layer of slow-moving air around each leaf of all terrestrial plants. While the term boundary layer suggests a zone of essentially stagnant air, this is not the reality. Leaf boundary layers are dynamic, being readily and frequently disrupted and swept away for brief intervals by atmospheric turbulence generated during windy conditions (Aylor, 1990). Such events have obviously important effects on the phyllosphere environment and its microbial inhabitants. Winds also constantly bombard leaves with soil and debris (and immigrant microbes) and may inflict physical damage via abrasion or flexing. Plant epicuticular waxes are a major component of blue haze associated with dense vegetation, and it is hypothesized that much wax enters the atmosphere in the form of wax crystals. Wind-generated turbulence likely contributes to exfoliation of wax crystals, and this process may impede microbial colonization of the phylloplane (Juniper, 1991; Beattie, 2002).

Moisture. The phyllosphere experiences extreme fluctuations in moisture conditions. There may be a surfeit of free water during rain or dew fall, and wind-driven rain subjects the phylloplane to severe wetting and shearing forces. These periods of extreme wetness are normally punctuated by periods during which the leaf surface is essentially dry (with little or no free moisture). Mean levels of relative humidity within the boundary layer are generally substantially higher than ambient; however, humidity levels also fluctuate widely under the influence of many interacting factors, including weather conditions, leaf architecture, hydrophobicity, location on the plant or in a plant canopy, moisture balance, and herbivore and epiphyte activity. Ventral surfaces usually generate a thicker zone of high humidity than dorsal surfaces due to greater densities of stomata and trichomes and entrapment of humid air by surface concavities. Willmer (1986) described the humid boundary layer of cabbage as extending at least 10 mm above and up to 25 mm below the leaves.

Solar Radiation. Solar radiation, including ultraviolet radiation (UVR), is potentially lethal to all phyllosphere microbes. Many epiphytes produce melanin and other pigments, thickened cells, or cellular aggregations to resist radiation. UVR is rapidly lethal to dispersed, unpigmented or lightly pigmented microbes, including most entomopathogenic fungi. Solar radiation usually impinges with greatest intensity upon dorsal surfaces of exposed leaves. Exposure to radiation

is greatly reduced on shaded interior- or lower-canopy leaves, and the body of even a single leaf is generally an effective attenuator of radiation, including UVR (Sundin, 2002). However, some UVR does reach the ventral phyllosphere and other shaded surfaces via reflection, via penetration (of thin leaves) or during brief intervals when the leaf is inverted by wind. Leaf topography, especially features such as trichomes, also affects radiation levels.

Temperature. Plants possess a considerable capacity for thermo-regulation. Solar radiation warms exposed surfaces, and during cool weather, leaves may orient to maximize irradiance and maintain leaf temperatures above ambient. As temperatures rise above the approximate maximum optimal temperature for photosynthesis and other physiological processes (H" 30°C), cooling mechanisms are activated (especially opening of stomata to increase transpiration), and with abundant water, leaf temperatures can be maintained as much as 10°C cooler than the surrounding air (Willmer, 1986; Salisbury and Ross, 1992). Temperatures are generally lower on ventral phylloplanes, which are usually shaded and have more stomata than dorsal phylloplanes. Cooling the leaf surface by even a few degrees can affect abvailability of free water by inducing condensation. Although plants will attempt to maintain leaf temperatures below ambient during hot weather, this is not always possible. During intervals of drought or extreme heat, stomata must be closed to conserve water. Under such conditions, leaf temperatures may exceed ambient by at least 4–5°C (Taiz and Zeiger, 1998).

Nutrients. The leaf epicuticle consists of waxes that are highly resistant to microbial degradation and thus do not constitute an important nutrient resource. Nutrients are deposited on surfaces of intact leaves via diffusion across the epicuticle. This process is normally slow and limited by the thickness of the wax layer. Under certain environmental conditions, greater amounts of of nutrients may be deposited via guttation fluids (Froussard, 1981). Sugars and proteins are also secreted from glands (e.g., from glandular trichomes). Nevertheless, nutrient levels on the phylloplane are normally quite low (Beattie, 2002) and this is considered a limiting factor in microbial colonization.

Allelochemicals. A broad range of chemicals that exhibit antifungal and other antimicrobial properties have been isolated from leaf surfaces. These include many terpenoid, alkaloid and phenolic compounds. Most of these materials diffuse onto the phylloplane from internal leaf tissues or are deposited by glands; however, some are associated with the epicuticular waxes. Many of these chemicals are volatile and diffuse into the phyllosphere.

Microbial epiphytes. As is the case with rhizospheres, the phyllospheres of most plants support diverse communities of microbes, dominated by bacteria, yeasts and filamentous fungi. Epiphyte populations are highly aggregated, with greatest densities on the ventral phylloplane and in other niches shaded from solar radiation or with high nutrient concentrations. Recent studies suggest that significant percentages of bacterial epiphytes are aggregated in biofilms (Morris et al., 2002). Many microbial epiphytes, especially in aggregations, produce compounds such as biosurfactants, plant growth hormones, or toxins in quantities sufficient to alter leaf structure and physiology in ways that increase nutrient concentrations on the leaf surface. The products of synergistic metabolism in biofilms can have dramatic effects on phyllosphere ecology. It is hypothesized that the leaf may play an important role in microbial genome evolution. Mutation rates are unusually high on phylloplanes due to UVR, and persistence of gene mutations may be enhanced in UV-resistant epiphytes possessing efficient DNA repair mechanisms (Sundin, 2002). Phylloplanes supporting microbial aggregations may also represent important sites for genetic exchange (e.g., Christensen et al., 1998).

Leaf-inhabiting invertebrates. The phyllosphere is inhabited by an enormous variety of arthropods and other small invertebrates. The mechanical damage inflicted by the feeding of phytophagous

species has obviously profound impacts on phyllosphere ecology. Impact may derive secondarily from defoliation that thins the plant canopy. Even minor wounds inflicted by arthropod feeding or oviposition may exude large quantities of nutrients onto the phylloplane, and many common phytophagous insects contaminate the foliage with nutrient-rich honeydews. These materials may stimulate profuse microbial growth. Toxins injected into leaves by many insects also affect leaf physiology in ways that alter the phyllosphere environment.

Agrochemical antagonists. The foliage of many crop plants is targeted by numerous and frequent applications of fungicides and other chemicals to control plant pests and diseases. These agents obviously have great potential to negatively impact EPF and other phyllosphere microbes. Discussion of the affects of these agents, however, is beyond the scope of this brief review.

Phylloshere exploitation by entomopathogenic fungi

Host inoculation. Terrestrial EPF generally initiate new infections via conidia that are widely dispersed by air. Thus, to infect leaf-inhabiting insects, EPF need not be established members of the epiphyte community; conidia need only be transported onto the leaf. The phylloplane is a much larger target than an individual insect, and fungal pathogens of phytophagous insects have evolved mechanisms to ensure deposition and at least short-term persistence on the phylloplane and subsequent inoculation of the host (thus exploiting the leaf as an infection court). Entomophthorales produce large, sticky conidia that adhere to leaves and are capable of several cycles of germination/sporulation to produce secondary, tertiary, and even additional stages of infectious conidia (see Hajek, 1997). These fungi may contact new hosts by actively ejecting conidia (ballistospores) from the substrate or by producing specialized structures to elevate conidia (capilliconidia) above the substrate for passive acquisition by ambulatory hosts. Most entomophthoralean conidia are highly susceptible to desiccation; however, the conidiogenesis and conidia germination processes of these fungi are extremely rapid, producing a high probability of host inoculation during a single night of moisture-saturated conditions. Studies also indicate that conidia of some species and the more resistant capilliconidia may survive many days in the shaded, humid environment of the ventral phyllosphere (Brobyn et al., 1985; Uziel and Kenneth, 1991). Hyphomycetes, though they lack the capacity for rapid sporulation and do not produce ballistospores or capilliconidia, are nevertheless also able to exploit the phylloplane for host infection. Many of these fungi produce great numbers of small, hydrophobic, desiccation-tolerant conidia that are readily dislodged from their conidiophores and carried long distances by wind. If deposited on a leaf, these conidia adhere to the waxy epicuticle via physiochemical forces that are not fully understood. Aylor (2002) suggests that small, hydrophobic conidia adhered to leaves are not readily removed by wind, and persistent conidia are known to survive many days in phylloplane niches protected from UVR (Wraight, unpublished). The forces adhering conidia to the leaf are not so great, however, as to preclude removal by rain (Inglis et al., 1995) or passive acquisition by hosts moving or feeding in the phyllosphere (especially thigmotactic insects that maintain close contact with the substrate). Conidia of some hyphomycete entomopathogens are capable of exploiting large deposits of nutrients (e.g., honeydew) and the low UVR-high moisture conditions of the phyllosphere to germinate and produce hyphal elements that in turn produce either blastospores or aerial conidiophores and conidia. Insects likely acquire conidia more readily from conidiophores than from leaf surfaces (see sporulation section). Growth of EPF under these conditions is usually limited, however, by competition from aggressive epiphytes.

Spore germination and host infection. The EPF universally require high levels of moisture (generally near 100% RH) to complete these processes. Such conditions only occasionally

prevail in the phyllosphere during the day; however, favorable conditions commonly develop at night when winds decrease (stabilizing the leaf boundary layer) and temperatures drop (often to dew point). As previously related, the Entomophthorales possess a remarkable capacity for rapid development and are thus supremely adapted to exploit these ephemeral conditions. The processes of conidiogenesis, germination, and host penetration can all be completed within hours (e.g., Wraight et al. 1998), and wet conditions need prevail only during the night to support development of entomophthoralean epizootics (even when the intervening days are characterized by high rates of desiccation and insolation) (Galaini-Wraight et al., 1991; Steinkraus et al., 1991). In contrast, hyphomycete species are normally unable to develop sufficiently rapidly to complete the infection process in a single night. Recent work by Fargues and Luz (2000) indicates, however, that germlings of these fungi are able to survive repeated intervals of low RH, renewing development with each return of favorable conditions until infection is achieved. Results from a number of lab studies also indicate that at least some Hyphomycetes, including *Beauveria bassiana* and *Metarhizium anisopliae*, are able to infect their hosts essentially independently of ambient RH conditions (see Inglis et al. 2001). The mechanisms for this are unclear; however, many have suggested that the small (< 3-10 μm) conidia of these fungi become lodged in intersegmental folds or articulations of the host body where moisture levels are high. However, the leaf as a source of moisture may play a critical role in creation of sufficiently humid sites for infection under dry conditions (Wraight et al., 2000). Low ambient RH was found detrimental to *B. bassiana* infection in studies in which blood-fed *Rhodnius* bugs were inoculated and held in ventilated glass vials with no source of water (Fargues and Luz, 2000). Little is known about the variability of phyllosphere moisture levels across host plants, but great differences certainly exist as determined by such factors as densities of stomata and trichomes and structure of the epicuticular wax layer. Interestingly, infection independent of ambient RH has not been demonstrated with the Entomophthorales, suggesting that the much larger conidia (mostly > 20 μm) of these fungi are not inoculated into the same microniches on the host as hyphomycete conidia or that under dry condition, these sites may not have moisture adequate to support the entomophthoralean infection processes. Effects of allelochemicals produced by the leaf or by microbial epiphytes inhabiting the phyllosphere on host infection by EPF is a greatly under-explored area of research. Studies have demonstrated inhibition of EPF by both volatile and nonvolatile chemicals produced by leaves (e.g., Brown et al., 1995; Lacey and Mercadier, 1998). Studies are also needed to elucidate effects of host-sequestered allelochemicals on EPF.

Parasitic growth. Recent studies indicate that temperature, in many cases, may be more important than moisture in limiting the biocontrol efficacy of EPF. Studies have revealed near complete inhibition and even curing of fungal infections in insects able to bask at will (Carruthers et al., 1992; Inglis et al. 1996). This result is typically attributed to direct inhibition of fungal development by high temperatures. Less attention has been paid to potentially equally important effects of high-temperature enhancement of host immune systems (James et al. 1998). Temperature optima for vegetative growth of EPF generally range from 24-28°C, whereas the optima for many insects exceed 30°C. Many plants are able to grow normally during periods when daily temperatures reach 35-40°C, but nearly all EPF are severely inhibited at these temperatures. In some cases, pathogenicity may be substantially reduced when infected hosts are exposed to temperatures near 40°C for only short, daily intervals (Inglis et al., 1996). Most plants do attempt, however, to maintain leaf temperatures near 20-30°C (the approximate optimal range for photosynthesis). This temperature range is also favorable to EPF, and many of the most spectacular epizootic fungal pathogens exploit hosts that inhabit the regulated environment of the ventral phylloplane.

Saprophytic growth and sporulation. As with the processes of germination and host infection, EPF, with few exceptions, require nearly saturated moisture conditions to produce conidia. In the case of most Entomophthorales, the processes of conidiogenesis and conidia release are as rapid as those of germination and host penetration, and these pathogens are thus able to exploit overnight periods of high moisture for this phase of development. These fungi can produce only a fraction of their potential conidial biomass during a single night but have the capacity to survive desiccation during low-humidity daytime periods as mycelia in host cadavers. These mycelia rehydrate and initiate additional cycles of sporulation during subsequent nights. *Zoophthora radicans* has been observed to undergo up to 8 such sporulation cycles on leafhopper cadavers (Wraight and Galaini-Wraight, unpublished). Therefore, in exploiting the leaf for sporulation (usually the ventral surfaces inhabited by the insect hosts), these fungi are not necessarily dependent upon moisture from the leaf. More importantly, the phylloplane serves as a highly favorable substrate shaded from lethal UVR, shielded from wind and rain, isolated from scavengers, and harboring generally low populations of saprophytic microbial competitors. In this situation, the most important adaptation of these fungi (after developmental speed) is production of rhizoids with specialized holdfast structures that secure the cadaver to the phylloplane. From this substrate, during humid nights, these fungi blast conidia directly onto new hosts or leaves in the surrounding habitat and into humid air currents that carry them longer distances to establish new infection foci. Many Hyphomycetes also complete their development on host cadavers on the phylloplane, receiving the same advantages just described. These fungi lack the developmental speed of the Entomophthorales, but nevertheless employ a similar sporulation strategy, producing conidia over multiple nights (Fargues and Luz, 1998; Wraight, unpublished). The desiccation tolerant conidia of these fungi readily survive the daytime conditions on the humid, UVR-shielded ventral phylloplane, and may accumulate in great numbers on or near the cadaver. From these substrates the conidia are entrained by wind turbulence (primarily during daytime) and are transported long distances. Spores are also dispersed by rain splash, and potential hosts (or vectors) moving across the phylloplane may contact conidia concentrated on killed host cadavers. Lacking a mechanism for active dispersal of conidia, Hyphomycetes also use the phylloplane as a substrate to support spreading growth of spore-bearing pseudorhizomophs or individual hyphae (e.g., Wraight et al. 1998). Conidia of some species (e.g., of *Verticillium* and *Hirsutella*) are borne in slime droplets. Production of sticky spores on aerial conidiophores enhances the "sit and wait" infection strategy of these fungi. Hyphomycetes do not produce rhizoids, but hyphae and pseudorhizomorphs produced by some species radiate out onto the substrate and serve to at least weakly anchor the cadaver. These fungi often attack homopteran hosts, which may die with mouthparts anchored in the plant. Because Hyphomycetes develop slowly, extensive sporulation and development of epizootics generally require prolonged periods of high humidity or wet weather (Fargues and Luz, 1998; Wraight, unpublished). Heavy rain, however, can be detrimental to both entomophthoralean and hyphomycete fungi by removing conidia and host cadavers from the phyllosphere.

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Endophytic fungi as agents for the biological control of insects

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Abstract

This review article describes an overview of interesting topics concerning the control of insects-pests by endophytic fungi. A great deal of information has accumulated in the past two decades that demonstrate the promising use of endophytes, mainly fungi and bacteria, as a tool to improve crop performance. The importance of endophytic entomopathogenic fungi for insect control is addressed. Other strategies displayed by endophytic fungi to control insect attacks are also discussed. Finally, recent advances in the characterization of the fungal endophytic communities colonizing tropical plants, including several Amazon trees, are described to show the potential of these microrganisms to improve the agriculture of areas severely affected by pests and diseases.

Introduction

The biological control of pests and diseases of economically important cultivated plants has gained much attention in the past decades as a way of reducing the use of chemical products in agriculture. The use of agrochemicals, although decreasing the attack of insects and phytopathogenic microorganisms, still represents a high risk to field workers and consumers. In addition, their use is, in certain cases, economically unviable. The control of pests and diseases by means of biological processes i. e., use of entomopathogenic microorganisms or those that inhibit/antagonize other microorganisms pathogenic to plants, is an alternative that may contribute to reduce or eliminate the use of chemical products in agriculture.

Endophytic microorganisms are those that colonize internally plant tissues, especially leaves, branches and stems showing no apparently harm to the host (Azevedo, 1998). Endophytes were initially considered neutral, not causing benefits nor showing detriment to plants. More recently, the nature of the endophytic interaction started to be better understood and nowadays, it is obvious that in the majority of cases, they have an important role in host protection against predators and pathogens. In addition, other effects on the host such as greater resistance to stress conditions and beneficial alteration in physiological properties are described as well as the production of phytohormones and other compounds of biotechnological interest (i. e. enzymes and pharmaceutical drugs) by the endophyte. Besides the economical aspects, the study of endophytes has strong academic interests, concerning the discovery of new microbial species, mainly when tropical hosts are investigated. The present review will focus on examples of associations between endophytic fungi and plants that result in insect control as well as in the description of recent studies involving new tropical hosts that may result in the discovery of new candidate agents of insect control.

Entomopathogenic fungal endophytes

Recent studies conduced by our team with tropical endophytes has shown a very delicate interface between endophytism and phytopathogenicity. Thus, the fungus *Guignardia citricarpa*, the causal agent of "Citrus Black Spot", may be an endophyte in certain citrus varieties that are known to be resistant to the disease. The opposite is true where the fungus *G. cameliae*

behaves as a pathogen of mango and can be isolated as endophyte from several other plant species. The same interplay can be observed regarding ecological niches and involving epiphytic and rizosphere microbial communities and endophytes. The transition from one state (mutualist or parasite) or niche to the other seems to be outspread in nature and have been also described with fungal endophytes and entomopathogens as well as phytopathogens and entomopathogens.

Fungal species such as *Metarhizium anisopliae* and *Beauveria bassiana*, among others, are well characterized in respect to pathogenicity to several insects and they have been used as agents for the biological control of agriculture pests worldwide (Alves, 1998). If these microorganisms colonize plant tissues as endophytes, they will obviously be able to act in insect control. Even in situations where they are not present in the plant, techniques may circumvent this obstacle. Pioneer works have shown that the fungus *Beauveria bassiana* can be artificially inoculated in corn by foliar application or injection and then detected endophytically (Wagner and Lewis, 2000; references therein). Inoculation by injection resulted in 95% colonized plants and aspersion in 98,3%. An interesting finding was that in 33,3% of the control plants (not treated), the fungus was present as a natural endophyte. The inoculated fungus was able to colonize several corn tissues, translocate, and persiste in the plants to provide season-long suppression of the corn insect-pest *Ostrinia nubilalis*, blocking the insect attack in some of its developmental stages. Light and electron microscopy were used to describe the mode of penetration of the fungus in corn. Conidia grew randomly across the leaf surface and penetration did not require specific topographic signals. *B. bassiana* did not exhibit especial penetration structures such as appressorium and a host response to infection was not evident. Fungal cells were localized in the apoplastic space and a few in the xylem vessels. Artificial inoculation did not affect the virulence of the fungus towards the insect. Production of conidia inside or outside the plant was not observed and thus, it is not known from these studies if *B. bassiana* may complete its life cycle in corn. It has been also speculated by the authors that the endophytic state of the entomopathogenic fungus *B. bassiana* in corn may explain the suppression of *O nubilalis* attacks in certain seasons, correlated with the presence of the fungus in the corn senescent phase. Other entomopathogenic fungi have been isolated from a few other plants. *Beauveria bassiana*, *Paecilomyces farinosus*, *Verticillium lecanii*, *Acrodontium crateriforme* and *Penicillium thomii* were found in bark of *Carpinus caroliniana*. It was proposed that bark play a role as reservoir for entomopathogenic fungi. Hence, these fungi might act as facultative saprophytes while not coming into contact with insects. A recent work undertaken in Brazil have identified endophytically entomopathogenic fungi in corn and soybean (Pimentel, 2001). *Beauveria* sp. and *Paecilomyces* sp. were found in corn whereas the later was present also in soybean. Preliminary assays have demonstrated that isolates of *Beauveria sp.*, currently identified as *B. bassiana*, were pathogenic towards ticks.

Colletotrichum comprises a variety of phytopathogenic species used as models for the studies concerning pathogenicity and fungal-plant interaction. Commonly they are also described as endophytes, establishing asymptomatic plant infections. A few studies have shown that some phytopathogenic isolates of *Colletotrichum gloeosporioides* may behave as opportunistic pathogens of insects. In Brazil, these fungal isolates have proved to be highly pathogenic to the citrus pest *Orthezia praelonga* and preliminary assays demonstrated their potential as biocontrol agents (Teixeira, 2000).

Other fungal endophytes in the control of insects

Webber (1981) was probably the first researcher to report an example of plant protection giving by an endophytic fungus, in which the endophyte *Phomopsis oblonga* protected elm

trees against the beetle *Physocnemum brevilineum*. Several other examples were then described establishing a clear connection between fungal endophytes and plant protection against insects (for a complete review see Azevedo *et al.*, 2000). Almost the totality of the research done has been performed with plants from temperate climate, particularly with grazing grasses such as the tall fescue, *Festuca arundinacea* Schreb. and *Lollium perenne* L. They are plants of great importance for countries in the Oceania, Europe and for the USA, where they are widely used to feed livestock. The symbiosis established between endophytes and grasses seems to be quite specific, revealing only a few fungal genera as the most frequently found. Among them *Acremonium*, nowadays classified as *Neotyphodium* (Redlin and Carris, 1996). The capacity of endophytic fungus to repel insects, induce weight loss, growth and development reduction and even to increase pest death rate was correlated with toxin production. In several cases, it was shown that the mode of action of certain fungi was based on the capability to render the plant unpalatable to pests like aphids, grasshoppers, beetles, etc. Larvae from the bluegrass webworm *Parapediasia teterrella* prefer diets with endophyte-free plants of *L. perenne* and *F. arundinacea*, to a point that the larvae would starve to death if only plants infected with *Acremonium* were available. In the field, endophyte-free species were severely attacked by insects, whereas those infected with *Acremonium* stayed almost free of insect larvae. The most important toxins found in *L. perenne* are ergot alkaloids of two types, ergopeptine and clavine and neurotoxines called lolitrems. In opposition to the ergot toxins that are isolated directly from the endophytic fungi, the neurotoxins produced by endophytes like *A. lolii* are only percursors of toxins like, for example, paxiline. It is not known if the precursor is converted in lolitrems by the plant or if the fungus is not capable of synthesizing it in pure culture but is able to do so while inside the plant. The associations between grasses and endophytes are complex and factors like the level of nitrogen fertilizers may as well affect insect attack frequency. Although the majority of works related to toxin production was performed in grasses, some of them have identified toxic products synthesized by endophytic fungi in woody plants and that were able to modify growth and death rates in larvae of the spruce budworm feeding on balsam fir. The endophytes in this case were identified as *Phyllosticta* and *Hormonema dematioides* and the toxic compounds were mainly heptelidic acid and rugulosine. Some tremorgenic toxins were identified in tropical woody plant infected with an endophytic fungus from the genus *Phomopsis*. The expression of insect resistance may be affected by several factors such as active amounts of allelochemicals, plant genotype, endophyte concentration, soil fertility and endophyte genotype. Hydric stress, temperature, soil pH, insect-pest resistance and other factors may also affect the endophyte concentration and toxin production. Endophytic fungi may indirectly affect seed dissemination by insects, especially ants. In *Festuca*, seeds infected with certain endophytes are discarded after being collected and, therefore, favor plant dissemination.

Endophytes from tropical plants

The role of endophytes in plant protection against insects and diseases has been quite well studied. However, the research is based mainly on endophytes from temperate hosts and, in addition, the work has been concentrated in some genera of grasses. Apart from isolated studies, only more recently, efforts are being directed to endophytes from tropical plant hosts. This is a broad field of investigation that is almost entirely open to new findings. Our group at the University of São Paulo has conduced a survey for endophytic fungi in several tropical plants such as citrus, banana, eucalyptus, cocoa, soybean, corn, etc. In citrus, isolation yielded no fungal endophytes from seeds of *Citrus* spp. and the most frequent genera found in leaves were *Colletotrichum* and *Guignardia*. In banana leaves, 16 fungal taxa were isolated and *Xylaria* sp. was the most frequent genus, followed by the species *Colletotrichum musae*

and *Cordana musae*. Other research groups found *Colletotrichum gloeosporioides*, *Pestalotia* sp., *Fusarium solani* and *Phomopsis* sp. as the most frequent endophytes in leaves of cashew tree (*Anacardium occidentale*). Several palm trees and forage plants were also subject of research. Isolation from young and old leaves of *Stylosanthes guianensis*, a leguminous genus widely distributed in the tropical and subtropical regions of South America yielded *Glomerella cingulata*, *Phomopsis* sp. and *Xylaria* sp. as the most frequently found endophytes. The frequency of infection of leaves, as expected, increases with the increase of the plant age. Overall, members of the genus *Xylaria* are the most frequent fungal endophytes reported from tropical hosts so far.

Several other studies are been carried out by the group of Dr. J. O. Pereira at the University of Amazonas involving Brazilian Amazon plants, most of them of economical importance. *Paullinia cupana* var. *sorbilis* (guaranazeiro), a Sarpindaceae collected in the Amazon Basin (Manaus and Maués), has been the first species studied. From *P. cupana* var. *sorbilis* is produced a soft drink, the guaraná, which has great acceptance in Brazil and several other countries. The four most common genera of endophytic fungi found in the host were *Guignardia*, *Phomopsis*, *Glomerella* (*Colletotrichum*) and *Xylaria*. Other genera less frequently found were *Fusarium*, *Dreschrella*, *Pestalotia*, *Curvularia*, *Humicola* and *Nodulisporium*. From *Theobroma gradiflorum*, a Sterculidaceae (cupuaçuzeiro) largely employed in the production of juices, sweets, ice cream, liquors and a kind of chocolate called cupolata, *Guignardia*, *Phomopsis*, *Colletotrichum* and *Pestalotia* were the most common endophytic fungi found. It is severely attacked by pathogenic fungi and insect-pests. This host plant was chosen in an attempt to find some possible endophytic fungi, which could be useful for biological control. Two other hosts were surveyed for endophytes, *Pueraria phaseoloides*, a leguminous plant originated from Asia and well adapted in Amazon and used as pasture and to increase nitrogen fertilization and *Scleria pterota*, an invading Cyperacea common in Tropical and Subtropical America. Similarly to *T. grandifolium*, the most frequently endophytes isolated were *Colletotrichum*, *Guignardia*, *Phomopsis* and *Pestalotia* besides *Xylaria* and *Curvularia.* From all these Amazon hosts, a number of unidentified fungi and micelia sterilia were found. Other plants under investigation are: *Dipteryix odorata*, an arboreal leguminous found in the low Amazon Basin and used in folk medicine where its seeds are employed in the production of coumarin; *Copaifera multijuga* (copaiba), a leguminous plant which produces a terpenoid resin mainly from its stem and leaves and the Copaiba-oil that is extracted from its seeds, with several industrial uses as for instance, the production of dyes and varnishes. The endophytic mycobiota from *Victoria amazonica*, an aquatic plant, and *Paulicourea marcgravii*, a Rubiaceae toxic for livestock, is now starting to be investigated.

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Microecology of entomopathogenic fungi from aquatic environments

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Introduction

Studies related to entomopathogenic fungi from aquatic environments had been reported mainly from the North hemisphere, (Apperson *et al*, 1993; Lucarotti, 1987; Golkar *et al*, 1993, Kerwin & Washino, 1986; Lord & Roberts, 1986). Although there is no much information reported about pathogenic fungi of insects from South America (García *et al*, 1994, López Lastra *et al* 1992; López Lastra 1999, López Lastra *et al.* 1999).

Field surveys over the past twenty years were conducted to assess the presence and incidence of pathogenic fungi of aquatic insects from Buenos Aires province, Argentina. The objective of this study was present an overall and up to date report of the research done about entomopathogenic fungi from aquatic enviroments in Argentina.

Materials and methods

Mosquito larvae were collected from temporary and permanent ponds and from artificial containers in and around La Plata, Buenos Aires province, Argentina.The survey had been done on weekly basis. Field collected mosquitoes were carried to the lab and checked for fungal infections under phase contrast microscope. Most of the entomopathogenic fungi identified were isolated in culture in artificial media and conserved at mycological collections (CEPAVE and Spegazzini Institute Culture collections at La Plata, Argentina and ARSEF Entomopahtogenic Fungal Culture Collection, Ithaca, NY, USA). Studies on virulence, seasonality, life cycles and natural incidence of the fungi identified were assesed.

From all the fungal species *Leptolegnia chapmanii* (ARSEF N° 5499) isolated from *Ochlerotatus albifasciatus* larvae from La Plata on 1996 was the more extensively studied. It was tested for its pathogenicity on *Aedes aegypti* larvae from a colony maintained at CEPAVE. The susceptibility for each larval instar of *Ae. aegypti* under laboratory conditions was assayed. For obtaining the fungal innoculum, *L. chapmanii* was cultured on Emerson YPSS media, incubated at 22° C during 7 days. From these cultures, small square blocks of media (0.7 mm ^{2}) were cut and deposited in 60 mm diameter Petri dishes containing 15 ml of sterile distilled water and incubated at the 22 °C for 3 days to allow zoospores developing. A batch of one hundred *Ae. aegypti* second instar larvae were innoculated with a suspension of 1x10 ^{4} zoospores/ ml, and at 24 hours, dead infected larvae were collected, the larvae were incubated at 22 °C for 24 hours, and after they produce externally zoosporangia with zoospores they were used as innoculum for the experiments. The zoospores produced by twenty infected larvae were counted (with a hemocytometer) obtaining a mean of 1.5 x 10 ^{4} zoospores/ml/ larvae. Those infected larvae were used as innoculum (it was used three larvae for each treatment). A total of 25 larvae of each instar I-IV (3 replicates for each treatment and controls, without fungal innoculum). Larvae were incubated at 25° C and 80 % humidity until larval death or pupation. Larval mortality was recorded at 12, 24, 48, 72 and 96 hours and dead infected larvae were observed for fungal infection and they were used for re-isolating *L. chapmanii*.

For the host range studies, four mosquito species, *Culex pipiens*, *Oc. albifasciatus, Oc. crinifer* and *Psorophora ferox*, where tested for susceptibility. Ten larvae (from intars II, III and IV as they were available) were exposed to 1x 10^4 zoospores/ml in sterile distilled water. Larvae were incubated at 25 ° C and 80 % humidity. Mortality was recorded at 24, 48, 72 and 96 hours.

Results

As the results of the twenty years survey fourteenth entomopathogenic fungal species from twelve species of culicids. (Table 1).

Fungal species	Hosts	Stage of development	Locations (*)
Aphanocladium album	*Ochlerotatus Albifasciatus*	Adults	La Plata
Fusarium oxysporum	*Psorophora ferox* *Culex sp*	Larvae and pupae	Punta Lara, Ensenada
Metarhizium anisopliae	*Oc. Crinifer*	Adults	Los Talas, Berisso
Tolypocladium cylindrosporum	*Cx. pipiens*	Larvae	La Granja, La Plata
Smittium morbosum	*Cx. dolosus, Ps. Ferox*	Larvae	Punta Lara, Ensenada
S. aff. morbosum	*Ae. Aegypti*	Larvae and adults	La Plata
Leptolegnia chapmanii	*Oc. Albifasciatus* *Ae. Aegypti*	Larvae Larvae	M. Romero, La Plata La Plata
Saprolegnia ferax	*Cx.dolosus, Cx. pipiens* *Cx. maxi*	Larvae	Punta Lara, Ensenada La Plata
Coelomomyces iliensis	*Cx. dolosus* *Oc. Crinifer*	Larvae Adults	Punta Lara, Ensenada
C. reticulatus	*Cx. dolosus*	Larvae	Coronel Brandsen
Coelomomyces spp	*Cx. chidesteri* *Uranotaenia nataliae*	Larvae	Coronel Brandsen

(*) All locations are situated at Buenos Aires province, Argentina.

Studies about the fungal lyfe cycles, seasonality and natural incidence had been done. From all of the fungal species detected from insects in aquatic environments, *C. iliensis* from *Cx. dolosus* larvae was present since 1990 to 1997, showing a natural incidence range between 1,2 to 25 % . Its ocurrence was only during the months of October and November (from 1990 to 1995). It was also detected in adult females of *Ae. crinifer*. *Coelomomyces reticulatus* and two others non identified species were also observed in three species of mosquito larvae (Table 1).

Leptolegnia chapmanii was collected at the first time in September- October on 1996 in one temporary pound and from larvae of *Oc. albifasciatus* and it was not registered any infected mosquitoes more, until October of 2000, when it was observed on *Ae. aegypti* larvae in artificial containers from another location .

From all the entomopathogenic fungi that were challenged against mosquitoes during laboratory assays, the more promisory was *L. chapmanii*. This isolate shows high virulence and it has

been easily culturable in simple media, under field conditions was causing larval death in pounds containing hig organic matter. Also the resistance of oospores could be probable be evaluated for being used on field applications. In preliminary research studies done at our lab we had been tested in vivo produced oospores for bioassays obtaining 100 % of larvae dead (3rd and 4 rd instars) Persistance of *in vivo* infection was estimated using cadavers of *Ae. aegypti* infected larvae and adding healthy larvae on a weekly basis, we observed up to 2 months of pathogenicity maintenance.

All four instars larvae of *Ae. aegypti* larvae were susceptible to *L. chapmanii* infection, dying between 24- 72 hours, depending on the larval age. Mortality of I and II instar larvae were 100 % after 24 hours, and 64 % of third instar died up to 48 hours, finally there was a decreased in larval mortality in fourth instar larvae, showing 45 percent mortality between 48 and 72 hours.

Host range studies demonstrated that all the four mosquito species tested demonstrated that *Oc. crinifer* was the more susceptible to *L. chapmanii.*. These studies are still to be complete with an abroad number of mosquito species .

Preliminary results shown that *L. chapmanii* is the most promising of the fungal species isolated and it needs to be further evaluated, in order to be possible to use as a potential candidate for mosquito control agent.

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Symposium (Virus 2) Prospects for the Use of Viral Pesticides

The successful use of AgMNPV for the control of velvetbean caterpillar, *Anticarsia gemmatalis*, in soybean in Brazil

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Introduction

The velvetbean caterpillar, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) is a key pest of soybean in Brazil. In the 1970's, a nucleopolyhedrosis of the insect (AgMNPV) was isolated in different regions of the country (Allen and Knell, 1977; Carner and Turnipseed, 1977). Initial field experiments with the AgMNPV indicated its potential to be used to control the insect in soybean integrated pest management programs (Carner and Turnipseed, 1977; Moscardi *et al.*,1981). In the beginning of the 1980's, research results generated at the Brazilian Agricultural Research Coorporation (Embrapa), National Center for Soybean Research (Embrapa Soja), in Londrina, state of Parana, were use to implement a pilot program for AgMNPV use in soybean growers' fields (Moscardi and Corrêa Ferreira, 1985; Moscardi, 1989; 1999). The early beginnings of this program, its consolidation phase, its current status, and the future perspectives and needs of the program will be discussed.

Early Implementation of the Program for AgMNPV use at farmer level

A pilot program for AgMNPV was conducted during the 1980/81 and 1881/82 seasons, on 21 farms in the states of Parana and Rio Grande do Sul, when one-hectare plots treated with the virus resulted in efficient control of *A. gemmatalis* populations compared to insecticide-treated and untreated paired plots. Implementation of the program began in the 1982/83 season, when ca. 2000 ha of soybean were treated. Initially, small amounts of AgMNPV were produced in host larvae reared on artificial diet. Frozen killed larvae were distributed to extension officers for treatment of demonstration plots and virus production in the field, which in turn provided inoculum to treat other areas in the same season or to collect and store dead larvae for use in the subsequent season.

Consolidation of the Program

AgMNPV use gained momentum with the development of a wettable powder formulation of the virus in 1986 (Moscardi, 1989; 1999). Production and formulation was initially performed by the Employees' Association of Embrapa Soja, Londrina, PR and the Research Station of the Farmers' Cooperatives for the State of Paraná (COODETEC), Cascavel, PR. At that time, COODETEC hired two technicians to work at Embrapa Soja, in Londrina, to perform the quality control of batches of formulated AgMNPV. In 1989 Embrapa started to negotiate

contracts with private companies interested in producing and commercializing this biological insecticide. From 1990 on, five companies signed the contracts with Embrapa. Through these Embrapa would transfer all the technology for AgMNPV production in the laboratory and in soybean growers' fields, as well as for formulation of the product, including the quality control of production batches. The companies would each pay to Embrapa US$ 40,000 initially, plus 5% of royalties of the AgMNPV sales each year, for a period of ten years. The products based on the AgMNPV of each company were registered according to the Brazilian policies for registration of plant protection insecticides. All tests related to physical and chemical aspects of the formulations, toxicological data, identity (characterization), etc. were performed and approved by the Brazilian agencies involved (Agriculture, Health, and Environment) (Moscardi and Sosa-Gómez, 1996).

Virus Production

Despite the efforts of Embrapa to develop and improve a technically and economically viable procedures for AgMNPV mass production in the laboratory (Moscardi *et al.*, 1997), it was seriously attempted by only one of the companies (Geratec, now Millenia), that was able to produce around 150,000 hectares equivalent of the virus per year. Due to high cost of labor and components of the insect diet, this company discontinued laboratory production of the virus. On the other hand, AgMNPV field production became widely adopted by all companies, as the best available method to obtain large quantities of virus-killed larvae at a low cost. Field production of the virus became a big business, involving different persons that have specialized in this type of production to sell over 35 metric tons of AgMNPV-killed caterpillars/year to the private companies (Moscardi, 1999). It involves impressive logistics. Growers' fields are contracted and the pest control in their fields is taken care off by the AgMNPV producers. Usually two to three fields are sprayed with the AgMNPV every day during the most prevalent *A. gemmatalis* larval occurrence (December and January). Peak collection occurs from the 8th to the 10th day in each selected field, and may involve from 200-300 "larval pickers"/day, requiring around 10 buses to transport the "pickers" for each collecting site. In a single day, production in one collection site may reach up to 600 kg of AgMNPV-killed larvae, enough to process the virus for treatment of ca. 30,000 ha. However, the annual AgMNPV field production has not been sufficient to attend the crescent demand for this biological insecticide. Furthermore, yearly production is too much dependent on natural incidence of the host insect that may occur in low numbers in certain seasons, thus reducing AgMNPV yield. Recent efforts are being undertaken to improve laboratory large-scale production of the virus, in order to complement field production and attend the crescent demand for the biological insecticide. Procedures for laboratory production have been developed or modified in order to increase efficacy and reduce costs. The cost of the artificial diet has been reduced to 1/5 and a pilot plant is being built at Embrapa Soja, Londrina, to test the new system (B. Santos and F. Moscardi, unpubl.). It is expected that the cost of the final product produced in the laboratory will be competitive with those of the chemical insecticides used to control the insect, allowing the private industries to also adopt this method for AgMNPV production.

AgMNPV Use

Details on technology of application (dosage, timing, equipment, etc) and evaluation for AgMNPV use can be found in Moscardi and Sosa-Gómez (2000). Effective use by growers started in the season 1982/83, when about 2,000 hectares of soybean were treated with the virus. In the early to mid 1990s AgMNPV use reached around 1,000,000 ha annually and, in the season 2001/2002, the treated area with the pathogen in Brazil was over 1,550,000 ha (more than 11% of the soybean cultivated area in the country). In the season 2001/2002 the demand for

the biological insecticide was sufficient for 2,300,000 ha, about 750,000 hectare equivalent (HEQ) over the availability of the product in the market. The increase in demand for the AgMNPV occurred mainly in the central region of Brazil (states of Mato Grosso-MT, Mato Grosso do Sul-MS, and Goiás-GO), where over 50% of the soybean is produced. The use of AgMNPV in this region used to be small three years ago, but increased dramatically in the last two seasons. This is illustrated in Table 1, which shows the sales of AgMNPV by COODETEC in the last three soybean seasons and the sales for the coming season (2002/2003). It is important to notice that this company was short of Coopervirus[a] (AgMNPV) to attend the crescent demand in the last season (2001/02) and for the upcoming season (2002/03). Also, its sales to the central regions, especially to Mato Grosso state, increased substantially from the season 1999/2000 (27,330 HEQ) to the season 2002/03 (222,500 HEQ), representing and increase from 9.2% to 46.3% of the total sale of AgMNPV by COODETEC, in this period. For the upcoming season of 2002/03, all the private companies have produced AgMNPV to treat about 1,700,000 ha, which will be ca. 800,000 HEQ of the actual demand for the biological insecticide (information obtained from the private companies producing the virus, by F. Moscardi). Use of the virus in Paraguay has also been significant, and it is currently estimated at 150,000 ha annually.

TABLE 1. Sales of Coopervirus® (AgMNPV) (hectare equivalent) by COODETEC (seasons 1999/2000 to 2002/2003 by region/state of Brazil and Paraguay

Region/State	1999/2000	2000/2001	2001/2002[1]	2002/2003[2]	Total
South					
PR	257,915	437,575	173,550	190,500	1,059,540
SC	9,650	6,450	2,180	500	18,780
RS	3,450	42,480	42,180	27,000	115,110
Sub total	271,015	486,505	217,910	218,000	1,192,430
Southeast					
MG	0	7,300	3,000	0	10,300
SP	0	800	8,200	0	9,000
Sub total	0	8,100	11,200	0	19,300
Central					
MT	17,200	51,650	174,440	194,750	438,040
MS	10,130	25,300	0	10,000	45,430
GO	0	31,680	12,900	17,750	62,330
Sub total	27,330	108,630	187,340	222,500	545,800
	(9.2%)	(16.9%)	(37.7%)	(46.3%)	(28.4%)
Paraguay	0	40,500	80,000	40,000	160,500
Total	298,345	643,735	496,450	480,500	1,919,030

[1] COODETEC could not attend a demand for 180,000 hectare equivalent of the AgMNPV .

[2] All of the AgMNPV production was sold up to April, 2002 for the season 2002/2003. An additional demand for 300,000 hectares could not be attended by COODETEC (MT, GO, MS, MG, PR and Paragua y), due to lack of the product.

Benefits of the program

The use of the AgMNPV in Brazil has generated substantial economical, ecological, and social benefits. At the soybean grower level, considering that the cost of the AgMNPV is ca. 20% to 30% lower than the average cost of chemical insecticides, and that the virus usually provides control of the insect during the season with only one application, compared to an average of

two applications for chemical insecticides, the economical returns from the use of the virus may reach up to ca. US$ 7.00/ha/season, including product cost and application cost (fuel, labor, etc.). That means that current annual savings at grower level, in the total area sprayed with the virus (ca. 1,550,000 ha in season 2001/2002) is over US$ 10,800,000. Since implementation of the program in the season 1982/83 up to season 2001/2002, it is estimated that cumulative use of the AgMNPV reached 17,000,000 ha, which represents savings to growers of about 120 million US$ in that period. Most importantly, since the beginning of the program for AgMNPV use more than 17 million liters of chemical insecticides were not sprayed in the environment, resulting in considerable benefits to society. Also, the program has contributed for socioeconomic improvement of poor families in AgMNPV field production regions in the south, as handpicking of virus-killed larvae during the soybean season has provided an additional and important income to these families. Another important aspect is that the program has contributed to change the profile of the insecticides used to control A. gemmatalis larvae in soybean. An example can be taken from the southern state of Parana, where the AgMNPV use corresponds to about 38% of the total use of this biological insecticide in the country. Through surveys conducted by the official extension of Parana (Emater), during five seasons (1992/93 to 1996/97), it was evident that the profile of the insecticides used to control A. gemmatalis changed substantially from wide spectra and highly toxic insecticides, such as monocrotofos, to AgMNPV-based products and insect growth regulators (IGR's) (Fig. 1). Currently, the AgMNPV is the most used insecticide against the target host, representing over 22% of insecticide applications in this state, with IGR's representing about 15% of the applications against the insect (Fig. 1). A three-year survey among about 1,000 farmer of Parana state, also conducted by Emater-PR (L. Morales, unpubl.), have evidenced the importance of choosing selective insecticides for the first application against A. gemmatalis on soybean (Fig. 2). When AgMNPV or IGR's were used in the first application on the crop,

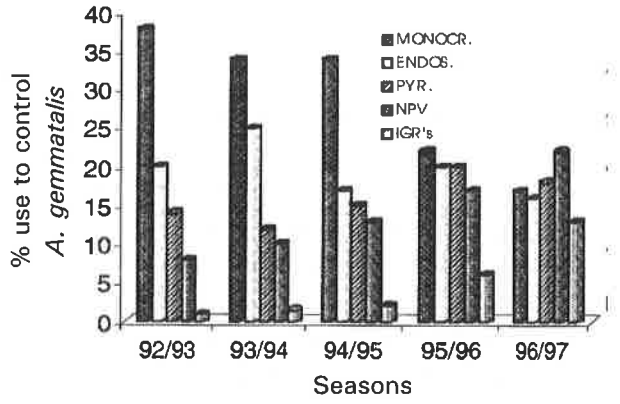

FIG. 1. Participation of insecticides (%) for the control of *A. Gemmatalis* in the state of Paraná, Brazil (L. Morales, unpubl.).

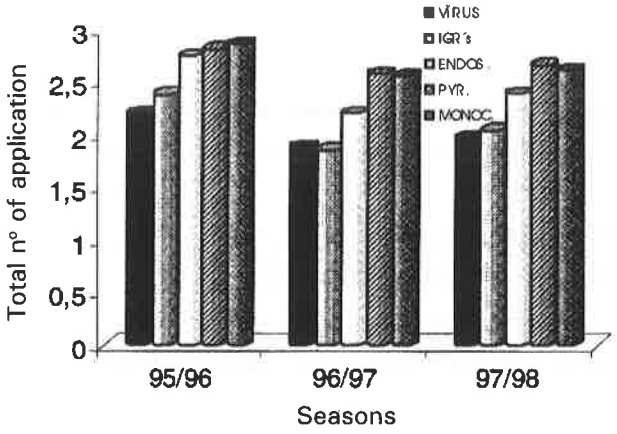

FIG. 2. Total number of insecticide applications in soybean as related to the type of insecticide used in the first application on the crop. Paraná state, Brazil (L. Morales, unpubl.).

the total number of application of insecticides (including all insect pests) were substantially lower compared to the first applications on the crop by the chemical insecticides monocrotophos, endosulphan, and pyrethroids.

Future perspectives and needs

The program for AgMNPV use in Brazil has been so successful due to various reasons: i) The implementation of a soybean IPM program in the country, in the 1970's (see Moscardi 1993), facilitating adoption of the AgMNPV by soybean growers; ii) The proactive activities of official extension services in transferring the AgMNPV technology, initially in the South and more recently to other regions; iii) High virulence of the pathogen to the host and efficient horizontal transmission in the host population by biotic and abiotic factors, allowing control of *A. gemmatalis* with only one application during the season (see Moscardi 1999); iv) As a defoliator, the host insect is continually exposed to the application of the AgMNPV; v) Soybean tolerates high defoliation (30-40%) (high economic injury level) without significant yield reduction; vi) Usually, in most regions there are no other simultaneous key pests, allowing use of a highly selective biological insecticide as the AgMNPV; and vii) The possibility of producing large quantities of the virus under field conditions at a very low cost. However, to attend the crescent demand for this biological insecticide, field and laboratory production procedures will have to be improved, as for the last season (2001/2002) the availability of the product was well short of the demand. This is the main factor limiting expansion of AgMNPV use nowadays. Current improvements in laboratory production of the virus (quality and cost wise) look promising, but its success will depend on adoption of the technology by the private companies producing and commercializing the virus. Problems related to the formulation of the product, as well as other factors that affect efficacy of the AgMNPV in the field have been worked out (Silva & Moscardi 2002). With the expansion of the area treated with the pathogen, there have been concerns related to the selection for resistance to AgMNPV by field populations of *A. gemmatalis*. High resistance to the pathogen has been detected through selection pressure in the laboratory (Abot et al. 1996), but has never been observed in field populations of the insect (Moscardi et al., unpubl.). However, there is a need of continuous monitoring of the host populations, especially in regions of extensive use of the biological insecticide, to detect possible cases of resistance and devise strategies for management of the resistance to the AgMNPV. Genetic engineering of AgMNPV is being undertaken in Brazil. An AgMNPV lacking the *egt* gene was developed, being more virulent to *A. gemmatalis* larvae and reduced the time to kill this host by at least by one day compared to the wild AgMNPV (Pinedo *et al.*, unpubl.). This and other recombinant AgMNPV will have to be tested for its usefulness in the context of the soybean IPM program and the environmental risks involved. Despite the problems and some limitations, the future perspectives indicate expansion of the AgMNPV use in Brazil and other South American countries.

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Development of *Spodoptera frugiperda* nucleopolyhedrovirus as a bioinsecticide in Mexico and Central America

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The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is the principal pest of maize and sorghum in the Americas. The damage caused by larvae feeding in the leaf whorl is very apparent and the majority of insecticide applications in maize are directed at controlling this pest. In Mesoamerica, insecticides are often applied manually, with no protective measures/ equipment, resulting in a very high prevalence of chronic intoxication of farm workers (Tinoco and Halperin, 1998). The multinucleocapsid nucleopolyhedrovirus of S. *frugiperda* (SfMNPV) has been observed to cause epizootics of disease in high density host populations (Fuxa, 1982). Such observations have motivated several attempts to develop this virus as a biological insecticide in the U.S. (Hamm and Young, 1971) and Nicaragua (Mullock et al., 1990) although the only successful programme is being run by Embrapa in Brazil, with up to 20,000 ha of maize treated annually (de Oliveira, 1998; I. Cruz , pers. comm.). In 1997, we began a collaborative project aimed at developing the SfMNPV as a bioinsecticide for small scale resource-poor maize farmers in Mesoamerica. The project involved David Goulson (Southampton University, UK), Primitivo Caballero (Universidad Pública de Navarra, Spain), Ronald Cave (Escuela Agrícola Pamanericana, Honduras) and Trevor Williams (ECOSUR, Mexico). I present an overview of the project with particular emphasis on the experiments performed in Mexico and Honduras.

1. Selection of isolates. The first step of the study involved a biological and genetic comparison of SfMNPV isolates from Argentina, Nicaragua and the US. The genetic differences among the isolates were relatively minor, whereas the differences in biological activity were marked. The isolate from Nicaragua had the lowest LC50 value and had the additional advantage of being native to the region (Escribano et al., 1999). All of the following work is therefore based upon the use of this isolate.

2. Production systems. The highly cannibalistic habits of S. *frugiperda* means that larvae must be reared individually, which increases the time required for setting up and harvesting virus-infected insects and, therefore, raises the cost of virus production. Virus was initially produced by infection of late fourth instars that were subsequently reared in plastic cups with semi-synthetic diet. This system has now been replaced by a tray of diet which is surface contaminated by virus and divided into cells by pressing a plastic grid into the diet to form 120 individual cells. A larva is placed into each cell and the unit sealed using a perspex lid, similar to the system described by Cherry et al. (1997) for the production of *S. exigua* MNPV.

3. Formulation. Baculoviruses do not behave like chemicals, so the formulation technology developed by the chemical industry is often not appropriate for these pathogens. From the very beginning of the work, it was apparent that formulation would be of key importance to the success of the project. There were two reasons for this. First, application of virus in simple aqueous sprays did not result in high prevalence of infection (less than 60%), even at high concentrations of virus (up to 6 x 1012 OBs/ha). Second, the complex structure of

the developing leaf whorl, in which the larvae feed, made it difficult to deliver the inoculum to the feeding site of the insect.

We therefore focussed on developing formulations to improve the activity and delivery of the virus. In particular, we evaluated the synergistic properties of boric acid, an optical brightener and Spinosad, and the phagostimulant properties of maize flour based granules.

(i) Boric acid. Boric acid has an established history as an insecticide for the control of cockroaches and ants. The LC50 value of SfMNPV applied to the surface of semi-synthetic diet was 114 OBs/mm2 diet surface for virus alone compared to 51 OBs/mm2 in the presence of 1% (wt/vol) boric acid. Increasing the boric acid concentration up to 4% resulted in an increasing prevalence of virus-induced mortality. Field trials indicated that virus applied in phagostimulant maize flour granules with 1% boric acid resulted in approximately 20% increase in virus-induced mortality of *S. frugiperda* larvae. Boric acid solutions sprayed onto maize plants at concentrations of up to 4% did not significantly affect the abundance of natural enemies or other non-target arthropods present in the crop. Boric acid has the advantage of being economical; the cost of including 1% boric acid in a virus application was estimated at just US¢48/ha (Cisneros et al., 2002).

(ii) Optical brighteners. Stilbene derived optical brighteners can greatly enhance the activity of certain baculoviruses because they inhibit the sloughing of infected gut cells and very much increase the porosity of the peritrophic membrane (Washburn et al., 1998; Wang and Granados, 2000). Different optical brighteners differ greatly in their synergistic qualities. The optical brightener Tinopal LPW caused a reduction of over x100 in the LC50 value of SfMNPV in second instars (Martínez et al., 2000). At a cost of US$44/kg, Tinopal CBS was identified as being the cheapest optical brightener available in Mexico, but including 0.1% Tinopal CBS in a 300 litre/ha application of virus would increase the cost of the application by US$13/ha, which was considered prohibitive. Unfortunately, Tinopal CBS has little potentiating activity towards SfMNPV (J. Cisneros, unpublished data). Furthermore, experiments in the UK and Mexico indicated that applications of optical brightener could adversely affect the foraging behaviour of pollinating insects (Goulson et al., 2000). Tinopal also increased the reflectance of the leaf surface, particularly in the region 420-470 nm, and reduced the rate of growth of maize and barley plants (Goulson et al., 2002), leading to a recommendation for further evaluation prior to the use of these brighteners on a wide scale.

(iii) Spinosad. Spinosad((Dow AgroSciences) is a mixture of two tetracyclic-macrolide compounds with unique neurotoxic properties, that are produced by fermentation of a soil actinomycete. Spinosad is primarily a stomach poison with particularly high toxicity to Lepidoptera but is virtually non-toxic to birds and mammals and is classified by the US Environmental Protection Agency as a reduced risk material (Bret et al., 1997). Slightly antagonistic interactions were observed in insect mortality when mixtures of SfMNPV and ultra-low concentrations of Spinosad (0.05 - 0.5 ppm) were offered to second instars, whereas mixtures of SfMNPV + 3 ppm Spinosad were generally synergistic in nature (Méndez et al., 2002). Field trials indicated that 1.2 x 1012 OB/ha + 3 ppm Spinosad gave a marked increase in the degree of pest control compared to applications of virus alone. This was due to a combination of Spinosad induced mortality shortly after the application, and an increased prevalence of virus-induced mortality observed in larvae collected at 5 and 10 days post-application and reared in the laboratory.

Additional trials indicated that the product label recommended concentration for control *of S. frugiperda* in maize (200 ppm, 60 g a.i./ha) had a similar impact on non-target arthropods as chlorpyrifos (Lorsban, 0.75 l/ha). Predatory earwigs (D. taeniatum), Orius spp. and staphylinid

and nitidulid beetles (Tachyporus sp. and Carpophilus sp.) were particularly sensitive to 200 ppm Spinosad and chlorpyrifos applications. In contrast, the effect of Spinosad at 3 ppm was minimal. The cost of including Spinosad in a virus application would be approximately US$1.00 for the treatment of 1 ha with 3 ppm Spinosad in 300 litters of water (Méndez et al., 2002).

(iv) Phagostimulant granules. The use of phagostimulant granules based on nixtamalized maize flour has recently been shown to improve bioinsecticidal efficacy and rainfastness, and to provide protection against UV inactivation of Bt and NPV (Tamez-Guerra et al., 2000). We have adapted this technology with the aim of reducing costs while maintaining the desirable phagostimulant qualities of maize flour based granules. A granule recipe was identified involving nixtamalized maize flour, cornstarch, maize oil, water and ground husk of the maize cob, that was highly palatable to *S. frugiperda* larvae. Multiple linear regression procedures were employed to analyze the results of 7 independent field trials involving spray applications and 8 independent field trials involving application of phagostimulant granules for control of *S. frugiperda* in maize. Virus-induced mortality was consistently greater when inoculum was applied in granular than in spray formulation. Persistence of activity of viral inoculum applied in spray or granular formulations was significantly greater when applied in the granular formulation, with approximately 23% of the original activity remaining at 8 days post-application compared to less than 1% in the spray-treated plants. The granular formulation caused a higher prevalence of infection in *S. frugiperda* larvae and persistence longer on crop foliage compared to an aqueous spray application (Castillejos et al., 2002).

4. Application. Most resource-poor maize farmers apply liquid insecticides using a manual backpack sprayer with a cone nozzle, whereas granular insecticides are sprinkled directly into the leaf whorl by hand. For applications involving SfMNPV in maize, the volume of water used for spray applications can have a significant effect on the efficacy of the treatment; higher volumes necessitate dilution of the inoculum but may be better able to deliver the virus to the feeding site of the insect pest (Hamm et al., 1994; J. Cisneros, Unpublished data). This was not the case for virus formulated as phagostimulant granules. Delivery of inoculum in small quantities of granules per plant (6 kg/ha) was as efficient as large quantities of granules (18 or 32 kg/ha), although an application rate of about 10 kg/ha was identified as being a practical quantity for manual applications (Castillejos et al., 2002).

5. Cost. Throughout the course of these studies, the cost of the bioinsecticide has been an issue of constant concern. The principal point of reference for cost issues has been that of the chemical control measures usually employed by maize farmers in the region; around US$13.50/ha for a granular insecticide and US$11.00/ha for a liquid concentrate (chlorpyrifos, methyl parathion, etc.). The cost of 10 kg of phagostimulant granules would be US$5.00 for the ingredients alone. The cost of producing 1.5 x 1012 OBs of virus on a laboratory scale in Mexico was recently estimated at US$12.10 (Williams et al., 1999) giving a total cost for the virus + formulation ingredients of about US$17. Large scale production of virus is likely to be cheaper than laboratory scale production leading to a lower cost bioinsecticide, but these calculations do not include the commercial costs involved in marketing, distribution, overheads, etc. (Castillejos et al., 2002).

6. Other considerations. (i) Interactions with natural enemies. Analysis of field trials involving applications of virus failed to reveal any detrimental effects on the prevalence of parasitism (typically ca. 20%) in *S. frugiperda* larvae collected from virus-treated plots (Martínez et al., 2000). However, when the prevalence of parasitism is high (ca. 50%), an inverse relationship between the prevalence of virus infection and the prevalence of parasitoid

emergence has occasionally been detected, suggesting that virus infection can have a significant impact on parasitized as well as unparasitized *S. frugiperda* larvae (Castillejos et al., 2002).

The earwig, Doru taeniatum, is one of the most abundant predators present in the maize crop and was shown to be capable of disseminating virus following consumption of virus-infected *S. frugiperda* larvae (Castillejos et al., 2001). Studies conducted in the Universidad Pública de Navarra revealed the importance of the interval between virus infection and parasitism on the probability of survival of two common parasitoids *Chelonus insularis* (Braconidae) and *Campoletis sonorensis* (Ichneumonidae). Parasitized and unparasitized larvae had similar susceptibilities to viral infection but viral production was significantly reduced in parasitized late instars compared to unparasitized hosts. The appearance of novel genotypic variants that differed in their infectivity and virulence was also observed in hosts parasitized by Ch. insularis (Escribano et al., 2000ab, 2001).

(ii) Integration with chemical insecticide treatments. Field experiments involving the spray application of virus or chlorpyrifos to early-whorl stage maize plants indicated that early virus application were preferable to chemical treatments because of their impact on natural enemy populations associated with the crop. In contrast, chemical treatment of late-whorl maize had a less severe impact on the abundance of natural enemies, probably due to the greater number of physical refuges available and the reduced penetration by the insecticidal spray applied at the late-whorl stage (Armenta, 2000).

(iii) Recycling of inoculum. Studies on the feasibility of recycling inoculum, by collecting the cadavers of infected insects several days after a virus application, indicated that this was not a viable technique for the SfMNPV-maize system due to low recovery and rapid liquefaction of virus-infected larvae (R.D. Cave, unpublished data).

7. Future prospects. I finish by reviewing the current situation in the development of this project, with emphasis on how the phagostimulant granular formulations may hold the key to the delivery of other biopesticides with a greater insecticidal capacity than SfMNPV.

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Development of wild-type and recombinant HaSNPVs as viral pesticides for the control of cotton bollworm in China

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Abstract

Cotton is one of the most important crops in China and plays a significant role in economic and societal developments. The Chinese textile industry needs stable production of cotton, and in the past 15 years, an average of 5.6 million ha were grown with cotton annually in China, yielding an annual production of 4.5 million tons of cotton. A single nucleocapside nucleopolyhedrovirus (HaSNPV) was first isolated from the diseased *Helicoverpa armigera* larvae and had been developed as a commercial pesticide for the control of the cotton bollworm in China. Recently, the genome of the HaSNPV had been entirely sequenced and several genetically modified HaSNPVs have been constructed and tested in the laboratory and in the field. In this paper, we describe the development of wild-type HaSNPV as a viral pesticide, including the process of viral production, formulation and application. In addition, the construction and evaluation of recombinant HaSNPVs are also presented.

Heliothine bollworm (*Helicoverpa armigera* and *H. zea etc.*) is one of the most serious pests around the world (Fitt, 1989). As one of the key pests ~~on~~ of important crops in China, the cotton bollworm (*H. armigera*) is widely distributed in the three major cotton-producing regions, which are the North China cotton region along the Yellow River, the South China cotton region along the Yangtze River and the Northwest inland cotton region in the Xinjiang Uygur Autonomous Region. In general, there are 4 or 5 generations within a year in the South China cotton region, while in the North China and Northwest China cotton regions, there are 3 or 4 generations annually. Control of *H. armigera* on cotton in South China depends almost exclusively on chemical pesticides such as esfenvalerate, endosulfan and pyrethroids. In North China, insect resistance transgenic cotton, based on genetic modification with the toxin genes from *Bacillus thuringiensis*, has been introduced at a substantial scale (~ 0.5 million ha in 2000). However, *H. armigera* has a strong ability to develop resistance to chemical insecticides (McCaffery and Walker, 1991) as well as to the toxins present in the genetically modified cotton varieties (Liu *et al.*, 1999; Shelton *et al.*, 2000). Therefore there are increasing and urgent demands for Integrated Pest Management (IPM) or biocontrol alternatives.

Two types of *H. armigera* NPVs, HaSNPV and HaMNPV were isolated in China (JSM & CCNU, 1975; Jiang *et al.*, 1978; WIV-CAS, 1979). Due to its high virulence, HaSNPV has been adopted for mass production as a viral pesticide and has been widely used to control the insect pests in China and in other countries (Jones, 1994). The morphology and pathology of HaSNPV has been described to some degree (WIV-CAS, 1979; Zhang *et al.*, 1983a; 1983b; 1983c). Eight HaSNPV genotypes have been isolated from wild-type HaSNPV isolation by using an *in vivo* cloning method (Sun *et al.*, 1998). There are differences of both median lethal dosage (LD_{50}) and median lethal time (LT_{50}) among some of these HaSNPV genotypes, but the differences are marginal (Sun *et al.*, unpublished data). Variant G4 has been selected as the type species. HaSNPV appears to have a host range restricted to members of the genus

Helicoverpa including *H. zea, H. virescens* and *H. assulta* (Ignoffo and Couch, 1981; Sun and Zhang, 1994).

The entire HaSNPV genome sequence was determined and it was found that the circular, double-stranded DNA genome contains 131,403 bp (Chen *et al.*, 2001). Of 135 potential ORFs predicted from the sequence, 115 have homologues in other baculoviruses, while twenty are unique to HaSNPV and are subject to further investigation. Phylogeny analysis indicated that HaSNPV belongs to the Group II NPVs (Bulach *et al.*, 1999).

Production and application of the wild-type HaSNPV insecticide

Based on perennial rearing of *H. armigera* on artificial diet, production technology of HaSNPV pesticide has been developed during 1970s

- 1980s. It included three main steps:
- Rearing of healthy host insect culture
- Multiplication of the virus
- Formulation of the insecticide.

Healthy *H. armigera* were reared on an artificial diet that has been optimized containing soybean powder, wheat powder, bread yeast, agar and vitamins (Zhang *et al.*, 1981). A special box was designed for moths mating and oviposition. Artificial diet is automatically dispensed onto plypropylene trays with 120 wells that are used for insect rearing. The trays are covered with papers of eggs and incubated at 28 °C. When larvae reach early fourth instar, 5% are set aside for further mass rearing while the rest are used for virus production.

Larvae were infected by contamination of the diet surface with 50ul of a solution containing 1×10^7 PIB (polyhedra) /ml. Normally, 75 ~ 90% of the treated larvae die within 6~8 days after infection. On average, one dead larva contains 6.5×10^9 PIBs.

Two types of formulations are made for the HaSNPV pesticide, a wettable power and an emulsifiable suspension. The infected larvae are first homogenized and insect debris is removed by centrifugation. To make a wettable powder, an aqueous suspension of PIBs is mixed with fine clay and then dried by spray-drying techniques. Thickening agents, enhancing agents, UV screening agents and wetting agents are then added. The powders are sifted ~~out~~ through 200 pores/inch sieves. One gram of the wettable powder contains 1×10^9 PIBs. To make an emulsifiable suspension of HaSNPV insecticide, thickening agents, emulsifiers, enhancing agents and UV-absorbing agents are added directly to the virus suspension. Normally, one milliliter of the suspension contained 2×10^9 PIBs. The quality of HaSNPV insecticide was controlled both by counting PIBs with a standardized staining procedure (Sun *et al.*, 1999) and by bioassays. The cost of one kilogram of wettable powder of HaSNPV insecticide is about $3.75. A one litre suspension costs approximately $6.2.

Application of HaSNPV insecticide

From 1988 to 1993, a total of 100 tons HaSNPV products had been produced and a total area of about 70,000 ha had been treated (Zhang, 1994). In 1993, the first HaSNPV pesticide was registered in China. It is estimated that in the last ten years, the annual output of HaSNPV products was about 200 tons.

It has been previously determined that HaSNPV insecticide is best used on eggs or early instars of pest larvae through either high volume spray or ultra low volume spray method. Depending on the size and density of the crop and the stage and density of the pest population,

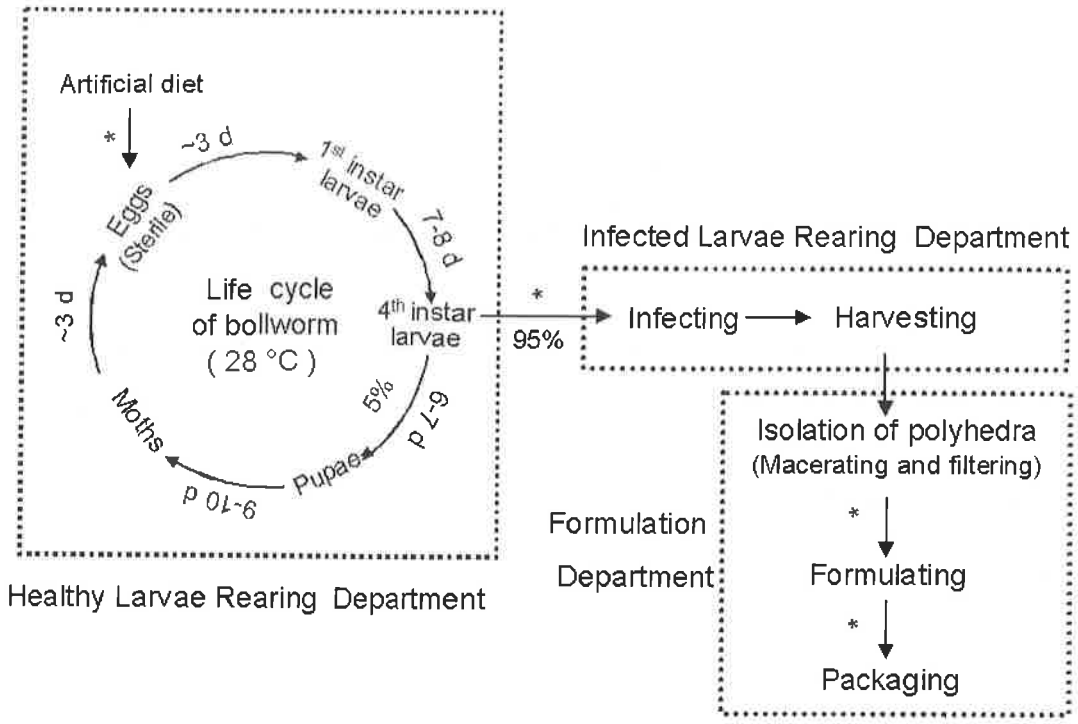

FIG. 1. Procedure of mass production of HaSNPV pesticide
(Steps with * could be done automatically.)

the dosage of virus application is 1.2 ~ 2.4 × 10^{12} PIB/ha, which is 1.2 ~ 2.4 kilogram of wettable powder, or 0.6 ~ 1.2 liter of emulsifiable suspension. In the South China cotton region, the viral pesticide is normally used to control the third and forth generations of *H. armigera*. In the North and Northwest of China, the second, third and fourth generations of the pest are needed to be controlled. Normally 2 ~ 3 sprays are needed to control one natural infestation with 4 ~ 5 days interval. The surviving number of cotton bollworm larvae was reduced by 83.7 ~ 91.7 % when cotton was treated with this spray regime (Zhang *et al.*, 1995).

Construction and evaluation of genetically modified HaSNPVs

HaSNPV pesticides is considered slow acting and requires about 5 days to kill lavae in the field which to a large extent precluded wide scale application of the pesticides. In order to compete with chemical insecticides, approaches are sought to improve the efficacy of HaSNPVs by genetic engineering. Three recombinant HaSNPVs have been generated. One was an ecdysteroid UDP-glucosyltransferase gene (*egt*) deletion mutant (HaCXW1), and the second was a recombinant (HaCXW2) where the *egt* gene was replaced by the *Aa*IT gene (Chen *et al.*, 2000). Recombinant HaCXW1 lacks the coding sequence of *egt* and contains instead GFP gene in the antisense orientation under the control of the polyhedrin promoter. Recombinant HaCXW2 is based on HaCXW1, keeps the GFP gene and contains the AaIT gene under control of a polyhedrin promoter, downstream from the *egt* promoter. The third recombinant HaWHL4A is also based on HaCXW1 and contains the AaIT gene under the control of a chimaeric promoter of the polyhedrin gene and the p6.9 gene (Sun *et al.* 2001).

Laboratory and field evaluation of recombinant HaSNPV

The infectivity and incapacitating speed of the three HaSNPV recombinants were compared with those of wild-type HaSNPV against *H. armigera* larvae by using a droplet bioassay method in laboratory. The LD_{50} values of these recombinants were similar to those of wild-type HaSNPV,

when tested on the 1st-5th instar larvae of *H. armigera*. The LT_{50} and FT_{50} (median time of feeding cessation) values of the recombinants were significantly shorter than those of wild-type HaSNPV (Chen *et al.*, 2000; Sun *et al.*, unpublished data).

Field release experiments indicated that the HaSNPV *egt* deletion recombinant did not show significant improvement as compared to wild-type viruses. However, the number of surviving larvae and the percentage of damaged squares in the plots treated with recombinants carrying AaIT were significantly lower than in plots treated with wild-type HaSNPV (Sun *et al.* 2001 and unpublished data). These results indicated that modification of HaSNPV by expressing an insect-specific toxin can significantly improve the control efficacy of the cotton bollworm and can be useful in controlling this pest in Chinese cotton in the future. Currently, the safety assessments of the recombinant pesticides are carried out.

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Use of engineered baculoviruses as biopesticides: reality and prospects

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Baculoviruses have been the focus of genetic modification to improve their speed of kill for over 10 years. A variety of genes have been inserted into baculoviruses with varying success. Laboratory studies with the most successful recombinants have shown that the time taken to kill (or paralyse) an insect can be reduced by up to 60%: whether further improvements can be made or whether this approach has reached its limit, remains to be seen. However, in controlled field experiments genetically modified baculoviruses have been shown to reduce crop damage and thus undoubtedly have potential for improve crop protection in certain situations. I will review current work on genetically modified baculoviruses, their prospects for use and future research directions. I will also discuss potential difficulties attached to their uptake and the assessment of environmental risks attached to their wide-scale release.

Symposium (Nematodes 1) Entomopathogenic Nematodes: Current Status

Worldwide production and use of entomopathogenic nematodes

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Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) are being produced in many different countries. The main commercial production occurs in Asia, Europe, and North America. Commercial production of entomopathogenic nematodes is carried out in vivo and in vitro. In vivo production is most often done with available insects that can be mass-produced cheaply such as *Galleria mellonella* or *Tenebrio molitor*. In vitro production involves solid culture (i.e., Bedding method) or liquid culture in bioreactors. Each production methodology requires separation of the infective juveniles from the growth medium. The infective juveniles are then formulated in various substrates. Formulations of entomopathogenic nematodes offer unique challenges because of the infective juvenile's demand for oxygen and moisture, sensitivity to temperature extremes, and behavior. Formulations include substrates such as sponge, vermiculite, alginate gel, and water dispersible granules. In some cases, unformulated nematodes are bulk shipped in refrigerated, aerated tanks to their final destination and used. Entomopathogenic nematodes are used primarily against insects that occur in high value crops. Target insects include the black vine weevil in nurseries and landscape, cranberry girdler in cranberry bogs, Diaprepes root weevil in citrus, fungus gnats in nurseries, sciarid flies in mushrooms, Western flower thrips in the greenhouses, hunting billbugs in turfgrass, white grubs in turfgrass, and mole crickets in turfgrass. The proper nematode species must be used against the target insect as all nematode species are not effective against a given target insect.

Entomopathogenic nematode diversity in South America: opportunities for exploration

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Most of our planet's biota consists of arthropods, nematodes, fungi, and other microorganisms. In spite of their abundance, these organisms are the least known or understood in the context of biodiversity and systematics. Indeed, for many groups of species, such as nematodes, what we know for certain is only that we do not know many, perhaps even most, of the species.

Almost 90 years ago, Nathan A. Cobb (1914), the father of Nematology in USA, assertively stated that "so little do we know of this vast multitude of soil-inhabiting nematodes that the first spadeful of earth we lift is practically certain to contain kinds never seen before" and "there exits... a greater disproportion between the known and the unknown than exists in almost any other class of organisms". Unfortunately, our knowledge of nematode diversity has not varied much during the last 90 years. According to Lambshead (1993), between 500,000 and more than 100,000,000 nematode species are believed to exist on Earth and fewer than 25,000 species have been described.

Among invertebrate parasites, hundreds of named nematode species in six orders are known to be associated with insects, with associations ranging from phoresis to obligate parasitism and pathogenesis. However, this probably represents a small fraction of the existing species considering insect diversity and nematode specialization on such hosts. It is very likely that this group of largely uncharted parasites is rich in biological control agents and management tools for medical, veterinary and agricultural practices.

Because of their biological control potential, entomopathogenic nematodes (EPN) *Steinernema* and *Heterorhabditis* and their symbiotic bacteria *Xenorhabus* and *Photorhabdus* spp, respectively, are the most studied group of insect parasites. Moreover, EPN and their symbionts are increasingly viewed as an exciting subject for basic research in ecology, evolution, biochemistry and molecular genetics. Yet, in spite of the progress that has been made during the last 20 years in understanding of the basic biology and genetics of EPN and their symbionts, knowledge on their diversity and geographic distribution is still scarce.

In this presentation, an overview of the present state of affairs in the systematics of the Steinernematidae and Heterorhabditidae will be discussed based on a combined re-examination of morphological attributes and interpretation of their recovered phylogenetic relationships. Additionally, the current status of EPN diversity, with particular reference to research conducted in South America will be presented.

Taxonomic status

When reviewing the history of EPN taxonomy we cannot overlook the contribution that three outstanding South American researchers made to this field. The first one in this list was the Brazilian nematologist, L. Travassos who in 1927 created the genus *Steinernema*. Another Brazilian researcher, C. Pereira (1937) described what probably was the first *Heterorhabditis* species encountered, *H. hambletoni* (=*Rhabditis hambletoni)* from the cotton borer, *Eutinobothrus brasiliensis*. In the 1980's, the late M. M. Doucet, from Argentina, was the first

to initiate studies on EPN and contributed with the description of two steinernematids, *S. rarum* and *S. ritteri* (Doucet, 1986; Doucet, 1990). Moreover, she was the first one in Argentina to promote the study of EPN by offering courses on this topic at the Universidad Nacional de Cordoba.

The most updated taxonomic account indicates that for the family Steinernematidae, there currently are 29 species described within the genus *Steinernema* and there is only one species within the genus *Neosteinernema*. However, these numbers are expected to increase in time. As for the family Heterorhabditidae, which only comprises one genus, *Heterorhabditis*, with 10 species described.

Most recognized EPN species have been described on the basis of phenetic and biological species concepts (revised by Burnell and Stock, 2000). However, with the increasing number of described species, traditional approaches, such as comparative morphology, have become of limited utility in EPN taxonomy. This is mainly due to two factors: 1) the lack of morphological variation, particularly for taxa that are too closely related (i.e. *Heterorhabditis* species), and 2) certain morphological characters are not phylogenetically informative due to homoplasy (caused by convergent or parallel evolution). Application of biological species concept via cross-breeding tests has also been questioned, mainly because of labor and time constrains. Moreover, the discovery of hermaphroditism in steinernematids (see Griffin and Callaghan, 2001), has set a "caution signal" for the consideration of hybridizations assays to test validity of biological species in this group.

Current trends in nematode systematics emphasize that discovery and description of new species require a phylogenetic context for many taxonomic decisions, including reevaluation of previously described taxa and consideration of their relationships. In this respect, *Heterorhabditis* spp. were the first group of nematodes that were delimited based on this evolution-based-approach (see Adams et al., 1998). The application of molecular data provided an independent source of evidence not only to assess validity of named *Heterorhabditis* species but for the discovery of new taxa.

Similarly, Stock et al. (2001) interpreted evolutionary relationships among 21 *Steinernema* species using a combined morphological and molecular approach. Although partially consistent with some traditional expectations and previous phylogenetic studies, the hypotheses inferred from molecular evidence and from combined analysis of morphological and molecular data, provided a new comprehensive framework for evaluating character evolution of steinernematids and assessment of known species and the discovery of new and/or cryptic species.

Richness and distribution of EPN in South America

Hominick et al. (1996) provided a list with the geographic distribution of described EPN species at both continental and national levels. This review and subsequent published studies indicate that EPN are a main component of the soil environment in most geographic regions of the world.

With respect to the Neotropical region, current knowledge of EPN diversity is very limited. Available records indicate that EPN have found in only in seven countries: Argentina, Brazil, Chile, Colombia, Suriname, Uruguay and Venezuela. However, motivation, geographic coverage and sampling design, have been different in all these studies. For example, in a survey conducted in the Pampean Region of Argentina, one of the most important agricultural regions of this country, the goal was to isolate native EPN which could be used to control insect pests of two major crops of this region, corn and alfalfa (Stock, 1995). Therefore, this study focused not

only on the collection of soil samples from several localities were these crops are cultivated, but also considered the capture of local alfalfa and corn insect pests.

Similarly, in the early 1980s, the IFAS at the University of Florida initiated a campaign to identify natural enemies that could control the introduced South American crickets in that region. A few years later, the presence of steinernematid nematodes naturally parasitizing field-collected mole crickets was reported in Uruguay (Lima Costa et al., 1984; Fowler and Garcia, 1988). This isolate was brought to Univ. of Florida and was posteriorly identified as *Steinernema scapterisci*.

As observed in other regions of the world, the available data suggests that in the Neotropical region, the diversity of steinernematids is greater than that of heterorhabditids. This could be true, but could also be an "artifact" due to the fact of difficulties in identifying *Heterorhabditis* spp. Moreover, and based on published evidence that heterorhabditids are more likely to be found in coastal regions, the fact that most surveys have been conducted inland, rather than in coastal areas, could also be an explanation for the prevalence of steinernematids over heterorhabditids.

In terms of species richness, at the national level, Argentina is the country with the most diverse account. With six *Steinernema* and two *Heterorhabditis* species, Argentina is followed by Brazil (3 *Steinernema* and 1 *Heterorhabditis* spp.), Venezuela (2 *Steinernema*, 2 *Heterorhabditis* spp.), Colombia (2 *Steinernema*, 1 *Heterorhabditis* spp.), Chile (2 *Steinernema* spp), Uruguay (one *Steinernema* species) and Suriname (one unidentified *Heterorhabditis* sp.). Of all these taxa, only four species, *S. rarum, S. ritteri, S. scapterisci* and *H. argentinensis* appear to be either endemic to the countries where they were originally isolated or have a wider but still restricted geographic distribution.

In terms of habitat preference, very little information is currently available. For instance, in the Argentine survey, natural permanent pastures adjacent to corn and alfalfa fields were also considered for sampling. These pastures were the most diverse in terms of number of species recovered (Stock, 1995). There are also reports of the presence of a new undescribed *Steinernema* species (isolate Qu-N820) from Puerto Cisne, Chile that was also isolated from natural pastures (France, pers. comm.).

These results agree with studies conducted in other parts of the world where it has been demonstrated that natural or less disturbed habitats are more likely to host greater diversity of species than human impacted areas. However, it is difficult to make any general statements for any of the EPN species reported in South America. Most surveys have been conducted in agricultural areas, targeting a specific crop, and with a limited geographic and habitat coverage, therefore no trends can be defined at this time.

Like in other survey studies, it is important to bear in mind that in all these correlations and habitat associations, it is prevalence what is being assessed, and concluding statements should be made cautiously. Consideration of measurement of population size and knowledge on the spatial distribution of these nematodes is should be necessary for a much more critical assessment.

South American EPN and their natural host range

Peters (1996) reviewed the natural host range of EPN at a global scale. At that time he indicated that information on this subject was rare. This is mainly due to fact that in most cases, EPN have been recovered using insect baits (mainly *Galleria mellonella*), rather than the

recovery of naturally infested hosts. This has also been the situation in South America, and the current knowledge of the natural host range of EPN in this region is very limited.

From a total of eleven EPN species reported, information on natural hosts is only available for seven of these species. For instance, the most updated records indicate that *S. carpocapsae* has been found to infect Coleoptera (Scarabaeidae and Curculionidae) and Lepidoptera, mainly Noctuidae. The Argentine strains of *S. carpocapsae* seem to be adapted to parasitize endemic scarab species such as *Cyclocephala signaticolis* and *Diloboderus abderus*. Fowler and Garcia (1988) also reported the natural parasitism of *S. carpocapsae* to the mole cricket, *Scapteriscus borellii* (Orthoptera, Gryllotalpidae) in Brazil. However, subsequent studies revealed that the identification of this nematode was incorrect, and that the reported steinernematid was *S. scapterisci,* another neotropical species, which natural host range is limited to Orthoptera, mainly Gryllotalpidae. This species is considered to be very specific to mole crickets of the genus *Scapteriscus*.

The neotropical isolates of *S. feltiae* have been found associated mainly to Coleoptera, Scarabaeidae. In addition to this, in a recent study conducted in the region of Cundinamarca, Colombia, *S. feltiae* has also been associated to the potato borer *Tecia solanivora* (Lepidoptera: Gelechiidae) (J. C. Parada, pers. comm.). *Steinernema glaseri* has, so far, only been found infecting larvae of *Migodulus fryanus* (Coleoptera, Cerambycidae) from Santa Rosa, Brazil (Poinar, 1990). Although this species has also been reported in Argentina, at present, there are no other natural-host records available. As for the Argentine *S. rarum*, this species was originally isolated from *Heliothis* sp (Lepidoptera, Noctuidae). Although several other strains have been collected in Argentina, no further records of naturally occurring hosts have been reported.

Heterorhabiditis bacteriophora was found associated to Hemiptera Cynidae, specifically *Cyrtomenus bergi* in cassava plantations in Colombia (Caicedo and Belloti, 1996). However the correct identification of this species is currently being reevaluated (Stock et al, unpublished). *Heterorhabditis argentinensis* has, so far, been found parasitizing *Graphognathus leucoloma* larvae (Coleoptera, Curculionidae).

Considerations for future documentation and interpretation of EPN diversity in South America

The study of EPN is still an unexplored territory in South America. So far, only a few autoctonous species have been found. This is likely a minimal representation of the overall diversity of EPN that are waiting to be discovered in this continent. Because of the great diversity of geographic regions, habitats, climates, and insect diversity in the Neotropics, it is reasonable to consider that there is also high diversity of EPN species. Indigenous species will undoubtedly be more efficacious in controlling native insect pests, and will also be better adapted to local environmental conditions. Thus, systematic surveys with appropriate sampling designs are urgently needed.

In contrast to human-modified areas, natural habitats are more likely uncontaminated by introduced species, and will offer a better chance for finding native species. Collection of data from these surveys, and the organization of this information in a form that best serves our needs, will imply a large amount of effort. Therefore, collaborative research efforts at the national and international levels should be sought to promote success and promptness of results.

It is obvious that accurate identification of EPN species/isolates is the "key" to correct interpretation of any future ecological or biological studies and will also have implications for

the successful implementation of EPN as biological control agents. In this respect, new technologies such as the application of molecular approaches (in combination with classical morphology) and phylogenetic interpretation are highly recommended.

Finally, the numerous attributes that EPN and their symbionts harbor not only as biological control agents, but also as bioprospecting sources, make them valuable organisms in both basic and applied biological disciplines. South America, as part of one of the Earth's biologically diverse hotspots, not only has an incredible pool of organisms to discover (including EPN), but also has the capability and enthusiasm of its scientific community... these two valuable resources should not be wasted!

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Development of entomopathogenic nematodes as a management tactic for citrus root weevils in Florida

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Several species of polyphagous root weevils, particularly *Diaprepes abbreviatus* L. and *Pachnaeus* spp., are important pests of citrus, ornamentals, and some agronomic crops in Florida (McCoy, 1999). Larval feeding injury to the roots by *D. abbreviatus* can have a devastating effect on citrus trees since all stages feed on the roots for most of the year. Root injury appears to be cumulative, and more importantly, feeding sites can serve as infection courts for root rot diseases (Graham *et al.*, 1996), thereby exacerbating economic loss. Tree decline can be particularly severe in poorly drained groves when flooding water stress affects root health. There is no estimate of the total economic loss to the growers from larval root injury to citrus, but the end result can frequently be tree death.

A number of citrus pests are susceptible to entomopathogenic nematodes that are potential microbial control agents against citrus root weevils (Shapiro *et al.*, 2002, McCoy *et al.*, 2000b). This paper presents a case history on the microbial control of larvae of *D. abbreviatus* in soil with nematodes.

Naturally occurring nematode species within the genera *Heterorhabditis* and *Steinernema* have been found in citrus groves throughout Florida infecting 38-68% of caged *D. abbreviatus* larvae in the deep sandy soils of the central ridge and the sandy clay loam soils of the coastal and interior flatwoods (Beavers *et al.*, 1983; McCoy *et al.*, 2000a). *Steinernema carpocapsae* was the first nematode shown to be pathogenic to *D. abbreviatus* (Lamond *et al.*, 1979) and to be developed commercially for citrus root weevil control (Smith, 1994). Inundative release of mass_produced entomopathogenic nematodes has been pursued by private industry for about 15 years (Duncan *et al.*, 1999; Schroeder, 1987) because the density and distribution of endemic nematode populations vary between groves, within groves and within seasons. Four nematode species have been sold in Florida to control *D. abbreviatus* in citrus: *Heterorhabditis bacteriophora* Poinar; *Heterorhabditis indica* Poinar, Karunaker and David; *S. carpocapsae* (Weiser); and *Steinernema riobrave* (= *riobravis*) Cabanillas, Poinar and Raulston (Shapiro and McCoy, 2000a). Currently, *H. indica* (Grubstake™ 100, Integrated BioControl Systems, Aurora, IN) and *S. riobrave* (Bio Vector 355, Certis Corporation, Columbia, MD) are sold commercially for use on Florida citrus.

The label rate for Bio Vector 355 is 4.9×10^8 viable infective juveniles (IJs)/treated hectare at 250 trees/grove hectare, that is, 2,000,000 IJs/tree (Knapp, 2000). The label rate for Grubstake™ 100 is one-half the Bio-Vector rate. These field rates can be highly variable since the area of soil treated per hectare can change depending on tree size and method of application. To circumvent this problem, private industry suggests that growers apply Grubstake™ 100 at 11 IJs/cm^2 and Bio Vector 355 at twice that rate. To date, no published field research has shown that these rates are effective against *Diaprepes*. Field trials by Bullock and Miller (1994), Bullock *et al.* (1999), and Schroeder (1990), showed that rates of 2-5 m Miller (1994), Bullock *et al.* (1999), and Schroeder (1990), showed that rates of 2-5 million IJs of *S. carpocapsae* or *S. riobrave* per tree applied within a ~0.3 m^2 area surrounding the base of the tree in the spring significantly reduced adult emergence of both *D. abbreviatus* and *Pachnaeus*

litus (Germar). In addition, field trials in groves on the central ridge using *S. riobrave* and *H. bacteriophora* showed that rates of 120-250 IJs/cm^2 reduced larval populations significantly within 4 weeks post-treatment (Downing *et al.*, 1991; Duncan *et al.*, 1996; Duncan and McCoy, 1996). In three separate trials, McCoy *et al.* (2000a) showed that nematode parasitism by either *S. riobrave* or *H. indica* at 22 IJs/cm^2 or less was no different than parasitism in the untreated control in a flatwoods grove. In fact, 108-216 IJs/cm^2 of *S. riobrave* were required to increase parasitism to 40-60%.

Although most published data cited above explicitly show that higher rates of entomopathogenic nematodes result in (i) higher parasitism, (ii) greater suppression of larvae in the soil, and (iii) reduced adult emergence from the soil, data also suggest that efficacy is influenced by other unknown factors relating to the nematode, host and/or environment (Kaya and Gaugler, 1993). For example, *S. carpocapsae* at rates greater than 100 IJs/cm^2 gave no control in central ridge and coastal flatwoods groves (Adair, 1994; Bullock *et al.*, 1999; Duncan *et al.*, 1996), suggesting that the nematode failed to find the host due to poor searching ability (Schroeder and Beavers, 1987). Laboratory studies have shown that *S. riobrave* was more effective at warmer soil temperatures, (Shapiro and McCoy, 2000c) and host age also affected susceptibility of *D. abbreviatus* larvae to the nematode (Shapiro and McCoy 2000b; Shapiro *et al.*, 1999). In addition, soil type affected virulence and persistence of *S. riobrave* and *H. bacteriophora* (Shapiro *et al.*, 2000), whereas culture and formulation method had no effect on larval mortality for *S. riobrave* (Shapiro and McCoy, 2000a).

Recent field and microcosm experiments designed to determine the effect of soils of different composition and texture on nematode efficacy, strongly suggested that some field failures were soil-related (Duncan *et al.*, 2001; McCoy *et al.*, 2002). When soil (entisol type) from a deep sandy ridge grove with a percent sand:silt:clay ratio of 97.6:1.5:0.9 was compared to sandy clay loam soil (alfisol type) from an experimental site with a ratio of 68.8:11.8:19.4, *S. riobrave* applied at 20 IJs/cm^2 killed 70-80% of the larvae of *D. abbreviatus* buried to a depth of 30 cm in the sandy soil, but only 4_17% of the larvae in sandy clay loam soil. Furthermore, larval mortality of *D. abbreviatus* was correlated positively with the proportion of sand in the soils, but was related inversely to the percentage of fine sand. The strongest correlation with efficacy was with percentage of medium and coarse sand in soils (Duncan *et al.*, 2001). Both nematode emergence from the insect cadaver and recycling in cadavers favored coarse sandy soils. This suggested that soil with finer texture reduced host contact or affected the infection process.

This marked difference in nematode efficacy between sandy and sandy clay loam soils was suggested in the published literature. For example, in two groves on the central ridge with a deep sandy soil (entisol type), *S. riobrave* applied at 108 IJs/cm^2 reduced larval populations by 75-90% after three weeks (Duncan and McCoy, 1996; Duncan *et al.*, 1996). Both tests were evaluated in the same manner as these trials using the tree removal/soil sieve methodology. In another series of field trials conducted in a flatwoods grove near Ft. Pierce with pineda sandy clay loam soil, larval parasitism by *S. riobrave* ranged from 40-45% when rates of 54-108 IJs/cm^2 were applied, suggesting soil-related inhibition (McCoy *et al.*, 2000a).

Shapiro *et al.* (2000) measured virulence and persistence of *S. riobrave* and *H. bacteriophora* in marl (high silt + clay), ridge (entisol, sandy), and coastal flatwoods (spodosol, sandy clay loam) soils in the laboratory. Although both nematode species were virulent to *D. abbreviatus* larvae in all soils, both virulence and persistence were greater in the heavier marl soil and virulence was greater in spodosol compared to entisol soil. These laboratory data appear contradictory to field results, however, physical properties of the soil were not a factor in the

laboratory. Shapiro *et al.* (2000) suggest that the chemical composition of the soils was not a deterrent to parasitism but physical properties of the soil, such as structure, compaction, etc. might contribute to the variation in field efficacy.

The importance of soil texture in relationship to soil compaction as determined by Duncan *et al.* (2001) was supported by our field observations on soil compaction within the tree rhizosphere at the time of tree removal. Soil surrounding the roots was very fine in texture resulting in extreme compaction on the roots and within the rhizosphere. Soil was so compact within the root crown of the tree, it was virtually impossible to remove with a probe. When larvae adjoining the roots were removed with a probe, invariably they were healthy suggesting the soil was so compact that nematode penetration of the soil was infrequent.

The results of these field studies supported by the studies of Duncan *et al.* (2001) pose important implications relating to the biological control of larvae of *D. abbreviatus* with entomopathogenic nematodes in Florida citrus. Foremost, soil characteristics appear to be important determinants of field efficacy. Current nematode products appear most efficacious in deep course sandy soils common to the central ridge of Florida; however, efficacy is reduced substantially by different soils, particularly the sandy clay loams. Optimal rate appears variable and is likely influenced by host age and edaphic factors. Finally, further research is warranted on nematode species selection and the dynamics of edaphic conditions in relationship to field performance.

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Advances in the use of entomopathogenic nematodes for the management of scarab pests

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White grubs, the root-feeding larvae of scarab beetles, cause significant damage to many agricultural and horticultural plants around the world. In the USA, larvae of the introduced Japanese beetle, *Popillia japonica*, are a major pest of turfgrass and ornamentals throughout much of the eastern states, and native masked chafers, *Cyclocephala* spp., larvae are major turfgrass pests and pests in ornamentals throughout the Midwest and western states (Potter 1998). In the Northeast and along the eastern seaboard of the USA, other species, especially the oriental beetle, *Exomala* (=*Anomala*) *orientalis*, the European chafer, *Rhizotrogus majalis*, and the Asiatic garden beetle, *Maladera castanea*, have become similarly or more important as turfgrass and ornamental pests as the Japanese beetle. Other crops that can be damaged by white grubs in the USA include vegetables (Asiatic garden beetle, May/June beetles), field, forage, and nursery crop (oriental beetle, European chafer, May/June beetles), and small fruit (oriental beetle, Japanese beetle). Introduced species, especially oriental and Japanese beetle, are also quarantine pests because they can easily be spread in potted plants and nursery stock.

Entomopathogenic nematodes (EPN) offer an environmentally safe alternative to chemical insecticides in the management of white grubs. EPN efficacy in the field, however, has been variable. Improper handling of these living control agents may cause some of this variability, and only persistent education efforts by extension personnel and industry may alleviate this limitation. EPN are also more susceptible than chemical insecticides to environmental factors that can affect their infectivity and persistence. These factors include abiotic factors such as extreme temperature and moisture conditions and biotic factors such as natural enemies that prey on or parasitize nematodes. A better understanding of these factors will enable us to optimize the use of EPN in pest management.

Here we will present and discuss other approaches that we have pursued to improve the efficacy of EPN for white grubs including the use of EPN synergists, understanding the effect of white grub stage on nematode efficacy, and developing new nematode species for more effective white grub control.

Nematode synergists

Previous studies have shown that combinations of EPN with the milky disease bacterium, *Paenibacillus* (=*Bacillus*) *popilliae* (Thurston et al. 1994), or the scarab specific Buibui strain of *Bacillus thuringiensis* ssp. *japonensis* (Koppenhöfer and Kaya 1997, Koppenhöfer et al. 1999) can have a synergistic effect on white grub mortality. However, the combination of EPN and milky disease is feasible only for long-term control in areas that can tolerate some damage whereas the combination of EPN and *Bt* Buibui is feasible only for white grub species that are sufficiently susceptible to this bacterium.

A more efficient combination with wider applicability may be EPN and the neonicotinoid insecticide imidacloprid (Koppenhöfer and Kaya 1998; Koppenhöfer et al. 2000a). Imidacloprid, a broad-spectrum neonicotinoid (chloronicotinyl) insecticide (Yamamoto and Casida 1999),

with relatively low vertebrate toxicity, low application rates, long residual activity, and a relatively small effect on beneficial invertebrates (Kunkel et al. 1999, 2001), is one of the most widely used agents for white grub control. Because its efficacy declines with advancing white grub development, it has to be applied preventatively, preferably before the presence of the first instar. However, white grub outbreaks are difficult to predict because they are localized and usually sporadic and because of the difficulty in sampling for white grubs in general and their eggs and first instars in particular. Therefore, the preventative approach involves treating large turf areas that otherwise may need only partial or no treatment at all. Curative spot applications against the easier to detect later white grub stages using the synergistic combination of imidacloprid and EPN could reduce cost and environmental impact, and offer an alternative to the more hazardous organophosphate and carbamate insecticides that are also threatened by the implementation of the Food Quality Protection Act of 1996 (Anonymous 1996).

In a series of greenhouse and field experiments, we tested the effect of application timing, imidacloprid rate, white grub species, and EPN species on the efficacy of imidacloprid-EPN combination for white grub control. We showed that imidacloprid interacted synergistically with the EPN *Heterorhabditis bacteriophora*, *H. marelatus*, *H. megidis*, and *Steinernema glaseri* in 3rd instars of the Japanese beetle, the oriental beetle, and the masked chafers, *Cyclocephala borealis*, *C. pasadenae*, and *C. hirta*. The degree of interaction varied with EPN species. The strongest and most consistent synergism occurred between imidacloprid and *S. glaseri*. Synergism between imidacloprid and *H. bacteriophora* was weaker and less consistent. Combinations of imidacloprid and *Steinernema kushidai* only resulted in additive white grub mortality. We observed synergistic interaction whether EPN were applied at the same time as imidacloprid or 14 days later. Although our study suggested that the synergistic interaction could occur at imidacloprid rates as low as 10 – 25% of the recommended field rate, the extent to which the chemical can be reduced seems to depend on a number of conditions including timing of application (earlier better) and white grub species.

We have expanded our observations on EPN-neonicotinoid interactions to include thiamethoxam (Koppenhöfer et al. 2002), a new neonicotinoid with very similar characteristics as imidacloprid that belongs to the subgroup of thianicotinyls. In a series of greenhouse experiments, we observed only occasional synergism between thiamethoxam and various *Heterorhabditis* species in oriental beetle 3rd instar larvae; only *S. glaseri* showed strong synergism with thiamethoxam. Imidacloprid showed consistent synergism with all EPN species in the same experiments. In a field experiment with oriental beetle 3rd instars, we also observed synergism between *H. bacteriophora* and imidacloprid and no synergism between thiamethoxam and this EPN.

We have also studied the mechanism responsible for the interaction between the imidacloprid and EPN against white (Koppenhöfer et al. 2000b). Exposure to imidacloprid facilitates EPN host attachment by inducing sluggishness and reducing defensive behaviors in the grubs. Because imidacloprid does not appear to compromise EPN infectivity and recycling in grubs (Koppenhöfer et al. unpublished), this combination is not only promising for curative white grub control but could also play a role in augmentative and inoculative approaches to white grub management, especially of scarab species that are not very susceptible to EPN.

Interactions between epn species and white grub species/developmental stage

Some of the variability in EPN efficacy may be attributable to limited knowledge about the effect of EPN species, white grub species, and white grub larval stages on EPN efficacy against white grubs. In laboratory (30-ml creamer cups filled with soil and grass) and greenhouse (1-Liter pots with soil and grass) studies we have compared the efficacy of different scarab-pathogenic *Steinernema* and *Heterorhabditis* spp. against the 3rd instar of 5 white grub species.

Generally, the Japanese beetle was the most susceptible species. The northern masked chafer, *C. borealis*, showed an intermediated level of susceptibility, whereas oriental beetle, European chafer, and Asiatic garden beetle were resistant to infection by all but one nematode species. *Steinernema* spec. (a putative new species isolated from Japanese and oriental beetle larvae in New Jersey, USA) was highly pathogenic to Japanese beetle, oriental beetle, European chafer, and Asiatic garden beetle, but its performance against the northern masked chafer was not significantly better than that of the other nematode spp. However, in microplot field trials (at 2.5 x 10^9 nematodes/ha and 21 DAT), *Steinernema* sp. provided good to excellent (71-100%) control of all 5 white grub species. On the other hand, *H. bacteriophora* provided excellent control only against the Japanese beetle (90%) but no to mediocre (10-50%) control of the other white grub species.

We are investigating the effect of white grub (primarily oriental and Japanese beetle) developmental stage on the efficacy of *Steinernema* sp. and *H. bacteriophora* under laboratory conditions. In oriental beetle, 2nd instars were more susceptible to *H. bacteriophora* than 3rd instars, but there was no difference for *Steinernema* spp. Similarly, small (< 100 mg) 3rd-instar oriental beetle were more susceptible to *H. bacteriophora* than large 3rd instars (> 175 mg), but there was no difference for *Steinernema* spp. Our observations also indicate that oriental beetle 3rd instars that have emptied their intestines in preparation for pupation as well as prepupae and pupae have low levels of susceptibility to *H. bacteriophora* and *Steinernema* sp. We are presently investigating whether the same trends occur in Japanese beetle as well. A recent study by Lacey et al. (2001) suggested that Japanese beetle pupae are more susceptible to EPN than 3rd instars.

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Entomopathogenic nematodes: research and implementation in South America countries

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Introduction

In the last 20 years studies on entomopathogenic nematodes (EPN) of the families Steinernematidae Chitwood & Chitwood, 1937 and Heterorhabdtidae Poinar, 1976 have received special attention from researchers throughout the world. Description of new species, genetics, biology, ecology, and effectiveness for the control of many insect pests have been emphasized.

In South America countries interest on these nematodes begun when Pereira (1937), in Brazil, reported finding an EPN species then described as *Rhabditis hambletoni* and later on transferred by Poinar (1990) to *Heterorhabditis hambletoni.* However, only in the seventies, work on EPN has resumed. Research has been focusing on surveys, description and characterization of native species and strains of species described elsewhere, concerning their biology, host range and potential against local insect pests.

An overview on entomopathogenic nematodes studies and use in South America is presented. Comments and suggestions on research lines are also presented.

Natural occurrence and description of new species

Entomopathogenic nematodes have been recovered in Argentina, Brazil, Colombia, Uruguay, and Venezuela. Few systematic surveys have been done, yet new and known species have been isolated.

In Argentina three new species were described: Steinernema. rarum (Doucet, 1986) Mamiya, 1988; S. ritteri Doucet and Doucet, 1990; and Heterorhabditis argentinensis Stock, 1993a. Other species recovered from the country were S. feltiae (Stock, 1993b, 1995); H. bacteriophora (Doucet and Bertolotti, 1996; Doucet et al., 1996; Stock, 1995); S. scapterisci (Stock, 1992, 1995); S. carpocapsae (Doucet, 1995; Stock, 1995).

In Brazil there is only one species described, *Heterorhabditis* (*Rhabditis*) *hambletoni* (Pereira, 1937) (Poinar, 1990). Other species recovered from soil are the following: *Steinernema (Neoaplectna) glaseri* (Pizano et al., 1985); *S. (Neoaplectana). carpocapsae* and *Heterorhabditis* sp (Fowler, 1988); S. carpocapsae (Schmitt, 1993). New EPN isolates belonging to the genera *Steinernema* and *Heterorhabditis* have been obtained from different states in Brazil and are presently being identified to species level (M. M. Aguillera, unpublished).

In Colombia, *Steinernema* sp was recovered from soil by Garzon et al., (1996) and *S. feltiae* by Saenz (1999). A soil survey revealed the presence of *Heterorhabditis bacteriophora* in the country (Caicedo and Bellotti, 1996).

In Uruguay, the species *S. scapterisci* was isolated and described by Nguyen and Smart (1990). The nematode was successfully introduced into the United States of America to control mole crickets (*Scapteriscus* spp).

In Venezuela, a survey carried by Rosales and Suarez (1998) led to recovery of *Heterorhabdtis* spp. Fan-Xue et al., (2000) also obtained a native strain of *Heterorhabditis* that has shown good control of *Tecia solanivora*.

Biology and Ecology

Life cycle of EPN from Argentina was studied by Doucet and Poinar (1985); Doucet et al., 1996. Nematode efficiency as related to temperature besides other parameters was determined by Doucet et al., 1996 (10). *Steinernema scapterisci*, species initially considered to be host specific to Orthoptera, in laboratory tests this nematode species has been shown to be also infective to and reproduce in some insects of the Orders Coleoptera, Diptera and Lepidoptera. Temperture requirements for the nematode invasion, establishment, infectivity and survival in storage were higher than those presented by *S. carpocapsae* which according to the authors indicate its probable adaptation to warm climates (Grewal et al. 1993). In order to add information about the species potential for exploitation *Steinernema rarum* had its physiological host range, host-foraging behavior, thermal activity range and soil moisture activity range determined by Koppenhöfer and Kaya (1999).

Other research lines

Host range of species isolated in South America has been tested against important insect pests in Argentina, Brazil, Chile, Colombia., and Venezuela. Efficiency tests of native and exotic EPN have been done with insects in a number of Orders, but especially with Coleoptera, Lepidoptera, Orhtoptera, Hemiptera and Homoptera.

Entomopathogenic nematodes mass rearing was achieved in Colombia and Brazil on *Diatraea saccharalis* and *Spodoptera frugiperda*. No attempt to rear EPN on culture medium has been reported.

Techniques for EPN isolation and multiplication have been developed in Argentina. In Brazil, the use of baiting for applying EPN in the field has been proposed.

Detailed reviews on EPN research in Argentina and Brazil was presented by Doucet and Doucet, 1996 and Grewal et al. 2001.

Final remarks

Research on EPN has been implemented little by little in some South America countries: Argentina, Brazil, Chile, Colombia, Uruguay and Venezuela. At this point there is a strong need for knowing the occurrence and distribution of steinernematids and heterorhabdits and exploiting them according to their potential for controlling local key pests. Systematic surveys as well as new research lines such as molecular approaches for identification and characterization of EPN species and populations are necessary. Training on EPN systematics is strongly urged.

Broader host range studies in the laboratory and field should be stressed if these biological control agents are to be properly and extensively exploited in those countries. Key pests which are possible targets for control by EPN were suggested by Nardo et al. (2001). Methodology for host range studies must be standardized in order to allowing comparison of effectiveness of the same species or strains. Parallel studies on environmental impact of native and exotic species are also necessary.

As it was emphasized by Koppenhöfer and Kaya (1999), new species should be also studied under an ecological point of view. This way not only the host range of native species should

be determined but also the nematode foraging behavior in relation to the host behavior, thermal and soil moisture activity ranges. These studies would lead to a selection of adapted and the most promising species or strains that should be further investigated

Knowledge about native EPN and their requirements can provide the chance of manipulating the environment to benefit natural populations and thus favor augmentative biological control. This way, the lag of exploitation of these biological control agents behind some other countries that have been successfully investing on the nematodes mass production, could be at least partially overcome. As nematode production is a desirable strategy to allow EPN application especially through inundative releases, production on culture media is strongly emphasized.

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Entomopathogenic nematodes: Research and implementation in Mexico and Central America countries

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Introduction

Entomopathogenic nematodes (EPN) are seen as promising control agents for insects pests in cryptic, moist environments such as soil and inside of vegetal tissues (Kaya and Gaulger; Georgis and Manweiler, 1994). Some of the main limitations for widespread use, however, are lack of consistency in the field (Franceschini, 2000, Alatorre-Rosas, 1999) and suitable mass production and formulation methods (Fridlender, 2000, Burges, 1998).

Several efforts to work with EPN in Latin American countries have been documented. The available reports are summarized as follows.

Surveys for entomopathogenic nematodes

In a survey carried out in Southern Mexico during 1998 and 1999, a total of 446 soil samples were collected in eight natural regions of Oaxaca State. The largest percentage of positive samples was obtained in medium textured soils, with year round adequate soil moisture, including irrigated zones. The promising isolates were identified as *Steinernema feltiae* Filepjev, *Steinernema* sp., and *Heterorhabditis* sp. (Ruiz *et al.*, 1998). It was recommended to carry out more surveys in the future, especially in temperate and irrigated areas.

The FCBA (Facultad de Ciencias Biológicas y Agronómicas), Universidad de Colima, has Strains of *Steinernema* spp. and *Heterorhabditis* spp isolated in the states of Colima, Jalisco and Michoacán (Lezama_Gutiérrez *et al.*, 2001), but the species have not been identified. In northeast Tamaulipas and southern Chihuahua, *Steinernema* sp and *S. carpocapsae* Mexica have been reported (Raulston *et al.*, 1992; Poinar, 1990). It is likely that the unknown *Steinernema* species was *S. riobravis* as Cabanillas and Turston (1994) isolated it just across the river.

Colegio de Posgraduados, Montecillo, Mex., has a collection of introduced EPN, but they have an ongoing subproject to find native EPN within a national project to control rhizofagous insects.

Since two years ago, CIBCM (Centro de Investigación en Biología Celular y Molecular), Universidad de Costa Rica has carried out surveys of EPN in protected zones and evaluations of their efficacy against insect pests in Costa Rica (Uribe-Lorío *et al.*, 2002). The genera *Steinernema* and *Heterorhabditis* have been found.

Mauleon *et al.* (1996) have reported the presence of the genus *Heterorhabditis* in Cuba, Brasil, Argentina, Guadalupe and República Dominicana. They also report the presence of *Heterorhabditis indicus* Poinar HV3 and HV6 strains in Cuba, Guadalupe, Jamaica, Puerto Rico, República Dominicana and Trinidad.

Bioassays for efficacy

Promising isolates of EPN collected in Oaxaca, México and other nematodes were bioassayed against 2nd to 3rd instar white grubs *Phyllophaga* spp. Harris to determine lethal dosages and times. Lethal mean dosages ranged from 87 to 105 nematodes per white grub for *Steinernema carpocapsae* Weiser and *S. glaseri* Steiner, to 146-263 nematodes per larva for the promising isolates *Steinernema feltiae* Filepjev, *Steinernema* sp., and *Heterorhabditis* sp. (Ruiz *et al.*, 1998).

Six EPN were evaluated against the fall armyworm *Spodoptera frugiperda* Smith. The most effective nematodes were *S. carpocapsae* and *S. riobravis*, reaching 73-79 % control in 72 hours. (Molina Ochoa *et al.*, 1996). LC_{50}'s were 1.5-20.6 nematodes / larva and 3.4 to 37.2 nematodes / prepupa. The less virulent species were *H. bacteriophora* against larvae and *S. feltiae* against prepupae.

Integrated control of the fall armyworm has been evaluated. Silks of "zapalote chico", a creole maize variety resistant to this pest, were added to the diet used to rear the larvae and then combinations of nematode dosages and rearing diets were tested. Both *S. carpocapsae* and *S. riobravis* nematodes were most effective at lower concentrations when combined with a silk supplemented diet (Molina-Ochoa *et al.*, 1999).

No synergistic effects were found with a combination of *N. rileyi* and *H. bacteriophora*, but when they were applied at the same time its effectiveness against fall armyworm larvae decreased (Lezama-Gutiérrez *et al.*, 1996). Earlier instar larvae were more susceptible, but pupae were not affected.

To evaluate the degree of control with local and introduced entomopathogenic nematodes and fungi on white grubs, laboratory experiments were conducted during 1999 in Oaxaca, México. The most effective nematodes were *Heterorhabditis* sp from Mexico and *Heterorhabditis bacteriophora* NC strain from USA, both giving 75 % control. When either fungus *Beauveria bassiana* or *Metarhyzium anisopliae* was combined with nematodes the LT_{50} was reduced in two days, especially with the combination *M. anisopliae* + *Heterorhabditis* sp. (Ruiz *et al.*, 2000).

CINVESTAV-Irapuato, in collaboration with ECOSUR, has initiated work on the control of fruit flies (*Anastrepha obliqua*) with *S. feltiae.* Earlier larval instars were more susceptible to the nematode and clayey soils appeared to increase the control percentage, but a higher water retention capacity in these soils may have increased parasitism. The optimum soil temperature for maximum parasitism was 26-27°C (Toledo *et al.*, 2000). Lezama *et al.* (1996) had previously found that larvae and pupae of *Anastrepha ludens* were highly susceptible to *S. riobravis* and *S. carpocapsae*.

H. bacteriophora (NC) was effective against larvae and prepupae of the grass looper *Mocis latipes* (González-Ramírez, 2000). Third and fourth instars showed the lowest LC_{50}'s.

Martínez-Tomas *et al.* (1998) applied pre-parasitic *Romanomermis iyengari* nematodes to *Anopheles pseudopunctipennis* larvae and found that the earlier instars were more susceptible, with a maximum penetration of 12-13 nematodes per each larva. A 100 % parasitism was reported when 1000 preparasitic nematodes were applied per square meter.

Field evaluations

Under semi-controlled conditions (using 5 liter plastic bags), entomopathogenic fungi alone produced the largest vegetative development in potted maize plants. However, the percentage of white grub control increased when the *Heterorhabditis* sp isolate was used either with *B.*

bassiana or *M. anisopliae*. It was concluded that combining fungi and nematodes, especially *Heterorhabditis*, increases their efficacy against white grubs (Ruiz *et al.*, 2000).

Under field conditions, in Oaxaca, México, maize yields were increased by 17 % in relation to the check when *S. carpocapsae* + *M. anisopliae* were applied, but using Diazinon increased grain yields by 25 % (Ruiz, 2002). In order to make the biological control alternative more attractive, better results are required.

A recent work in Guatemala in broccoli fields with non-specified species of *Steinernema* and *Diplogasteritus* nematodes against white grubs reports non-significant differences between these nematodes and the check treatment (Orellana *et al.* 1998). Estimated crop damage in two localities ranged from 21 to 66 %, obtaining the higher yields with imidacloprid.

Perez-Pacheco *et al.* (1998) applied *Romanomermis iyengari* to *Anopheles pseudopunctipennis* in ponds at three localities and found 73-100 % parasitism. This nematode is being currently evaluated in a largest scale in Tampico, Mexico.

According to Federici (2000), special expertise and methods are required to evaluate the performance and efficacy of entomopathogenic control agents in the laboratory, but this should be applied to field evaluations also, where a standard methodology is not practiced. Besides, there are not many field studies for the evaluation of EPN effectiveness carried out in LA.

Mass production and formulation

In vivo mass production of *S. feltiae* and *Heterorhabditis.* sp., is carried out in the CIICA (Centro Internacional de Investigación y Capacitación Agropecuaria), Tapachula, Chis., but there is little demand for the bioinsecticide. They are sold embedded in a polyurethane sponge, requiring refrigeration.

At CINVESTAV-Zacatenco liquid fermentation has been evaluated for production of *S. feltiae.* The requirements of oxygen (Suarez, 2002) and effects of the hydrodynamic conditions (Chavarria,. 2001) have been determined.

Conclusions

Given the high biodiversity of this area, it is advisable to carry out more surveys for native EPN, but standardized bioassays and systematic field studies are required to increase our understanding of the ecology of EPN and host-EPN relationships.

Mass production and formulation, as well as erratic results, remain as the main limiting factors for widespread use of EPN. Therefore, local efforts to adapt low cost production and effective, virulence-enhancing formulation methods, should be encouraged. Also, more striking field results are required to convince the farmers to adopt this technology.

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Workshop
The Future of Scientific Publications

The future of scientific publications: introduction and the scientific society's viewpoint

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There is no doubt that, with the advent of modern electronic technology, the future of scientific publications is at a crossroads. Ever since the advent of modern science, scientists, scientific societies, librarians, publishers and administrators have grappled with the dilemma of bringing scientific information to the public and scientists alike, in the most efficient, timely and economic manner possible. The importance of scientific publications cannot be overemphasized; a scientific study remains virtually worthless unless it is published and made available to others to peruse and build upon. The scientific paper has been and remains the backbone of scientific progress. And the scientific publication has been used as the yardstick of a scientist's productivity and worth. The saying "publish or perish" holds true to this day.

Traditionally, scientific societies established and published their own journals, in the interest of ensuring publication of articles in their own scientific area. But the ever increasing number of scientific publications meant an ever increasing number of scientific journals and books and an escalating cost of publishing. Scientific societies have grappled with this dilemma for years. Many responded by increasing page charges. Others just sold the rights to their journals or entered into agreements with private publishers. And it seems that with the ever increasing control of scientific information by private publishers, the cost of publications has been skyrocketing even more, leading to allegations of exorbitant profits for publishers (Hagedorn, 2001?) and the establishment of institutions, such as the Scholarly Publishing and Academic Resources Coalition (SPARC) advocating high-quality economical alternatives, such as electronic only journals and books, to existing high-priced publications(SPARC, 2001, 2002).

This workshop was inspired by a series of editorials published in the SIP Newsletter (Goettel, 1998, 2000, 2001) which culminated with numerous "Letters to the Editor" published in 2001 (SIP Newsletter, 2001) and a suggestion by Denis Burges to convene a Symposium on the subject. Furthermore, the Publications Committee has recently conducted a survey to gage its members opinions regarding scientific publications (see below).

We have invited viewpoints of a librarian, a scientific publisher, an organization involved in electronic publishing and a scientist. We, the conveners are representing the viewpoints of the scientific society, with our personal views and biases, which of course, lean towards the Society for Invertebrate Pathology.

The Society for Invertebrate Pathology chose not to launch a journal directly on its own, but to have the Journal of Insect Pathology (later renamed the Journal for Invertebrate Pathology) published by Academic Press "Under the Auspices of the Society for Invertebrate Pathology" because its founder, Ed Steinhaus had earlier convinced Academic Press to launch the journal in the first place (Steinhaus 1975). During difficult and turbulent times under this arrangement,

the Society had on several occasions investigated the possibility of launching a journal of its own. Each time such an undertaking was considered as too expensive. Members have also had several bad experiences whereby scientific textbooks authored or edited by SIP members were priced well above what the editors were first led to believe would be the cost of the books, and at prices that the editors, and members feel seriously limit the "reach" of the books (see SIP Newsletter, 2001). And the frustration is further increased due to the fact that most books are now presented to publishers in a "camera ready" format. The SIP Publications Committee has started to ponder the Society's future role in disseminating scientific information. This Workshop is a starting point.

The SIP Publications Committee emailed a survey to over 630 members in March, 2002. The committee wanted to learn how the membership felt about publications and electronic publishing. Within 1 month, over 176 members or about 25% of valid email names responded. We initially divided the set into two groups: 142 from Europe, Canada, USA, and 38 from other regions of world.

We looked at the smaller set first. Everyone stated that they could access the World Wide Web (not simply email) with a web browser. However, 20-25% stated that their computer technology (computers, printers and internet connections) make it difficult to use electronic publications. Based on these results, we did not compile the answers to these questions for the much larger group.

About two-thirds stated that their primary journal is published electronically. About 10% did not know if it is or not; 76% of respondents stated that either they or their library subscribes to the Journal of Invertebrate Pathology.

Forty-four percent prefer to read paper publications, 28% prefer electronic, and 28% like both or have no opinion. 81% have daily access to an adequate scientific library; 66% would submit a paper to an electronic-only peer-reviewed journal, 59% would consider publishing a book chapter in an electronic-only book.

Only 34% stated that their library had recently been unable to purchase invertebrate pathology books due to budget limitations.

We discovered that 18-25% of members use databases on the World Wide Web.

20% ARSEF fungus germplasm database
19% Canadian Bt toxicity/bioassay database
22% Ecological DB of the World's Insect Pathogens (EDWIP)
18% Viral Diseases of Insects in the Literature (VIDIL)
28% Other db; probably less if we omit bibliographic db

Note that more than one database could be marked on the survey form. These percentages indicate that databases of all kinds will play an important role in scientific publication in the future.

Our society serves our members by helping to promote (1) the dissemination of scientific knowledge, (2) archiving and storing knowledge, and (3) a process for determining the priority

(order of discovery) of scientific research. We work with publishers and libraries to accomplish these goals. Now we have the opportunity to collaborate with organizations disseminating publications through the Internet. We also may explore expanding our efforts to publish scientific information beyond the few items available on the SIP web site. For instance, just under half of our members prefer to receive the SIP newsletter electronically (actually they receive a

notice about the newsletter being published on the SIP web site). The variety of options includes DVD, CD, web sites, books, manuals, journals, and databases.

Walker (1998) presents an interesting scenario for publishing electronic journals. He compares commercial and society-based publishing and then promotes an inexpensive approach to publishing by societies that permits free internet access to the electronic journal articles by the scientific community. His comments relate directly to the issues of archiving, dissemination and priority.

If the Society for Invertebrate Pathology follows the needs and wishes of its members according to the survey, we would promote the publication of both paper and electronic versions of all journals and probably books. We would find ways to disseminate publications to members who do not have convenient access to high-quality libraries. We could accomplish this by publishing either electronic copies or inexpensive paper copies. Even though 67% know that their primary publication is published electronically, no more than 56% prefer or are neutral about reading electronic publications in 2002. Our results may not be valid for non-email members (about 100). In other words, some questions had biased outcomes given that the survey was disseminated via the Internet.

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The future of scientific publishing – the publisher's viewpoint

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The nature and needs of scientific information

Science progresses through research, but is mediated through the publication and distribution of the results of that research. These results are presented in individual articles, often summarized in review papers, and are hence fragmentary in nature. Some organization is therefore needed, both in terms of style and presentation and in terms of grouping like papers into subject classified collections. In addition, articles need to be evaluated and accredited through a peer review process. And finally they need to be distributed to readers and securely held in a permanent archive.

The full realization of the value of research results, both now and into the future depends on each of these simple processes being integrated, well performed and delivered at reasonable cost.

The age of paper

Traditional print on paper publishing has served the community well in the past. Scholarly publishers see themselves both as a service provider and as an integral part of and collaborator with the scientific community. Publishing is a service to both writers and readers and an essential and valid link in the chain whereby research leads to new ideas and to further research.

But paper publishing carries costs due to the medium and is increasingly unable to properly organize and facilitate the management of the burgeoning output of modern research to a widely dispersed research community. It has limited search and linking ability and can only show data in such forms such as print on paper can allow. And the growth in scientific output has increasingly outstripped the growth in library budgets, leading to the widely discussed "serials crisis".

The Web based future

The advent and development of the WorldWideWeb opens up a diversity of new possibilities while retaining the traditional strengths of paper publishing to display written and illustrative material. It can be electronically indexed and searched, linked to other www material, include tabular elements previously too large to print economically, and include video and sound clips – and can be delivered to a researcher's desktop anywhere in the world without the need for physical proximity to a library or other source of paper archives

Web archives are not cheap to create, maintain or to manage, but offer hugely increased functionality. No one doing research today would willingly dream of a return to the limitations of a print on paper only world.

A case in point is the situation in the developing countries. Even today people ask me about the research worker in countries where PCs are in limited supply and working telephone lines are a luxury. But many scientists in such places do enjoy increasing access to these facilities

in situations where a fully stocked and managed library would be a total impossibility. Electronic subscriptions to Elsevier journals are available free to low-income countries through the WHO initiative. Complete and up to date scientific information is fast becoming a global resource, available globally at last.

However, the demand for print on paper survives alongside the growth of Web archives and will presumably continue for as long as some users perceive a need for it and librarians can justify the undoubted extra expense of housing and managing it. Figures show that libraries may spend 70-75% of their budget on infrastructure and only 25-30% on actual acquisitions (Odlyzko 1999). Presumably, paper has its own place and will survive in whole or in part for so long as it fills a need. However, these figures do also serve to emphasize the benefits of electronic archives, whereby any extra money that librarians spend on electronic access can reap manifold savings in terms of reduced infrastructure costs.

Why publishers?

The processes of review, preparation, distribution and archiving of material, outlined above, have a cost and constitute a job of work that must be done. In the jargon, Core Business has always been a guiding principle in wise allocation of scarce resources – generally time and money. The core business of scientists is to do science, and their time and money is released to follow this goal if the job of publishing is delegated to experts whose core skill is publishing. It is sensible to allocate specialist jobs to experts. And publishing is more than just printing a manuscript on paper – or posting it onto a website. Crucially, it has always been primarily the task of organizing, presenting – and above all – distributing this material effectively. In a print world a research scientist could merely have nailed his or her articles to their office door - now they can be posted on a home page. But readers want more than to hunt the university corridors of the world – either in reality or virtually - seeking information of interest to them. Large, inclusive databases, peer reviewed, organized, globally distributed, properly archived and constantly developed and upgraded are essential to progress: a task requiring expertise and sufficiently large and costly to be worth delegating.

This delegation has traditionally been to publishers and the system has worked well for many years. It is worth reiterating that scientists decide on what is written and what is submitted for publication. Through the peer review system they also decide what is actually published. The task of publishers is to serve the scientific community and to compete for this work in a market where various other means of distribution and archiving may already exist or be developed. Their strategy is to always to try and provide the best service combined with the best value for money.

Of course, publishers do generate a financial surplus from this activity. Employees and suppliers must be paid for this valuable work and surplus must be accrued to pour back into developing newer and better services, and for sharing with learned societies where publications are collaborative. Any provider of publishing services must spend money and seek payment for their work. The question is less one of the actual cost of commercial publications than of whether this is the lowest or best value price to be paid for the level of service that scientists require or find useful.

The financial interest of publishers drives innovation and good stewardship, and the need to remain competitive focuses their activities and reduces waste. An added advantage of the independent status of the commercial publisher is their freedom from any political or philosophical interference, whether from governments or other research funding agencies. Again, Science decides on what is valid content and publishers manage its presentation and distribution.

Elsevier Science and ScienceDirect

At Elsevier, we are proud to be the world's largest single provider of scientific information and are committed to developing our ScienceDirect (SD) online platform as our central means of information distribution. Our size gives us the resources to focus on and fund new developments of SD, building ever better functionality for our users. Following the acquisition of Academic Press and the other Harcourt scientific imprints, Elsevier publishes some 1,500 journal titles, a 20% percent share of the global science information market. In addition, Elsevier's capacity allows display of many additional titles that have previously ceased publication or changed their titles.

And SD is a highly successful platform. According to a recent independent report it has the best design, functionality and value for money compared to its major competitors. It is also one of the most reliable in terms of performance, with very little "downtime".

Some 6000 institutions have licenses to its holdings in some form or another. Licenses are flexible, giving access to the entire collection, particular subject areas, groups of titles or single titles. Access to journal tables of contents and abstracts is free. Pay-per-view facilities, so that non-licensed users can purchase selected downloads of particular titles of interest, are soon to be introduced. And the system is heavily used. The average SD article was downloaded 328 times in 2001; with some review journal articles receiving up to 1000 downloads.

With so many options, the license provisions and their pricing are necessarily complex as we work with users to provide best value for money for their needs. Licenses are negotiated on an individual basis to try to provide the best package for that users circumstances. But the overall value for money is evident. The average article download costs licensees only $7 or less compared to $35 for an interlibrary loan. And we are constantly exploring new pricing and licensing models to cater to an ever-wider range of user needs.

The issue of cost to subscribers is a thorny one. For many years all publishers were trapped into a spiral of increasing prices as the amount of scientific output rose while library funding did not. Dwindling subscriptions fuelled higher unit costs and depleted library holdings, creating friction in a community that should have been working together on solutions. The development of SD has allowed Elsevier to take a broader view and to focus on moderate annual price rises – we have pledged annual increases of no more than 10% and have in the last five years held this to 7% per annum – while adding hugely to the functionality of SD and sinking millions of dollars into its development.

So what are the current and soon to be launched functionalities of SD and how do these exemplify our vision of future participation in the global distribution of scientific material?

- Of course SD incorporates millions of individual research articles, letters, reviews and so on, organized into journal titles, all fully searchable in PDF format. Acrobat formatted journal pages appear on screen, on the desktop, as well as full HTML articles with full internal links and user functionality providing an impressive degree of integrated information and functionality.

- CrossRef links from article reference lists take users to material on other publisher's sites within the CrossRef partnership.

- Elsevier seeks to foster ever-wider links between SD and other related scientific databases elsewhere, e.g., third party databases such as Inspec and PsycInfo.

- Article by Article posting. We recognize that speed is of the essence in scientific publication. Author's submitted manuscripts are posted on SD within two weeks of Editorial acceptance, clearly marked as uncorrected proofs. Once edited proofs have been approved the updated article is posted as a corrected proof pending assignment of the final article to a numbered journal issue.

- ContentsDirect is our email alerting service, whereby scientists can register for the tables of contents of new issues of selected journals to be emailed to them on a regular basis, removing the need to browse favourite journals

- SD provides electronic sample copies of our journals.

- Subject Area Portals / Gateways. A new and growing development. With such a large body of information, effective search and navigation can define the usefulness of a database. Journal title search and key word search can be tools too blunt and too sharp for efficient navigation. Subject Gateways will direct users to the core titles in their area of interest. While retaining all the links to the entire archive, they will be a convenient starting place for an article or subject search.

- Author Gateways. Soon, authors of articles in the Elsevier production system will be able to directly query the status of their own manuscript – whether it has yet been typeset, assigned to a journal issue, etc. A direct link to the Author Gateway also allows manuscript submission. Soon all authors will also be emailed when their article is published on SD.

- AP IDEAL. Following the acquisition of the AP journals, these are being integrated into SD during this summer. The IDEAL platform will remain in existence until 31 December 2002. In the meantime we will be working with IDEAL subscribers to migrate them into the SD license best suited to their needs, so as to maintain or broaden their journal access, as they wish and to enjoy the improved functionality of SD.

- Backfiles. We are in the process of scanning all Elsevier and AP journals published prior to 1995 so as to provide searchable backfiles to users as a one-off purchase -the entire archive, or by journal subject packages. This is an enormous task – costing $40 million for the scanning alone – all Elsevier journals back to volume 1 will be covered by spring 2003. AP/ Harcourt backfiles will be processed in a separate phase soon after. The complete current and backfile archive will be equivalent to 3.8 kms of shelving and will allow libraries to save huge amounts of storage space and shield them from loss of their holdings through fire, earthquake and the like ! Not to mention the savings in cataloguing, filing and administration tasks no longer necessary in the digital library world.

- Books, Major Reference Works, etc will be incorporated into SD increasingly in the future as our plans for their presentation and pricing models develop

In summary, Elsevier's vision of the future is simply to be a major contributor in a global scientific world in which a variety of databases, large and small, commercial and independent, are linked together into a true global information store. We will strive to develop ScienceDirect, incorporating ever more sophisticated and useful facilities to meet the changing needs of readers and writers and to increase value for money for subscribers, by increasing the value proposition of each journal title. Further, we will continue to work with subscribers to develop economical and appropriate pricing and access models. We fully recognize that science publishing is a competitive market and that our future in it firmly rests on service and value.

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Electronic publishing: open access, integration and interoperability

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Researchers' main objective when publishing their work is to share their knowledge and experience in order to promote further scientific and technological development and to enrich education. Developing countries have enormous barriers concerning the access to information and the publication of the results of their scientific findings.

Access to scientific journals is limited due to rising subscription costs and, in some cases, also due to lack of full Internet connectivity.

There are also many barriers to overcome in order to increase the participation of developing countries in internationally recognized journals. Barriers include language, quality and relevance. Quality standards and relevance differ from country to country, but, in order to publish scientific research information in recognized international journals, first world quality standards must be met and the work presented must also be relevant to the main target users, which again, are scientists from the most developed nations.

The result is intense information traffic and interactivity between researchers from more developed nations and a one-way flow of information from developed to developing nations, especially in printed media. This increases the information gap between rich and poor nations and deprives the international scientific community from essential knowledge generated in developing countries. This leads to a critical information gap, especially in the fields of public health and environment.

New information and communication technologies have promoted a breakthrough in the dissemination of scientific research, and may represent a solution to bridge the gap.

Bioline International[1] is an electronic publishing service that was established in 1993, operated by bioscientists who believed that scientific information could be widely distributed, more cheaply and with added scientific value, using electronic means. Its overall goal is to help promote the exchange of research information between developing countries and between these nations and the more developed ones. Its initial content consisted of online versions of mainstream, printed bioscience journals from the developed world. As these journals setup their own information system, Bioline focused its activities on the distribution of peer-reviewed but less well known journals from developing countries, and to explore the potential for online-only journals.

Bioline International is a collaborative initiative between the University of Toronto Libraries, Canada (management office), the Reference Center of Environmental Information[2] (CRIA – Centro de Referência em Informação Ambiental), Brazil (host computer and software development), and Bioline/UK (liaison office). Published material relate mainly to the fields of biotechnology, biodiversity, environmental and ecological sciences, food, agriculture, veterinary science, medicine, microbiology and taxonomy.

[1] *www.bioline.org.br*
[2] *www.cria.org.br*

The system has recently been upgraded to XML, which opens a whole new perspective concerning interoperability. An interface that provides direct links to several important resources was developed in order to add value to the existing information. Figure 1 illustrates a Journal "Insect Science and Its Application" published by the International Centre for Insect Physiology and Ecology (ICIPE), Kenya. All species names have been tagged in order to "link" the name to other information sources. Figure 2 shows the links of *Arachis hypogaea,* to other sources of information.

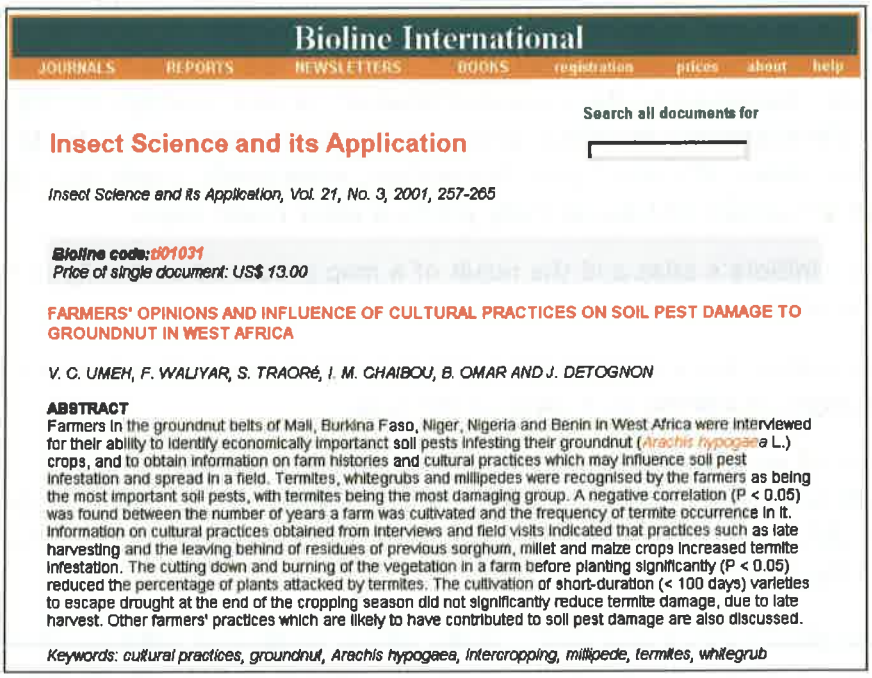

FIG. 1. An example of xml tagging within a journal

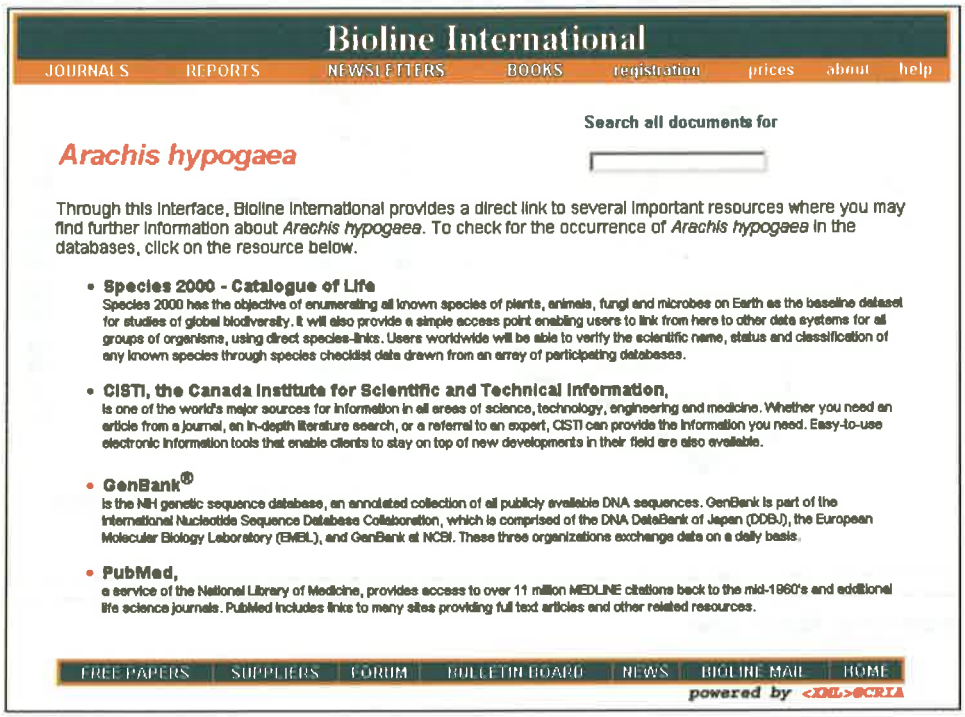

FIG. 2. Linking the species name to other information sources.

This experience gave CRIA the necessary expertise to develop new systems using innovative tools for cross-content and web document searching. An excellent example is the journal "Biota Neotropica"[1], which is part of a very challenging research program "Biota/Fapesp - The Virtual Institute of Biodiversity"[2]. This program is the result of the articulation of the scientific community of the State of São Paulo and aims at characterizing the state's biodiversity and defining mechanisms for its conservation and sustainable use.

The first step was the establishment of minimum standard data fields that all inventories should record in order to enable the development of an information system (SinBiota[3]) that would accept the input of data of all research groups through the Internet. The database, developed in collaboration with the UNICAMP[4], includes 168 different taxonomic groups. A digital map base, developed by the Instituto Florestal[5], is also available on the Internet. The result of these developments (database and mapbase) is the Atlas of the State of São Paulo, where users can select different layers (vegetation, watersheds, roads etc.) and search for the occurrence of species and dynamically produce tailor made maps.

Figure 3 shows SinBiota's atlas and the result of a map produced on-the-fly of the vegetative coverage of the state.

Figure 4 shows part of the collection form, the associated list of species and the distribution of *Annona coriacea*, presented as a layer of the map.

CRIA is developing all systems based on open protocols and, when possible, using free non commercial software. The journal Biota Neotropica, developed basically to publicize research results, uses XML technology and is able to link information back to SinBiota and other internet based systems.

Figure 5 shows an inventory of stream fishes where all species mentioned are wrapped in XML tags. This enables different outputs of the information. In this case all species mentioned are listed after the abstract and can be linked to other information systems.

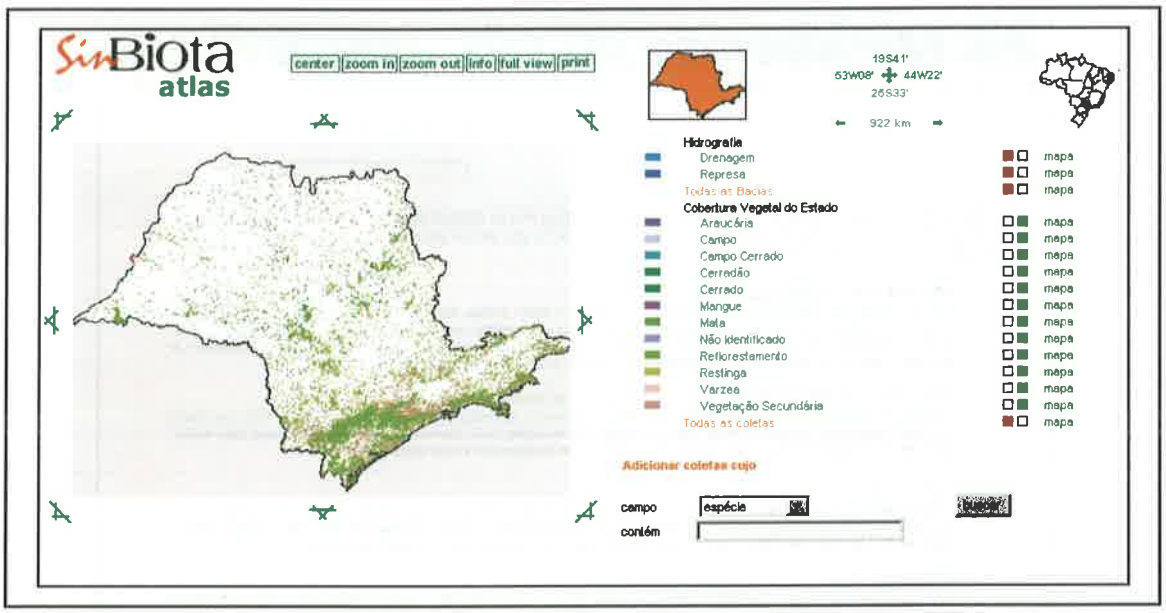

FIG. 3. Example of SinBiota's atlas

[1] *www.biotaneotropica.org.br;* [2] *www.biota.org.br;* [3] *sinbiota.cria.org.br;* [4] *State University of Campinas (www.unicamp.br);* [5] *www.iflorestsp.br/*

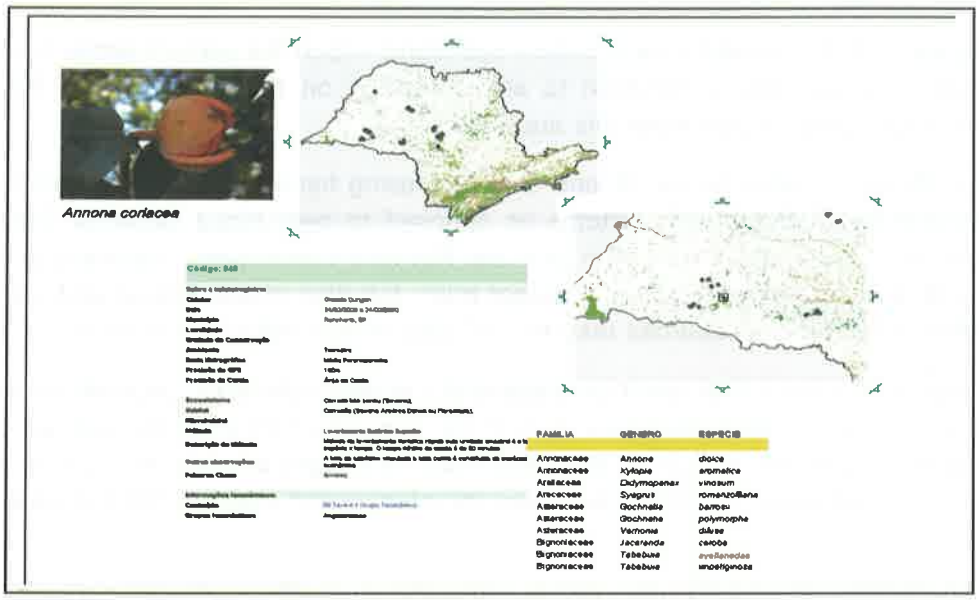

FIG. 4. Example of *Annona coriacea* distribution

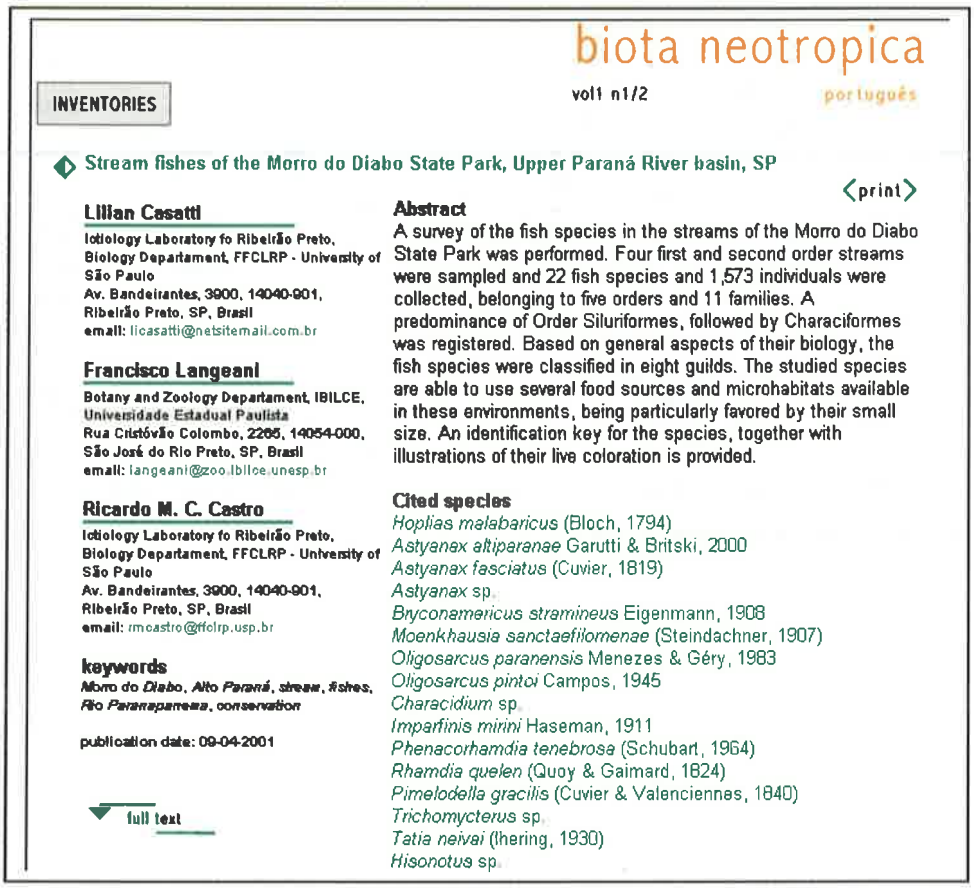

FIG. 5. Inventory published in the Biota Neotropica

Figure 6 shows a distribution mapped for *Astyanax fasciatus*, also produced dynamically when "clicking" on the name under the list of cited species of the abstract.

The examples shown illustrate cross-content searching within the same internet node but this is irrelevant. One can link information to any computer on the internet. This link may be dynamic when standard open protocols are used.

With the evolution of open protocols and ever increasing bandwidth, the tendency seems to be the integration of distributed systems as opposed to centralized systems. This offers a whole new set of possibilities for online journals. Not only can images, maps and photographs be more widely used when compared to paper print, but also sound, movement and dynamic links to other information resources may be included to add value to the work.

Another important aspect that must be addressed concerns electronic journals sustainability. The wish of the scientific community is to have this information freely and unrestrictedly available online. Alternative sources of funds must be sought as price is a barrier to access and there is a substantial cost involved when structuring and maintaining such a system.

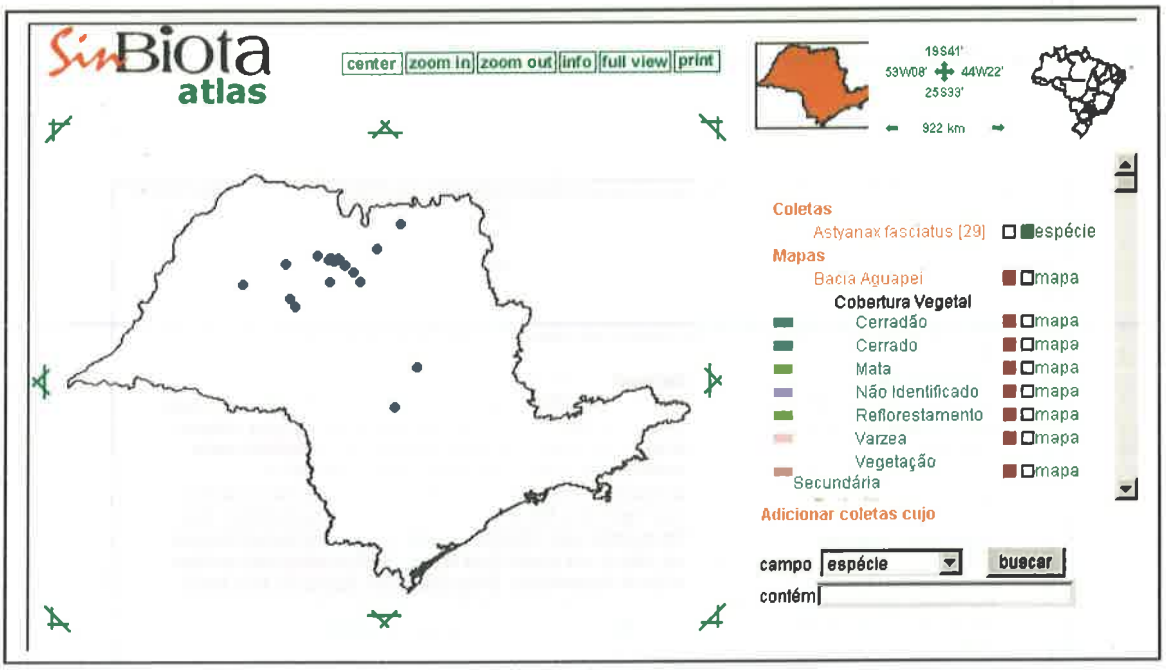

FIG. 6. Distribution map for *Astyanax fasciatus*

The future of scientific publications: one scientist's perspective

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The recent explosion of information, especially in digital form, is revolutionizing many fields of scientific endeavor, including the assorted venues scientists use to disseminate their research results. The question is not *whether* scientific publications will be digitized but rather *how* and *when*. Scientists should take a keen interest in this nascent paradigm shift – it is already having a profound impact on all aspects of our work.

Here I confine my considerations to publications in refereed journals. For my purposes, a refereed journal is one which enlists one or more specialist reviewers for each manuscript submitted to it. The authors, in turn, are often able to revise the manuscript or respond to reviewers' comments. An editor, or editorial board, serves as final arbitrator to determine acceptability. This process is meant to ensure a level of scientific rigor, originality, repeatability, relevance, significance and impact of the work published within the journal. Journals vary widely in perceived quality or impact. Nevertheless, articles in these journals are the coinage of our scientific realm. The complexities surrounding the future of the publication of books, reviews, and the assortment of non-refereed technical works are manifold and will not be addressed here. However, many of the issues described are common to the range of scientific publications.

Scientists have needs, now and into the foreseeable future, related to publishing their own work as well as obtaining access to the publications of others. The requirements are simply summarized. Scientists need: 1) a publication process that is as rapid as possible, while maintaining 2) high editorial quality and scientific rigor; 3) assurance of permanent availability of published work; 4) a wide audience for their work together with 5) broad availability of the works of others, including access to abstracts, indexes, and the use of robust searching tools.

The availability of on-line publishing requires scientists to evaluate carefully the relative merits of various outlets for their research results. On-line publishing seems to offer some advantages over traditional print publications, but whether these are fulfilled remains to be seen. Here is a selection of relevant issues:

Electronic manuscript submission and distribution to reviewers can greatly facilitate the steps of the pre-publication process, simultaneously improving the **speed of publication** (no snail mail) and reducing costs (no postage). For journals distributed on-line only, speedier publication seems to be a given: no printer delays – simply edit the copy and post it. On-line distribution promises a wider audience, but the size of the audience depends upon access (see below).

Many traditional print journals are now exploring some type of hybrid form, i.e., offering both printed and digitized versions. These **hybrid journals** can offer a blend of traits advantageous to scientists. For example, the on-line supplementation of illustrations and data contained in the print version (through color graphics, gene sequences or statistical results) would elevate the demonstrated rigor or impact of the journal. The use of emerging multilingual software could make translations available to a true worldwide audience, fostering better communications among scientists.

The digital age will lead scientists, librarians and publishers to consider what form "**permanence**" will take. What technology offers permanence? Printed and bound? Microfilm or microfiche? Zeroes and ones? We have been part of a paper paradigm for hundreds of years and, for the foreseeable future, a printed on-paper copy will continue to represent the ultimate archive. However, digital archives are already part of a new publishing paradigm. Who needs to maintain them? At what cost will future access be offered? What are the attendant limitations of digital archiving?

Scientists based at institutions will still depend upon institutional libraries for **access** to the breadth of scientific literature. Within the print paradigm, when a scientist's own library lacks a resource, an interlibrary loan can often provide it. For materials available only on-line, will such library-to-library transfers still occur or will publishers try to limit redistribution of their digitized information? How do scientists blend their own printed reprint collections with newly-distributed digital reprints? How can we search both efficiently?

The issue of **cost** is complex. Under the current model for distributing costs, taxes, grant overhead or tuition support libraries. Libraries in turn subscribe to journals (printed or on-line) at inflated rates and thereby help support publishers. Page charges to authors also defray publishers' costs. Libraries struggle with the duty of maintaining permanent printed records and providing on-line access to the increasing number of journals. Publishers struggle with a new set of costs and many now face dual overheads associated with hybrid publications. Under a new model, might publishers pay libraries to make their publications available to users (scientists) who would then have to pay access fees?

Many highly-respected scientists and organizations of scientists have undertaken the establishment of their own on-line publications. This **self-publication** endeavor surely comes with the attendant risk of turning scientists into publishers. Nevertheless, the attraction is clear: given widespread digital access available to scientists, it may be possible to circumvent profit-driven publishers and put research results directly into the hands (or rather, in front of the eyes) of other scientists. In order to meet the costs associated with any type of publication, even non-profit scientific societies must generate revenue. This can still mean charging libraries, members or individual users for access.

Finally, we seem to be drowning in a rising tide of journals. This ongoing increase in the number of journals has only accelerated with the advent of additional, on-line publications. Obviously, an increasing risk of dilution of quality attends this growth. *Caveat lector!* (Let the reader beware!)

The future of scientific publications: the librarian's viewpoint

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Abstract

Scientific publishing is undergoing a great deal of change. Authors, publishers, librarians, and readers all have a stake in the future of scholarly communications and may have different plans for the future. This presentation discusses some of the most prominent scholarly communications initiatives from the viewpoint of a librarian at a large research institution.

Introduction

The future of scientific publishing is uncertain. We are moving away from a model based on peer-reviewed print journals that we all (authors, publishers, librarians, and readers) understand and are moving into an unknown future. It may look rather like the present, or it may be radically different. We may simply replace existing print journals with enhanced electronic versions, or we may find ways of disseminating information that are almost unimaginable right now. Some of the more radical models might even do away with publishers and librarians as intermediaries between authors and readers.

The present model of scholarly communication is breaking down for a number of reasons, cost among them. In the past fifteen years, the general inflation rate (i.e., the Consumer Price Index) for the United States went up 57%, serials prices shot up 226%, and monograph costs increased 66% (Kyrillidou and Young, 2001). Library budgets could not keep up with subscription rates that climbed 10-20% per year, and so cancellations became an almost annual ritual at many libraries. The inflation rate for science journals has decreased in recent years but is still about 8-9%. The journal inflation rate is still far higher than libraries can afford, and there is a distinct tension between publishers and librarians. Some librarians are passionately opposed to all commercial publishers, and some publishers have equally negative responses to librarians. The president of the Association of American Publishers, Patricia Schroeder, described librarians as one of the chief enemies of publishers because we give away information for free (Weeks, 2001), a designation that both amused and outraged librarians.

Libraries have long known that change needed to happen, but as long as authors were willing to publish in expensive journals (and readers asked for those journals), there was little that we could do to encourage change. In the past few years, authors and readers have begun to understand the magnitude of the problem and are beginning to seek changes. The explosive growth of the Internet has also encouraged people to think of new mechanisms for disseminating information. As a result, there is a passionate debate going on about the future of publishing. The initiatives that are underway or are being proposed fall into two broad categories, designated "evolutionary" and "revolutionary" in this talk. The proponents of the milder evolutionary changes are largely publishers while the proponents of the more extreme revolutionary changes are often, but not exclusively, authors and librarians.

Evolutionary changes

Many of the evolutionary changes merely take advantage of the speed and ubiquity of the Internet to improve access to the existing journal literature. By now, most of the major

scientific journals are available both electronically and in print. Publishers are trying a number of subscription models, and prices for e-journals range from perhaps 10% cheaper than print to ten times more expensive. Societies have also moved into the e-journal realm. Some of them use the services of organizations such as Stanford University's Highwire Press (http://highwire.stanford.edu/) or BioOne (http://www.bioone.org/bioone/). Initiatives such as the Association for Research Libraries' SPARC (the Scholarly Publishing and Academic Resources Coalition, http://www.arl.org/sparc/) provide support for the development of less-expensive print or electronic journals which directly compete with commercially published titles. There are a few electronic-only journals, but for the most part they have a hard time getting high-quality articles due to the conservative nature of tenure and promotion decisions.

Even readers with no philosophical concern about the future of journals have begun to appreciate the speed and ease of use of electronic journals and are demanding them. Due to the pressure from users, libraries have been pushed to purchase e-journals in increasing numbers, but most have not canceled print subscriptions due to concerns with archiving. Most of the libraries that have gone to electronic-only access did so either because of severe budget crises or because of natural disasters that wiped out their print collections. Many publishers are offering large package deals containing most or all of their journals, which decreases libraries' fiscal flexibility though it improves access for users. A side effect of the general fondness for electronic access is a dramatic drop in the number of visitors to libraries that have many e-journal subscriptions.

Revolutionary Ideas

All of the projects listed above use the same old peer-reviewed journal as their model. There have been a number of more revolutionary initiatives as well. In 1999, Harold Varmus, then head of the US National Institute of Health (NIH), suggested a radical new idea (Marshall, 1999). All biomedical journals would deposit articles on a central Web site that anyone around the world could use for free. The site would be sponsored by NIH and would also include preprints and other non-peer reviewed items. This e-BioMed proposal was immediately attacked on a number of fronts and was dramatically scaled back and renamed PubMed Central (http://www.pubmedcentral.nih.gov/). A similar European site called E-Biosci (http://www.e-biosci.org/) was launched September 2001.

This proposal was only the first of a series of initiatives attempting to develop freely accessible archives for scientific research. While the controversy raged over e-BioMed, Highwire Press announced that many of its journals would provide free access to older articles and is now the second largest free article archive in the world. In March 2001, the Public Library of Science open letter (http://www.publiclibraryofscience.org/) asked researchers around the world to pledge not to publish in journals that did not freely archive their articles and asked publishers to develop such archives. The initiative gained many signatures but seemed to have little effect on publishers, but then in February 2002 the Soros Foundation came out with the Budapest Open Access Initiative (http://www.soros.org/openaccess/) and the race was on again. *Nature* and *Science* have both covered scholarly access issues, and their debate sections (http://www.nature.com/nature/debates/e-access/ and http://www.sciencemag.org/cgi/eletters/291/5512/2318a) provide an introduction to the debate.

The most extreme form of the open archives movement would do away with both journals and peer review. Authors would post their own works on an institutional Web site where they would be permanently archived and available at no charge to the entire world, and the researchers' peer communities would comment on them. Copyright would remain with the

authors, and metadata harvesting software would provide indexing. This system could co-exist with the existing journal system, or it might replace both publishers and librarians.

General issues with the electronic future

It is apparent that the future is an electronic one. Print will be around for many years, but even now articles often have supplementary material such as data sets or video clips that are available only as electronic files. Soon the electronic version will be the version of record, though there are some serious issues that need to be examined before this is universally accepted. First, will the electronic version be properly archived, and by whom? Also, will archiving bodies accept the responsibility of keeping their files readable by whatever new technology comes along?

Another issue deals with the ease of removing electronic files. Recently, a controversy erupted over a paper published in the journal *Human Immunology* based on political statements made in the introduction (Klarreich, 2001). The paper was then retracted and librarians and subscribers were asked to ignore or physically remove the article from the print journal, an unprecedented act. This being the digital age the publisher also removed the electronic file from the journal's Web site so that no one could view it. Regardless of the merits of this particular case, it demonstrates how easily the record can be re-written if only one copy of a file is held. And, of course, if we are to rely on electronic files as the official record, we must have confidence that no one can hack in and change or delete the file itself.

Another issue that we face when we move away from the traditional journal-based publication model is locating materials. At the present, researchers find out about new research in several ways, including searching indexes or article databases such as *Biological Abstracts* (BIOSIS) or PubMed. It is then a relatively simple matter to locate the journal at your own institution or request a copy of an article from another institution or colleague. At present, several databases such as PubMed allow one-click access to electronic versions of articles from their citation in the index. Abstracts and indexes provide a major service to users, though their producers have generally not been a part of the discussion of future trends in scholarly communications, but clearly if articles are posted to institutional archives they will need some sort of indexing if anyone is to find them.

The issue of copyright in the digital world has great relevance to the future of scholarly publishing. A number of recent legal decisions in the US and elsewhere seek to limit the concept of "fair use" in digital media, variously forbidding unauthorized linking on the Web, extending the length of time before a publication enters the public domain, forbidding the copying of software and music CDs even by the owner for his own use, and more. At the same time, a growing number of authors are chafing against the need to get permission from publishers to use their own articles for teaching purposes and are seeking to keep the copyright themselves rather than handing it over to their publishers.

Finally, the debate about the future of scientific publishing assumes that all potential users of the literature have easy access to computers, high-speed Internet connections, and good quality printers. While the Web is becoming more ubiquitous as time goes by, universal access is not available even in the United States. And while electronic access has advantages over physical libraries in some ways even in areas of desperate poverty or civil unrest, users still need electricity and computers.

Book publishing

The above discussion centers on journal publishing, the area that is of most interest to most scientists. Book publishing is also suffering a crisis. Book prices have also increased faster than the overall inflation rate, and libraries are buying fewer of them. Electronic versions of books, or e-books, have long been hyped but have failed to take hold among general readers. A recent poll found that only 4% of Internet users were "very likely" to buy an e-book (Bandler, 2002), and many e-book publishers have gone out of business. Reading an e-book just isn't as comfortable as reading a best-seller.

However, there are several possible niche markets for e-books. Electronic reference sources such as encyclopedias or manuals can be updated frequently and the full text searched. Textbooks are another obvious niche market for some of the same reasons and would have the extra advantage of portability. In fact, many new textbooks are published as CDs and/or have associated Web sites. A number of commercial publishers as well as PubMed Central and BioOne are all trying out scientific e-book publishing on the Web, so that this area of e-book publishing is flourishing in a limited way.

Conclusion

Scientific publishing is a field undergoing a great deal of change. Even if only the most modest of the proposed changes occur, the future will look very different from the present. There are many issues which need to be settled before we can move ahead with confidence. The change to an open archives model is threatening to all of the present stakeholders (authors, publishers, librarians, and readers). There are a number of legitimate issues that various stakeholders have raised. For authors, peer review, tenure decisions, copyright, and workload issues are all of concern. Publishers have concerns about revenue. Librarians face dramatic changes in the way we obtain material, organize information, and answer questions and may even become redundant. Readers will need access to a computer and a fast Internet connection rather than access to a library, and will need to learn new methods for finding research of interest.

Librarians' concerns about the new proposals relate to our experience with providing information to users. Librarians ask questions such as how much each new proposal will cost, whether it will retain our traditional rights to free dissemination of information, whether the information will be available in the future, and how users can locate the information. As an intermediary between authors and readers, our task is to make sure that scholarly communications in the future continue to thrive and meet the needs of scholars and students, and as such we take an active interest in the debate.

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Symposium (Bacteria 1) Bacterial Insecticidal Proteins: Specificity, Improvement and Novel Toxins

The diverse armoury of the Bt crystal

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Introduction

As of April 1st 2002 one hundred and sixteen different Cry or Cyt proteins had been characterized through gene cloning and sequencing. The vast majority of these proteins having been isolated from crystals of naturally occurring strains, with just a few expressed artificially from cryptic genes. In addition several of the toxins were isolated from non-Bt species but had sufficient similarity to the Bt toxins to be included in the nomenclature. By definition these hundred and sixteen proteins share the characteristics of being part of a crystal and possessing pesticidal activity. Within this group there is considerable variation in sequence and structure, suggesting a strong evolutionary pressure to produce pesticidal, crystalline bodies. The role of the crystal in the ecology of Bt is a highly debated subject and unfortunately outside the scope of this short review.

The crystals

It is remarkable that such a diverse collection of proteins are all capable of forming crystalline bodies within the sporulating Bt cell, especially when there are few other examples of proteins crystallizing in vivo. The ability to crystallize does not appear to be reliant on the Bt environment since toxin genes expressed in other bacteria, and even plants, are capable of forming crystals morphologically similar to those formed in Bt (De Cosa et al., 2001; Oeda et al., 1989). In contrast to the inclusion bodies formed when over-expressing proteins in *E. coli*, the crystalline toxin proteins are correctly folded and readily soluble at an appropriate pH in the absence of denaturants. Various factors have been considered as determinants of toxin crystallization including the C-terminal extension found associated with many of the toxins (Park, Bideshi, and Federici, 2000), however it must be noted that many of the other toxins lack this extension yet are still capable of forming crystals, examples include Cry3Aa and Cry11Aa. For other toxins such as Cyt1Aa and Cry2A accessory factors have been implicated in the crystallization process (Crickmore and Ellar, 1992; Wu and Federici, 1993). A suggestion has also been made that the presence of DNA in the crystals is important for crystallization (Bietlot et al., 1993), however others dispute this (Walters, Roquain, and Crickmore, 2002).

Toxin nomenclature

In 1989 Hofte and Whiteley (Hofte and Whiteley, 1989) proposed a nomenclature for the Bt toxins that was based largely on insecticidal activity. As more toxin genes were cloned it became apparent that this early nomenclature had limitations and there was confusion over the naming of novel toxins. Thus in the mid 1990s a new system was devised that was based

purely on amino acid similarity (Crickmore et al., 1998). A basic phylogenetic tree was derived from the amino acid sequences (figures 1,2) and the location of the node at which a toxin joined that tree was used as the means of naming that toxin. The boundary locations defining different ranks within the nomenclature were initially chosen to minimise changes between the old and new nomenclatures. A general decision was made to set the boundary between tertiary and quaternary ranks at a fairly high % sequence identity (approximately 95%). Thus two toxins with 94% sequence identity would be designated different toxins. Although one could argue that two such toxins are essentially the same, there are examples of two toxins varying very little in sequence but having sufficiently different biological activities to justify this 'splitting rather than lumping' approach. A decision was also taken to give each independently sequenced toxin gene a unique name, even if its sequence was identical to a previously sequenced gene. Doing this gives researchers useful information, largely because of the fact that it is often difficult to decide whether differences between sequences are real or due to sequencing errors. Furthermore it has been reported that a variant of the lepidopteran toxin Cry1Ab (Cry1Ab7) differing in only four amino acids from the consensus has an additional dipteran activity (Haider and Ellar, 1988), a good reason for giving this toxin a unique name.

Toxin diversity

Figures 1 and 2 show that many of the toxins show significant similarity to those encoded by the first cloned toxins genes, *cry1Aa*, *b*, and *c* from Bt *kurstaki*. The toxins shown in figure 1 are all believed to have the same basic three-domain structure that has been determined for Cry1Aa, Cry1Ac, Cry3Aa, Cry3Bb and Cry2Aa. Despite possessing the same protein fold there are significant differences in the specificity and efficacy of these toxins. There are various reasons for these differences including specificity of binding to the insect midgut and stability of the toxin. Figure 2 shows that in addition to the three-domain toxins there are many with little or no sequence similarity, these include the Cyt toxins that possess a largely beta sheet based structure (Li, Koni, and Ellar, 1996). An interesting recent discovery is the set of Cry34/Cry35 binary toxins (Ellis et al., 2002). The two components of the binary toxin act together to cause the pathogenic effect. The larger (44kDa) Cry35 toxins show significant sequence similarity to the mosquitocidal BinA and BinB toxins, which themselves act as a binary toxin. The smaller (14kDa) Cry34 toxins do not however show any sequence similarity to either Cry35 or BinA/B. A separate class of mosquitocidal toxins have been isolated from *Bacillus sphaericus* and named Mtx1, Mtx2 and Mtx3 (Delecluse, Juarez-Perez, and Berry, 2000). The latter two appear to be distantly related to a subset of the Bt Cry toxins, indicating that the distinction between the Bt and *B. sphaericus* toxins is not as clear as one might have thought. Comparisons have been made between domain I of the three domain toxins and other pore-forming toxins such as colicin (Parker and Pattus, 1993). Similarities can also be seen between the Cry23/37 binary toxin and aerolysin (Rydel et al., 2001) and between the Mtx2/3 toxins and the epsilon toxin of *Chlostridium perfringens* (Delecluse, Juarez-Perez, and Berry, 2000). These similarities might suggest common ancestry between these various bacterial toxins, but as mentioned above the Bt toxins appear to have evolved in a convergent manner to produce the characteristic crystalline toxins.

The future

Figure 3 shows the rate of discovery of novel Bt toxins, and suggests that the discovery of new toxins is likely to continue at least into the near future. Various techniques are being employed in this discovery process including high throughput bioassays (Ellis et al., 2002), multiplex and degenerate PCR (Mahadi et al., 1998) and genomic sequencing. A paper elsewhere in these proceedings describes the discovery of a bizarre toxin from Bt subsp *israelensis*

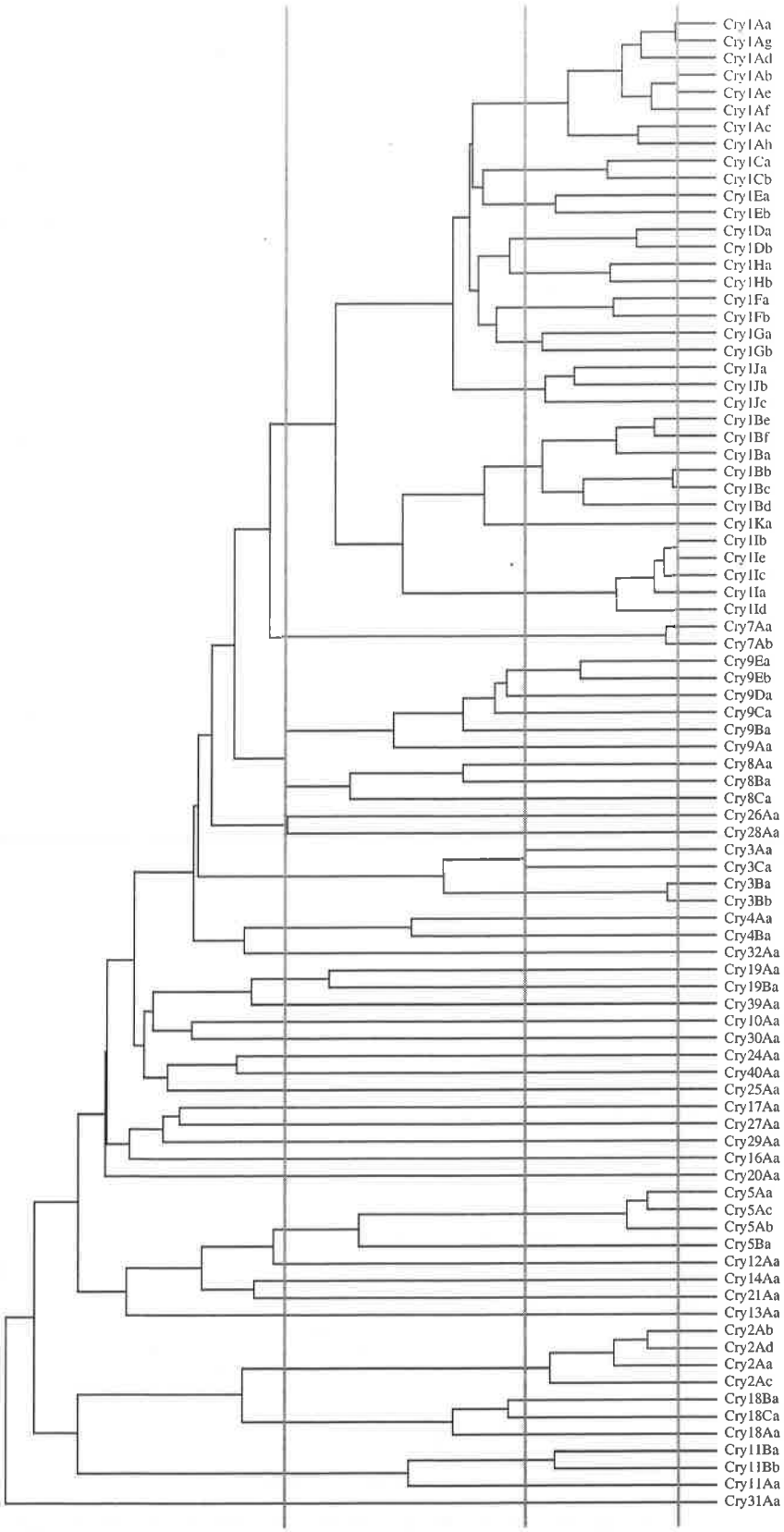

FIG. 1. Sequence similarity between the three-domain toxins, taken from http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/

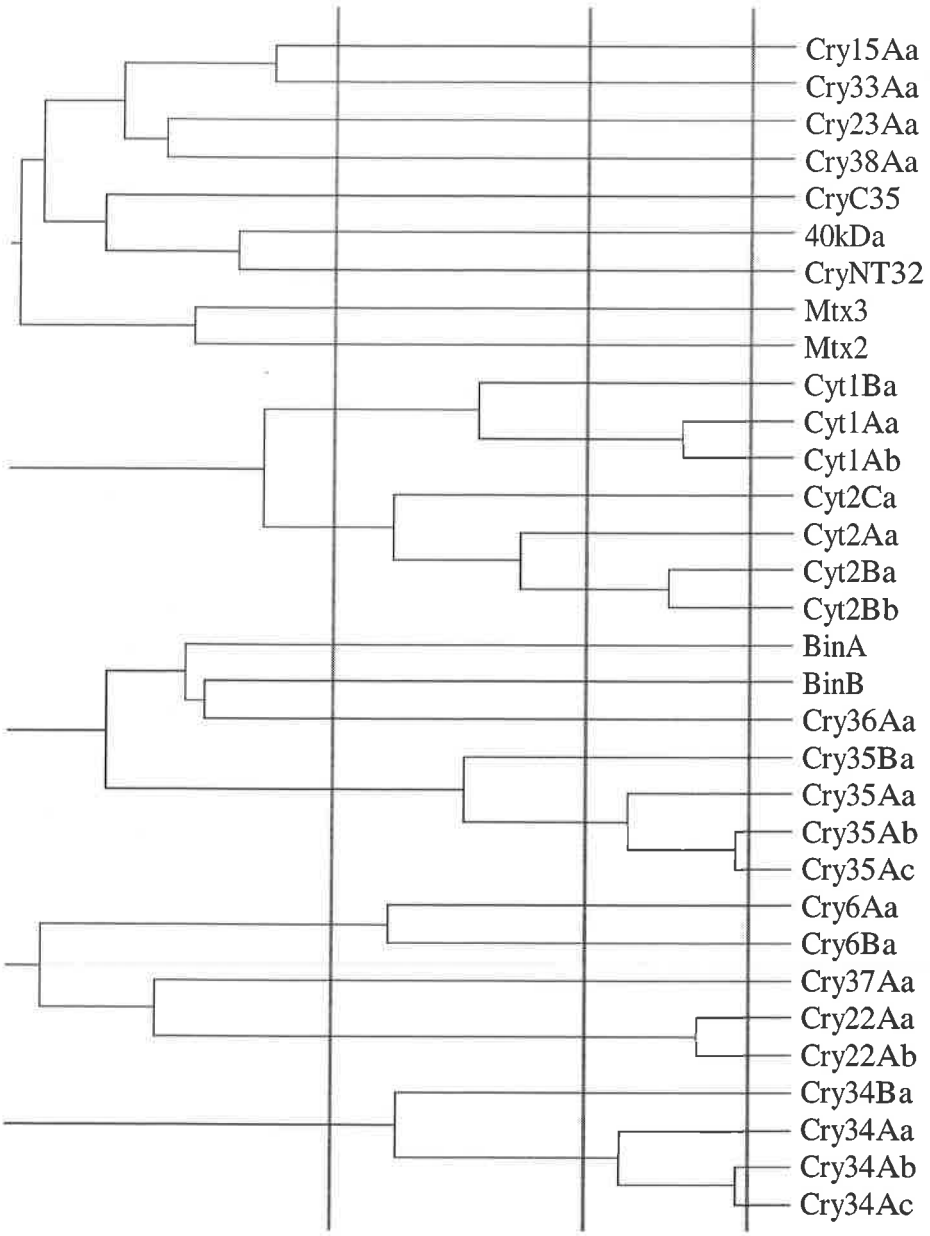

FIG. 2. Sequence similarity between other Bt and related toxins, taken from http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/

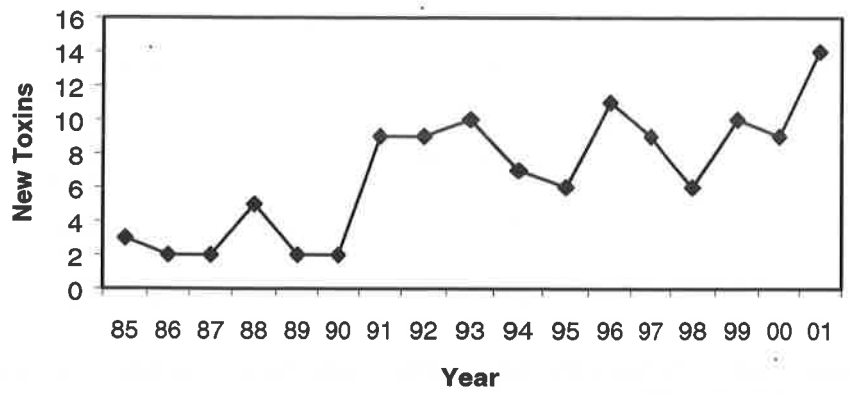

FIG. 3. Rate of discovery of novel Cry or Cyt toxins

through a large-scale sequencing project (Berry, 2002). In addition to the discovery of new native toxins a whole new armoury of novel toxins are being created through genetic engineering. Approaches used include site-directed mutagenesis (Audtho et al., 1999), domain swapping (Bosch et al., 1994) and DNA shuffling (Yamamoto, 2002).

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The toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis:* host regulation and a new toxin gene

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B. thuringiensis subsp. *israelensis* (Bti) is the bio-insecticide of choice in programmes worldwide to control mosquitoes and blackfly vectors. The insect pathogenicity of this bacterium depends on the presence of the pBtoxis megaplasmid that encodes all six of the previously-described toxins in this isolate (Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba). In addition, the plasmid carries several insertion sequences and encodes two further proteins (P19 and P20) with roles in promoting crystal formation and enhancing cell viability, probably by acting as chaperones. The pBtoxis plasmid has been partially mapped already but nucleotide sequence is limited to toxin genes and their flanking regions. Since toxicity of the Bti crystal is greater than that of any combination of the known toxins derived therefrom, it seems that other toxins or virulence factors may play a role in the activity of wild type crystals. One possible source of such additional factors is the approximately 80% of the pBtoxis sequence that has not previously been analysed. In order to understand fully this highly important virulence plasmid, we have therefore determined its entire 127,923 nucleotide sequence.

The full length 127,923 bp pBtoxis sequence was processed *in silico.* Restriction analyses of the complete sequence agree with the previously published map, except that all of the predicted restriction fragments are slightly smaller than previously estimated, consistent with the slightly smaller overall size of the plasmid (128 kb compared to the 137 kb proposed). Placement of genes on the restriction map agreed with those detected in the sequence, with the exceptions of *cyt2Ba, cry4Ba* and *cry10Aa,* which are in the same position but inverted in order and orientation.

In addition to the previously recognised toxin genes, pBtoxis coding sequence (CDS) pBt054 is a previously uncharacterised CDS that encodes a protein of approximately 60 kDa, which is related at its N–terminus to the known Cyt toxins of *B. thuringiensis.* Comparison of this region of the CDS to known Cyt proteins indicates that it could represent a new subdivision of this family, Cyt1Ca according to the conventional *B. thuringiensis* toxin nomenclature, although confirmation of this provisional name awaits further experimental evidence of its properties. The pBtoxis CDS is, however, unusual in another way. Whereas previously recognized members of the Cyt family are proteins of approximately 26-28 kDa, pBt054 represents a fusion between the Cyt1Ca-like region at the N–terminus with an extra domain at the C–terminus. The last 280 amino acids of this C–terminal domain appear to be tandem beta-trefoil modules such as those found in other bacterial toxins such as ricin, *Clostridium botulinum* neurotoxin and the mosquito larvicidal Mtx1 toxin from *Bacillus sphaericus.* This superfamily of motifs is implicated as likely carbohydrate binding moieties so that one possible function for the C–terminal region of pBt054 could be recognition of carbohydrate groups on toxin receptors.

In addition to the complete toxin CDSs, pBtoxis also contains short sequences encoding remnant fragments of toxins. pBt025 and pBt026 encode two segments with homologies to the centre region of a Cry28Aa-like toxin whilst pBt053 appears to encode a sequence with homology to the extreme C–terminus of a Cry26Aa-like protein. In addition, the amino acid

sequence encoded by pBt055 is similar at its C–terminus to proteins encoded upstream of toxin genes (e.g. a hypothetical 29.1kDa protein in the *cry2Aa* 5'–region of *B. thuringiensis* subsp. *kurstaki*) whilst its N–terminus is similar to that of the Cry11Bb. These apparent *cry* toxin gene remnants suggest that during its evolution, the ancestors of pBtoxis have been host to other toxins now lost. This suggests that toxin composition is a dynamic factor and may help to explain the great diversity in toxin composition observed in *B. thuringiensis* isolates. The fact that these remnants are located close to CDS with possible roles in transposition (pBt052 is similar to IS240-A; pBt027 & pBt028 are similar to IS231W sequences) implies that transposition is the most likely mechanism for this effect and this is consistent with previous observations that *B. thuringiensis* toxin genes may be flanked by transposase sequences. In total, over 23% of the genes on pBtoxis show similarity to transposon-related genes, indicating a considerable amount of DNA exchange has occurred in the evolutionary history of pBtoxis. As previously reported, the *cry10Aa* gene (pBt047) is similar to the 5'–end of other *cry* genes and encodes an approximately 78kDa protein that would appear to be truncated with respect to related Cry proteins. This gene is followed by a second CDS (pBt048) with similarity to the 3'–end of other *cry* genes. The intervening 67bp contains at least two stop codons in each of the three reading frames and causes disruption of what may once have been a single CDS to produce two CDSs. However, protein derived from *cry10Aa* (pBt047) has been identified in Bti inclusions, indicating that this CDS is not a pseudogene remnant.

Bt strains, like the closely-related *Bacillus cereus*, are known to produce other potential virulence factors including phosphatidyl inositol-specific phospholipase C that may contribute to the role of the spore in overall toxicity. It appears that the pBtoxis plasmid encodes a separate, extra-chromosomal copy of a phosphatidyl inositol-specific phospholipase C (pBt087) although the presence of an in-frame TGA stop codon indicates that this is either a pseudogene, or is expressed by translational read-through.

Analysis of the plasmid revealed many other genes that may have significant effects on several aspects of the phenotype of the host organism, the most striking of which are potentially involved in (i) sporulation and germination, (ii) transcriptional regulation, (iii) possible production and export of a peptide antibiotic, (iv) amino-acid metabolism, and (v) plasmid replication and partition.

Possible similarities between pBtoxis and other *B. thuringiensis* plasmid sequences in the database were analysed by BLAST comparisons. The only significant match was between pBtoxis pBt010 and an unannotated CDS of unknown function in pTX14–3 from *B. thuringiensis* subsp. *israelensis* (44% id in 84 aa). No other database matches for these sequences exist so the physiological function, if any, of these sequences cannot presently be judged.

Overall, 29/125 predicted pBtoxis proteins show detectable similarity to predicted proteins from pXO1, the virulence plasmid from *Bacillus anthracis*. Excluding transposon or insertion-sequence related proteins, just 17 of the predicted pBtoxis proteins are similar to predicted proteins from pXO1. This corresponds with the results of a previous study looking at conservation of pXO1 genes in a variety of *Bacillus* species, where between 1 and 53 pXO1 genes were found to be present in different *B. thuringiensis* strains using hybridisation and PCR experiments.

Most isolates of *B. thuringiensis*, like subsp. *israelensis*, encode their insecticidal toxins on extrachromosomal elements. Since pBt007 was found to be conserved between pBtoxis and pXO1–16 (96% identity in 569aa), its distribution in other *B. thuringiensis* strains was also investigated by PCR. As expected, no amplicons were produced from the primers when the negative control Bti strain 4Q7, a strain cured of pBtoxis, was used. PCR also produced no

product from the following *B. thuringiensis* isolates: *dakota* (Oats43(4R1)), *kyushuensis* (HD541(4U1)), *morrisoni* (HD12(4K1)), *tenebrionis*, *tohokuensis* (78-FS-29-17(4V1)). This may reflect the absence of homologous sequences in these strains or could be a result of an alteration in nucleotide sequence in the regions corresponding to one or both of the test primers. However, the existence of pBt007–homologous sequences was revealed by the production of ~600 bp amplicons in the following *B. thuringiensis* isolates: *aegypti* (from commercial Agerin powder), *aizawai* (HD133(J3)), *galleriae* (HD155), *indiana* (HD521(4S2)), *israelensis* (IPS70(4Q3)), *israelensis* (HD500(4Q2)), *israelensis* (HD567(4Q1), *jegathesan* , *japonensis* (T23OO1(4AT1)), *kenyae* (HD136(4F1)), *kumamotoensis* (HD867(4W1)), *kurstaki* (HD1(4D1)), *kurstaki* (HD73(4D4)), *medellin*, *thuringiensis* (HD2(4A3)), *tochingiensis* (HD868(4Y1)), *tolworthy* (HD125(4L1)) and *wuhanensis* (HD525(4T1)). This indicates that the pBt007/pXO1-16-like sequence is widespread in *B. thuringiensis* isolates and we speculate that it is likely to be associated with the virulence plasmids in all of these strains. In addition, an amplicon of the same size was also produced from the house fly-toxic *B. cereus moritai* (originally named *B. moritai*), perhaps indicating that this isolate should again be reclassified as *B. thuringiensis moritai.*

DNA shuffling of *Bacillus thuringiensis* crystal proteins

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Bacillus thuringiensis (*Bt*) produces intracellular crystals consisting of one or more proteins called crystal proteins. Since the first discovery of this bacterium as an insect pathogen (Ishiwata, 1901), a large number of isolates showing various insect host specificities have been reported. In most cases, the crystal proteins, being toxic to insects, are responsible for the pathogenicity of this bacterium. The first gene encoding the crystal protein was cloned by Schenpf and Whitely (1981) and designated as *cry1Aa*. Since then, numerous *cry* genes have been cloned, and their amino acid sequences determined. These crystal proteins have been classified into some 30 classes based on sequence homology (Crickmore, 2000).

Several *Bt cry* genes have been used in transgenic crops such as cotton, corn and soybean. The *cry1Ac* gene was introduced into cotton for *Helicoverpa virescens* control (Perlak, *et al.,* 1990), and the *cry1Ab* gene was successfully used in corn to control *Ostrinia nubilalis* (Estruch, *et al.,* 1994). In both cases, the proteins encoded by these genes have very high activity against the primary insect target. However, the Cry1Ac and Cry1Ab proteins are less active against secondary targets such as the *Spodoptera* complex. For example, LC_{50} of Cry1Ac against *H. virescens* is as low as 0.05 ppm (Lee, *et al.*, 1995), but the protein has no significant activity against *S. exigua*. Certain crystal proteins such as Cry1Ca and Cry1Fa are known to have certain activity against the *Spodoptera* complex. Although Cry1Ca appeared to be the most active protein against *Spodoptera*, its LC_{50} is about 10 ppm (de Maagd, *et al.,* 1996). The level of activity found with *Spodoptera*-active crystal proteins is presumably not high enough for transgenic crops.

Attempts have been made to improve crystal proteins against various insect targets by protein engineering. Bosch *et al.* (1994) and de Maagd *et al.* (1996) made hybrid proteins by substituting Domain III of Cry1Ab and Cry1Ea with the corresponding domain of Cry1Ca and found notable activity improvements against *S. exigua*. For example, the Cry1Ab-1Ca hybrid showed more than a 60-fold activity enhancement over its parent Cry1Ab protein. These results indicate that domain exchange is a powerful tool to improve the activity of *Bt* crystal proteins. It appears that *Bt* evolves its crystal proteins by exchanging domains among different proteins as indicated by Crickmore (2000).

Mutagenesis, either random or site-specific, can improve the activity of crystal proteins. Jellis *et al.* (1989) created several thousand mutants of Cry1Ab Domain I by chemically induced random mutagenesis and identified a number of mutants showing improved activity against *H. virescens*. Wu and Dean (1996) reported that the activity of Cry3Aa against *Tenebrio molitor* was increased by mutating a block of amino acid residues in a loop in Domain II. These reports indicated that mutations made in Domain I loops connecting various alpha-helices and Domain II loops connecting beta-strands could increase the activity of *Bt* crystal proteins (Yamamoto and Dean, 2000).

Random mutagenesis can generate a large number of mutants at a time. These mutants must be screened by insect assay in order to find ones with increased activity. Insect assay is time consuming and labor intensive. On the other hand, site-specific mutagenesis creates a limited number of mutants. So far, most studies have been conducted by site-specific mutagenesis. Although 3-D structures of several crystal proteins have been determined and intensive studies

have been conducted on the mode of action, it is still not easy to design improved crystal proteins efficiently by site-specific mutagenesis.

Currently, the agricultural chemical industry practices high throughput screening of a massive number of chemical compounds. We have applied this practice to *Bt* crystal proteins with a technology called DNA shuffling. DNA shuffling, described by Stemmer (1994), is a powerful technology that has been used to improve the biological activity of many proteins. DNA shuffling produces a large number of recombinant genes from one or several parent genes. These recombinant genes are screened for desired traits. To apply the technology to *Bt* crystal proteins, we developed a cloning and expression system in a *cry*-minus *Bt* strain and a high throughput insect screening protocol that is capable of handling 10,000 samples per week. In our model system, we shuffled the *cry1Ca* gene (Honee, *et al.,* 1988) and made substantial improvements in its activity against *S. exigua*.

Shuffling of *cry1Ca* was performed as described by Stemmer (1994). A portion of the gene from the translation start to the *kpn*I site was shuffled under certain mutational conditions. This shuffled portion contains the sequence coding for the mature toxin. In order to clone and express the shuffled gene library, we constructed an *E. coli-Bt* shuttle vector that contains a tetracycline-resistant gene and two replicons for both hosts. The vector also contains the remaining (not shuffled) 3′ portion of the *cry1Ca* gene from the *Kpn*I site to the translation end along with the *cry1Ca* transcription promoter and *cry1Ac* terminator. When the shuffled gene library was cloned in this vector, full-length 135-kDa proteins were produced.

The shuffled gene library was expressed in a *cry*-minus *Bt* host, which was derived from the HD1 strain by plasmid curing. A selection was made to assure a high transformation competency required for making a diversified shuffled library. The selected host, *Bt*-G8, showed a level of competency of over 10^6 transformants per mg DNA.

A shuffled gene library was made by sequentially transforming *E. coli* XL-1 Blue, *E. coli* GM2163 and *Bt*-G8 by electroporation. XL-1 Blue was used for the high transformation efficiency. The plasmid was prepared from transformed XL-1 Blue cells, and a small portion was examined by gel electrophoresis to ensure no visible amount of vector molecules without the shuffled DNA. GM2163 was used to prepare unmethylated DNA required for *Bt*-G8 transformation. The transformed *Bt*-G8 cells that grew on tetracycline plates were picked onto 96-well plates by robot. These plates were incubated until sporulation and were used as seeds for assay sample production.

We developed a two-tier insect screening to obtain high throughput. The first tier was to eliminate clones without any detectable activity. The first tier assay samples were produced in CYS liquid medium (Yamamoto, 1990) in shallow 96-well plates. At this stage, the culture broth containing crystals and spores was assayed in neonate *S. exigua* larvae in 96-well plates containing an artificial insect diet. The clones showing the activity were selected for the next step. For the second tier screening, the crystal proteins were purified from 1 ml culture broth produced in deep 96-well plates by differential solubilization between pH 10.5 and pH 4.4. The crystals were solubilized at pH 10.5 with 2% 2-mercaptoethanol, and the solubilized crystal proteins were precipitated at pH 4.4. After protein concentrations were determined, serial dilutions were made and assayed against *S. exigua* larvae. We have developed an automated assay system which utilizes robots for different tasks such as diet filling, sample loading and scoring. We used an automated scoring system consisting of a plate-handling robot, a high-resolution camera and an image analysis computer. Since our scoring was based on insect growth and diet consumption, the activity was expressed in EC_{50} rather than LC_{50}.

Our goal was to find a shuffled gene or genes that showed improved activity over the wild-type gene quickly and accurately.

After screening several thousand clones, we found a substantial number of proteins showing improved *S. exigua* activity up to four-fold over the parent Cry1Ca protein. When the amino acid sequences of these improved proteins were determined, one to two mutations were found in each protein at various locations suggesting that the activity improvement can be attributed to multiple mechanisms.

The results we obtained demonstrate that we can improve insecticidal activity of *Bt* crystal proteins by DNA shuffling and high throughput insect screening. Through DNA shuffling, we hope to create *Bt* crystal proteins that not only have higher specific activities against their original insect target but also have activities against a broader range of targets.

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Symposium (Fungi 3) Genetic Structure of Fungal Populations

The genetic structure of members from the *Entomophthora muscae* species complex proposes high host specificity and clonal life history strategies

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Summary

Molecular methods can together with morphological and pathological observations be used to elucidate different aspects of fungal biology. Within the Entomophthorales molecular characters have added significant information which enlighten areas as phylogeny, taxonomy and geographic distribution (Jensen *et al.*, 1998; Jensen and Eilenberg, 2001; Nielsen *et al.*, 2001), and most recently population genetic studies of fungi from the *Entomophthora muscae* species complex have given insight about their life history strategies.

Introduction

Fungi from the *E. muscae* species complex are important for the natural population regulation among adults from of several Diptera (Cyclorrhapha) families. The epizootic potential of these fungi has been demonstrated in several pest species and they therefore posses a potential to be used in biological control (Mullens *et al.*, 1987; Steinkraus *et al.*, 1993; Watson & Petersen, 1993; Klingen *et al.*, 2000).

The *E. muscae* species complex includes four recognized species, which are separated mainly based on characters of their primary conidia and host range. The four species are the low nucleate *E. schizophorae* Keller & Wilding (Keller, 1987), the high nucleate *E. muscae* s.str. (Cohn) Fresenius (Keller *et al.*, 1999), *E. scatophagae* Giard and *E. syrphi* Giard (Giard, 1888). Molecular characters support the separate origin of *E. schizophorae*, *E. muscae* s. str. and *E. syrphi* (Jensen & Eilenberg, 2001). The complex is currently being further subdivided into more species (Keller, in press).

The host specificity within the *E. muscae* species complex is thought to be high, but to better predict the interaction between flies and fungus and eventually epizootic developments, knowledge of the exact host range of each specific fungal species or genotype is important.

Physiological host range

In the laboratory the physiological host range can be tested by transmission experiments. Transmission of fungi from the *E. muscae* species complex has been challenged several times, and transmissions of fungi within and between fly families were achieved (Baird 1957; Kramer

& Steinkraus 1981; Eilenberg *et al.* 1987; Mullens 1989; Steenberg *et al.* 2001; Jensen *et al.* 2001). One study showed futher that transmission between host orders (from Hymenoptera to Diptera) was possible (Eilenberg *et al.*,1987). Generally receptor flies from other host genera are less susceptible than the original host flies or closely related flies.

The physiological host range does not necessarily reflect the ecological host range. The latter is, among others, dependent of both the spatial and temporal distribution of the pathogen and the host. This is exemplified with *Entomophaga maimaiga* were 7 out of 10 lepidopteran superfamilies were susceptible in laboratory tests, but only very few infected lepidopteraen larvae except *Lymantria dispar* were found in nature (Hajek *et al.*, 1995; Hajek *et al.*, 1996).

Ecological host range / population genetic structures

We conducted a study on the genetic structures of *in vitro* isolates of *E. muscae* s.str.. Two multi loci (unspecific PCR) and a single locus (PCR-RFLP on ITS II and LSU rDNA) approach showed that *E. muscae* could be divided into several different genotypes, and that each of the genotypes were restricted to a single host taxon (Jensen *et al.*, 2002). This shows that the genotypes within *E. muscae* s.str. have a very narrow host range and suggests that in this case as well, the physiological host range can not be extrapolated to account for the ecological host range.

Hardly any genetic variations were detected within the *E. muscae* s.str. isolates originating from the same host species though the isolates were sampled on different dates and different locations throughout Denmark between 1986 and 2000 (Fig. 1). Therefore it seems that the different genotypes consist of populations of the same clone (Jensen *et al.*, 2002). A similar clonal distribution has also been reported within the *Entomophaga grylli* species complex (Bidochka *et al.*, 1995).

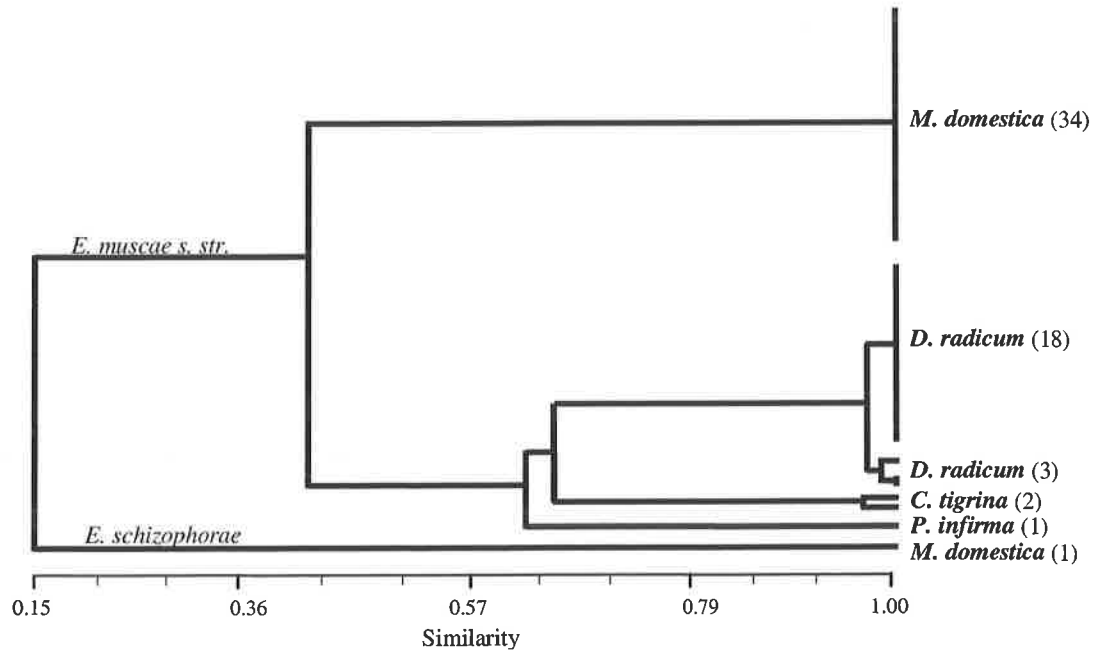

FIG. 1. Dendrogram based results obtained by RAPD of 59 *E. muscae* s.str. *in vitro* isolates.

Resting spores

Most fungi from the *E. muscae* species complex survive periods with unfavourable weather conditions and/or shortage of hosts as thick-walled resting spores. Resting spores can if they are zygospores present the sexual structure within Entomophthorales, but *E. muscae* resting spores are azygospores (Thomsen *et al.*, 2001).

Resting spores from the *E. muscae* species complex are spherical and surrounded by a thick cell wall, though the different species can not be distinguished morphologically. A nested PCR-RFLP approach were used to characterize resting spores genetically and by comparing them to known isolates the resting spores were determined to species level (Thomsen & Jensen, in press). It appeared that the resting spores as well as conidia-producing cadavers showed the same host related and clonal pattern as for the *E. muscae* s.str. *in vitro* isolates.

Conclusion

In conclusion it seems that different members of the *E. muscae* species complex are able to complete their lifecycle in one host species, and that each pathogen-host system is independent. It further seems that they have abandoned sexual recombination and posse a clonal lifehistory strategy. The high host specificity probably selects against genetic recombination since different genotypes will not meet in the same host. This led us to the question on how these specialized fungi have coped with the evolving insects over an evolutionary timescale.

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Parasexuality and its significance in natural populations of entomopathogenic fungi

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Diversity is essential for survival and evolution among and whithin species. This diversity is achieved from modifications in DNA, the genetic material of living organisms through a mechanism designated mutation. However, only mutations are not enough to provoke all variability necessary for the living beings to cope with the constant alterations in their habitats, mainly in the environment as well in other surrounding organisms also including anthropogenic changes, that is, alterations caused by man. A second process called genetic recombination acts , amplifying species variability, by combining in one individual two or more mutations. This junction may enable one individual to survive in conditions that their parents would not been mantained. In fungi, many species can produce recombinants through a sexual cycle.

Sexual recombination can be found in four out of the five main groups of fungi: Ascomycetes, Basidiomycetes, Zygomycetes and Oomycetes. On the other hand , species belonging to the fifth group that is, the group of Deuteromycetes also known by the common name of Imperfecti Fungi , does not present the sexual cycle. . In this last group many important entomopathogenic fungi are included as is the case of the genera *Metarhizium* and *Beauveria* .Sexual reprodution in fungi is fundamentally similar to that in other organisms . It involves the fusion of two haploid nuclei to form a diploid nucleus. However, in the majority of the fungi the diploid phase is very short lived , in opposition to what occurs in plants and animals. So, shortly after being formed the diploid nucleus undergoes meiosis and the recombinant nuclei have mixtures of the parental nuclei. In this way different genotypes are originated contributing to increase variability in species which reproduces by sexual recombination. To achieve nuclear fusion, many fungi can form heterokaryons in which genetically different haploid nuclei may co-exist in the same cytoplasmic system. Heterokaryons are originated by migration of nuclei through fusion points between different hyphae. Heterokaryons also can be formed by mutations in a homokaryon hyphae. In the same way, heterokaryons can also occur in Imperfect Fungi , but since they do not have sexual stage , no recombinants are produced by nuclei fusion followed by meiosis.

In 1952, Pontecorvo and Roper were able to show that an alternative process of recombination was taking place in the Ascomycete fungus *Aspergillus nidulans*. Using appropriated strains with different colour and auxotrophic genetic markers they were able to show that in the heterokaryons formed from two of such strains, nuclear fusions occurred in very low frequencies in hyphae regions which were not able to proceed sexual reproduction. In this way, the diploid formed suffered mitotic divisions producing diploid conidia. These conidia germinating on appropriated media originated diploid colonies but such colonies were unstable, producing sectors .These sectors proved to be recombinants between the two employed parental strains . Such recombinants were produced by two events : haploidization and mitotic crossing-over. The process has been termed parasexual cycle . It has the same effect as a regular sexual cycle in accomplishing genetic recombination and thus increasing the available genetic recombination. Soon after the discovery of the parasexual cycle in *A . nidulans* the same cycle was also described in Deuteromycetes as *A . niger*, *Penicillium chrysogenum* and some plant

pahogenic fungi as *Fusarium oxysporum* and *Verticillium albo-atrum* . The importance of this alternative to the sexual stage was shown by producing new races of plant pahogenic imperfect fungi. Also the parasexual cycle proved to be an important and valuable tool for genetic studies and for the improvement of fungi , especially the imperfect ones. Today parasexuality processes have been demonstrated in many fungi including several imperfect species (Azevedo, 1998).

Among entomopathogenic fungi Messias and Azevedo (1980) have demonstrate the occurrence of parasexuality in *Metarhizium anisopliae.* Heterokaryons between colour and auxotrophic mutants derived from a strain isolated from an adult insect of the genus *Deois* (Homoptera , Cercopidae) produced very unstable diploids. From the diploids , recombinant haploid sectors were recovered. These findings were important for the developent of basic genetics and applied studies in this species. Silveira and Azevedo (1987) tried to obtain diploids and recombinants using various combinations of *M. anisopliae* strains collected from different regions of Brazil. In many instances , these combinations did not form heterokaryons, probably due to genetic incompatibility between strains preventing in this way hyphae fusions and consequently heterokaryon formation. However successful heterokaryon formation was achieved by protoplast production and fusion between these incompatible strains. As a result , many recombinants were obtained but in all cases the diploid stage was not detected . Conidia from heterokaryons formed between well-marked mutant strains, when plated onto appropriate selective media, resulted in the recovery of recombinants, some unstable ones and others consisting of stable haploids. The same results were obtained by Bagagli et al (1991) with conidia derived from non-protoplast derived heterokaryons of *M. anisopliae* The emergence of haploid recombinants from heterokaryons without the visible intermediated diploid stage had already been observed in other imperfect fungi. This alternative variation of the parasexual cycle named parameiosis was first described by Bonatelli Jr. et al. (1983) in *A. niger*. Paccola-Meirelles and Azevedo (1991) described a similar parasexual process in the entomopathogenic fungus *B. bassiana*. Also in this fungus, no diploids were isolated but haploid recombinants were directed recovered from heterokaryons. It is interesting to note that in sexual reproducing fungi as *A. nidulans*, no parameiosis was found. In imperfect fungi as *A. niger* both relatively stable diploids and the parameiotic process co-exist. On the other hand, in the imperfect fungus *M. anisopliae* , diploids are very unstable and in some instances only the parameiotic mechanism is responsible for the producion of recombinants. Finally, in *B. bassiana* no diploids were dettected, but haplois recombinants were frequently obtained from conidia recovered from heterokaryons. These findings are shown that the parameiotic process is probably absent in sexual fungi. On the other hand in imperfect fungi there is a correlation between the rate of fungal growth and frequency of recombinants. In *A. niger*, a rapid and agressive filamentous fungi, both typical parasexual cycle and the alternative parameiosis co-exist. In *M. anisopliae* diploids are rarely found and in *B. bassiana* a slow growing fungus the diploid stage was not isolated but haploid recombinants are frequently found. Besides transient diploids, several other mechanisms may explain the emergence of recombinants through parameiosis. A mechanism of intertransformation as suggested in *Trichoderma* (Stasz and Harman,1990), a primitive meiosis (Bagagli et al 1991) or transposons may also be responsible for the produced recombinants. In any way, the process may play an important role in the natural variability found in fungi including the entomopathogenic ones.

Variability in fungi as *Metarhizium* and *Beauveria* was detected by several ways. In *B. bassiana* a variety of techniques including esterase polymorphism (Paccola-Meirelles and Azevedo, 1990; St. Leger et al., 1992), electrophoretic protein fraction patterns (Goristein et al., 1996) mitochondrial DNA (Hegedus and Khachaturians, 1993), random amplified polymorphic DNA (RAPD) markers (Bidochka et al., 1994; Urtz and Rice, 1997; Berretta et al., 1998; Castrillo

et al., 1999), Pulsed Field Gel electrophoresis (Pfeifer and Khachatourians, 1993), ITS sequencing and PCR-RFLP (Coates et al., 2002) have shown there is much variation among strains, possibly due to parasexual recombination. In *M. anisopliae* the described processes of recombination also may be explain the extensive variation that occurs in nature. In this species, Leal-Bertioli et al., (2000) suggested that recombination may occur during insect infection. This was detected by several techniques including esterase electrophoresis, restriction digestion of total genomic DNA and RAPD-PCR.

The role of parasexuality in imperfect fungi increasing natural variability is of paramount importance for evolution and survival of these species. In fungi of applied importance as the entomopathogenic fungi parasexuality has already been used to produce improved strains for biological control. These techniques allied to the DNA recombinant methodology may lead to less empirical and more rational ways to produce strains of entomopathogenic fungi more effective for insect biological control.

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Symposium (Cross-Division 1) Bacteria/Insect Interactions: Virulence Aspects

Environmental sensing in *Bacilli*: a basis for host specificity

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Introduction

Pathogenic members of the *Bacillus cereus* group are important as the principal biopesticide in insect control (*B.thuringiensis)* and as agents of human disease (*B. anthracis* and *B. cereus*). The emerging chromosomal similarity of the three organisms has resulted in claims that they are one species (Helgason *et al.,* 2000). If this is true, how can they differ so widely in their host targets? First their host range may depend upon additional virulence factors on extrachromosomal elements. Some support for this comes from the identification of crucial *B. anthracis* and *B. thuringiensis* virulence genes on resident plasmids. Second, differential gene expression in response to host-specific sensing may explain/influence different pathotypes. Investigation of both these possibilities at the genetic and molecular level will contribute significantly to our fundamental understanding of pathogenesis (Kotiranta *et al.,* 2000).

Comparison of *B. thuringiensis* and *B. cereus* virulence factors can reveal both general and host-specific factors required for infection of insects or humans respectively. Identification of factors vital for pathogenesis would also allow the directed identification of the most entomopathogenic *B. thuringiensis* strains for use in insect control and also identify novel targets for the chemotherapeutic treatment of *B. cereus* infections of humans.

Bacteria continuously monitor their environment and modulate their gene expression in response to changes in growth conditions. When a bacterium invades a host organism, whether insect or mammalian, it will experience several changes in its environment. The supply of many nutrients/minerals is likely to be depleted and there may be changes in growth temperature, CO_2 concentration and oxidative stress (Mekalanos, 1992). By replicating these environmental changes *in vitro*, variations in gene expression which may well occur inside the infected host can be detected and genes important in adapting to environmental changes and ensuring a successful host colonisation can be identified. A combination of *in vivo* and *in vitro* methods can provide a fuller picture of the pathogenic mechanisms of *B. cereus* and *B. thuringiensis*.

In vivo identification of *B. thuringiensis* virulence factors by STM

Signature-tagged mutagenesis (STM) was originally developed by Hensel *et al.*, (1995) to screen large numbers of mutants simultaneously for attenuation in virulence in an infection model. Following infection, surviving (unattenuated) mutants can be identified on the basis of a unique DNA "tag" incorporated into the transposon used to generate the mutants.

We used STM to discover genes involved in *B. thuringiensis* virulence. Many known *B. thuringiensis* virulence factors are either secreted from the cell or synthesised during sporulation. STM does not identify mutants disrupted in secreted virulence factors because the phenotype is transcomplemented by other mutants in the infection pool. To avoid finding virulence factors expressed during sporulation, vegetative cells were used in the infection model. Hence, it was anticipated that previously described virulence factors would not be "re-found".

1152 *B. thuringiensis* MEX312 (Guttmann & Ellar,2000) mutants were generated using the suicide delivery vector pID408 (Mei *et al.*, 1997) containing the transposon Tn917. The mutants were screened for attenuation of virulence in *Manduca Sexta* (tobacco hornworm) upon injection of vegetative cells into the haemolymph of 5th instar larvae. The mutants were injected in pools of 72, each mutant in the pool bearing a unique 40 base pair DNA "tag". Mutants whose tags were present in the inoculum used to infect the larvae, but not in the larval haemolymph 24 hours post-injection, were classed as attenuated. Following two screens 23 attenuated mutants were identified; further screening may identify yet more attenuated mutants. Cloning and sequencing of the transposon-flanking ends of the attenuated mutants revealed 12 to be disrupted in unique positions in the *B. thuringiensis* MEX312 genome. To confirm attenuation of virulence, *in vivo*/*in vitro* competition assays versus wild type *B. thuringiensis* MEX312 were performed according to the method of Darwin and Miller (1999) (with modifications) on 3 mutants. The results confirmed attenuation of all three mutants in comparison to the wild type since they were out-competed in the infection model but not in liquid culture. Sequence analysis of the disrupted DNA using FASTA and FASTY implicated the involvement of a transcriptional regulator, a histone-like DNA-binding protein, two bacteriophage-like genes, a transposon and several genes of no known function (Table 1) in virulence of *B. thuringiensis* MEX312 towards *M. Sexta*.

Two mutants, 1A9 and 1A12, were found to be disrupted in neighbouring open reading frames (orfs) in the genome. These two orfs were shown by reverse transcriptase polymerase

TABLE 1. The programs FASTA and FASTY were used to identify similarity between DNA disrupted in the 12 attenuated *B. thuringiensis* MEX312 mutants found using STM and sequences held in the public databases. For each mutant the organism and function of the database sequence with the highest similarity is shown, as well as the accession number, percentage identity and the number of nucleotides (nts) or amino acids (AAs) in the overlapping region. Mutants not attaining an e-score of <0.001 in the similarity searches were considered to show no significant similarity (NSS) to any sequence on the databases.

Mutant	Organism	Function	% identity	No. of nts or AAs in overlap	Accession number
1A7	NSS				
1A9	*C. acetylbutylicum*	Hypothetical protein	29.55	88 AAs	AAK79880
1A12	*B. anthracis*	PXO1-138	54.9	82 AAs	Q9X394
1B11	*B. subtilis*	Complete genome non-coding region	77.9	190 nts	BSUB0003
1D10	*B. thuringiensis*	Tn4430	100.0	105 nts	BTTN4430
2C3	NSS				
5A1	*B. halodurans*	Hypothetical protein	38.9	72 AAs	Q9KE96
6F8	*S. pyogenes*	Bacteriophage-like hypothetical protein	50.0	90 AAs	Q9A0Q5
6F9	*Lactobacillus* phage phi g1e	Hypothetical protein	35.9	117 AAs	O03921
8E3	*B. thuringiensis*	Tn4430 transposase	100.0	684 nts	BTTN4430
9D8	*B. subtilis*	Histone-like DNA- binding protein	67.4	89 AAs	PQ8821
10C11	NSS				

chain reaction to be transcribed as one (data not shown) and together with downstream sequences, may constitute a virulence-associated operon. The orf disrupted in 1A12 is related to the ArsR family of transcriptional regulators. Interestingly, the closest homologue of this orf found on the public databases was a putative gene on the virulence plasmid, pXO1, of *B. anthracis*.

None of the 12 sequences identified were previously known to be involved in virulence. To confirm that the transposon insertions are responsible for the attenuation, complementation and inactivation of the disrupted genes will be carried out.

In vitro identification of *B. cereus* and *B. thuringiensis* virulence factors by RAP-PCR

RAP-PCR was developed for use in *B. cereus* by a modification of a method utilised in *Enterococcus faecalis* (Shepard and Gilmore, 1999). The technique enables gene expression to be monitored under varying *in vitro* conditions and differentially expressed genes to be cloned and sequenced. Gene expression was investigated in *B. cereus* 11145 by RAP-PCR at 30°C and 37°C, a change in growth temperature that may occur when *B. cereus* enters a human host. A homologue of *B. subtilis* PerR (Peroxide regulon repressor) that is down-regulated in response to higher growth temperature was identified and an equivalent homologue was found in *B. thuringiensis*.

Changes in expression of a PerR homologue in response to growth temperature was a novel and very interesting result. PerR is a member of the Fur family of transcriptional regulators controlling a number of genes involved in regulation of intracellular ferric iron concentration, zinc concentration and resistance to peroxide stress (Bsat *et al.,* 1998). Iron homeostasis is of critical importance to growing bacteria as excess iron can cause uncontrolled production of superoxide via the Fenton reaction that would be detrimental to the cell (Hantke and Braun, 2000) Intriguingly, Fur also plays play a role in regulating the expression of virulence factors in a range of other species, including haemolysin production in *Escherichia coli* (Panina *et al.*, 2001). In order to investigate this family of regulators further and probe the role they may play in *B. cereus* and *B. thuringiensis* virulence, both PerR and Fur genes were cloned from *B. cereus* 11145 using sequence data from *B. anthracis* and *B. cereus* genome sequencing projects (Preliminary data obtained from http://www.tigr. org and http://www.integratedgenomics.com).

B. cereus fur was predicted to encode a 17.5 kDa protein with 95% sequence homology to *B. subtilis* Fur. The BcFur protein was over-expressed and purified to allow further study of its function. The protein selectively bound a 17bp DNA sequence (known as a Fur box) identical to the *B. subtilis* Fur binding region. *In vitro* experiments demonstrated that *B. cereus fur* was auto-regulated via an upstream Fur box from which expression of the *fur* mRNA was initiated.

To identify other genes whose expression is repressed by Fur, the confirmed Fur box sequence was searched against the available *B. cereus* genome sequence. Sequences matching the Fur box sequence were checked to confirm their position upstream of an identified orf. Results were only accepted if they were within 250 bp of an orf and the Fur box sequence was no more than 2 bp divergent from the *in vitro* confirmed sequence. Identified orfs were searched against the public databases to confirm their identity. The results of this computer analysis are shown in Table 2.

It can be seen from the table that a wide range of genes is predicted to form part of the Fur regulon. As expected, a number of genes involved in siderophore synthesis, bacterial iron uptake and storage were identified. Control of these genes by Fur allows bacteria to respond

TABLE 2. Genes identified in the available *B. cereus* genome sequence data as possessing an upstream Fur binding box. Orf identities are those assigning by the genome-sequencing project or the result of a FastA search against the available public databases. Fur box positions are given as base pairs upstream from the orf start codon.

ORF N°	Gene Identity	Position of Fur box	Fur box sequence	Function
05624	*fer*	113	TaattGATAAGCATTATCATTAtaatt	Ferredoxin
00055	*fhuB*	76	AaaatGATAATTATTATCAATAataat	Ferrichrome ABC transporter (permease)
05678	*ygfV*	86	AaattGATAATTATTCTCTCAAAagtat	Fur homologue
01567	*iucA*	86	TaattGATAATGATATTCAATTcaata	Siderophore biosynthesis
05783	*hemH*	86	GtaaaGATAATTATTCTAAATAaggat	Ferrochelatase
00032	*dhbA*	51	TgattGATAATGAAAATCAATATcatt	Siderophore synthesis
05890	*inlA*	75	AcaatGATAATGAAAATCATTATcatt	Internalin
00545	*yadT*	33	TaattGATAATGATAATCATTAActac	Fe(III) dicitrate binding precursor
04080	*pls*	50	AaaatGATAATTATTCTCAATTcataa	Surface protein- adhesion
02695	*araC*	188	TtattGATAATGATTTTCATTGTagtt	MSM operon regulatory protein
03340	*ykuP*	126	AaattGATAATTATTATCAATAGgatc	Probable Flavodoxin I
05173	–	74	CatttGATAATCATTTTCACTGTaact	Hypothetical
04469	*ywbD*	223	CaaatGATAATGAAAATCATTATcatt	Methyltransferase
02143	*feuA*	173	GattcGATAATCATTATCAATGAaaat	Iron uptake binding precursor
04714	*inlA*	49	TaaatGATAATGAATCTCATTATcatt	S-layer precursor
05482	*yviB*	105	AaaatGATATTTATTATCAATActata	Cytochrome p450
01123	*hlyII*	174	AtgttGATAATAGTTATCAATAatagt	Haemolysin II

to changes in intracellular iron concentration and to scavenge more iron from the environment when free iron concentrations are reduced. Genes involved in central carbon metabolism and energy generation were also identified as having upstream Fur box sequences. When carrying out a pathogenic response, the invading bacterium has to increase rates of protein synthesis to allow production of secreted enzymes in order to be successful in its pathogenic strategy. A higher growth rate would also allow the bacterium to rapidly take advantage of any available niche within the animal host which may be a short-lived. Limitation of the iron supply is likely to occur when the bacterium invades a eukaryotic host. Further RAP-PCR analysis of *B.* cereus gene expression under iron-limited conditions (data not shown) demonstrated that several genes involved in the central carbon metabolism are up-regulated in response to iron deprivation, suggesting a regulatory role for Fur. Table 2 also shows that Fur box sequences are upstream of several genes with potential roles in virulence, including genes involved in cellular adhesion and the invasion of eukaryotic cells. Other virulent bacterial species have been shown to activate transcription of similar genes in response to a limitation of the iron supply. By invading a eukaryotic cell, the bacteria gains access to a larger supply of nutrients and also gain protection from the host immune system and other defensive mechanisms. These results suggest that Fur may play an important role in regulating several important virulence factors in *B. cereus* and probably *B. thuringiensis*. The creation of a series of knockout mutations whose virulence can be measured will confirm this fact.

Conclusions

The results presented here demonstrate that virulence in *B. thuringiensis* and *B. cereus* is likely to be a multifaceted response involving a large number and wide range of bacterial genes. Our work has led to the identification of at least four transcriptional regulators likely to be involved in the virulence of these two organisms. When combined with previously identified

virulence regulatory genes such as PlcR (Agaisse *et al.,* 1999), it is clear that the bacterium has a variety of regulatory systems controlling virulence genes. By having a complex regulatory system the bacteria ensures that virulence gene expression is tightly controlled and only stimulated when conditions are favourable for a successful colonisation of the infected host. As is commonly found in other bacterial pathogens, we have demonstrated that a number of genes previously regarded as having only "house-keeping" functions are involved in the virulence of *B. thuringiensis* and *B. cereus*. It is clear that for a bacterial pathogen to successfully colonise a eukaryotic host, it not enough to simply increase the expression of secreted enzymes but it must also modulate its entire central metabolism. It would appear that in common with virtually all other pathogenic microbes, a virulent phenotype in *B. cereus* and *B. thuringiensis* is the result of a large number of interacting and overlapping factors and not the product of a single gene.

The identification of novel transcriptional regulators that play a key role in virulence opens many opportunities for development of both improved biopesticides and chemotherapeutic agents. Modulation of the expression of these transcriptional regulators in recombinant *B. thuringiensis,* strains may lead to more virulent strains which would be of higher efficacy as biopesticides. Conversely, the same transcriptional regulators could prove to be ideal targets of chemotherapeutic drugs as they are unlikely to have structural homologues in eukaryotic cells. Structural studies of Fur, ArsR and related regulators could lead to the rational design of small molecule inhibitors which could function as effective anti-microbial agents.

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Identification of new *Bacillus thuringiensis* virulence genes by genetic approaches

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In addition to the well studied d-endotoxins, *B. thuringiensis* spores and vegetative cells also have insecticidal activity. Indeed, mutant strains lacking crystals are highly pathogenic when injected into certain Lepidopteran larvae (Zhang *et al.*, 1993). Moreover, the addition of *B. thuringiensis* spores or vegetative cells strongly increases the insecticidal activity of Cry toxins against some insect species that are weakly susceptible to the ingestion of crystals alone (Li *et al.*, 1987; Salamitou *et al.*, 2000). *B. thuringiensis* cells have been shown to produce a variety of putative virulence factors that are speculated to facilitate the development of these bacteria within the host. These include phospholipases, enterotoxins, hemolysins and proteases, encoded by genes forming the PlcR regulon (Agaisse *et al.*, 1999). However none of these factors has been confirmed to be essential for the pathogenic mechanisms leading to systemic septicemia when bacteria are injected into the insect host. In this study, we are interested in identifying new genetic determinants responsible for the virulence of *B. thuringiensis* in insects. We focused on two *B. thuringiensis* protease families, known or suspected to be virulence factors in various pathogenic bacteria: the extracellular zinc-requiring metalloproteases and the intracellular Clp proteases.

The zinc metalloprotease InhA2 is required for pathogenicity of *B. thuringiensis* in insects, *via* the oral route. *B. thuringiensis* secretes a neutral zinc metalloprorease termed InhA (immune inhibitor A). This protease has been shown to specifically degrade antibacterial proteins from the silk moth *Hyalophora cecropia* and to be toxic to several other insect species (Dalhammar and.Steiner, 1984, Siden *et al.*, 1979). The *inhA* gene has been cloned and sequenced. The study of the regulation of this gene showed that *inhA* expression starts at the onset of sporulation and is activated by Spo0A, *via* AbrB (Grandvalet *et al.*, 2001). In the acrystalliferous *B. thuringiensis* strain 407 Cry$^-$, we identified a second copy of the *inhA* gene, named *inhA2*. The *inhA2* gene encodes a putative polypeptide showing 66.2% overall identity with the InhA protein and harboring the zinc-binding domain (HEXXH), which is characteristic of the zinc-requiring metalloproteases. Expression of a transcriptional *inhA2'-lacZ* fusion shows that *inhA2* transcription is induced at the onset of the stationary phase and is overexpressed in a Spo0A minus background (Fig. 1). The presence of a reverse Spo0A box in the promoter region of *inhA2* suggests that Spo0A directly regulates the transcription of *inhA2* (data not shown). Δ*inhA* and Δ*inhA2* mutant strains were constructed to determine the role of the InhA and InhA2 etalloproteases in pathogenesis. Spores and vegetative cells of the mutant strains were as virulent as those of the parental strain in immunized *Bombyx mori* larvae infected by the intrahemocoelic route (data not shown).

We investigated the synergistic effect of the mutant strain spores on the toxicity of Cry1C proteins against *Galleria mellonella* larvae infected *via* the oral pathway. The spores of D*inhA2* mutant strain were ineffective in providing synergism whereas those of the D*inhA* mutant strain were as active as the wild-type strain (Fig. 2). These results indicate that the *B. thuringiensis* InhA2 zinc metalloprotease has a vital role in virulence when the host is infected via the oral route (Fedhila *et al.*, 2002. In press).

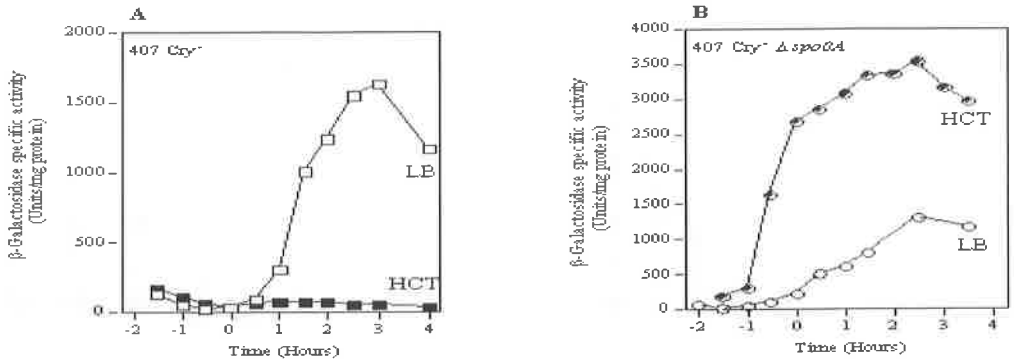

FIG. 1. Spo0A negatively regulates the transcription of *inhA2*. Expression of *inhA2'-lacZ* in strains 407 Cry⁻ (A) and 407 Cry⁻ Δ*spo0A* (B) at 30°C. Open and solid symbols indicate β-galactosidase activity expressed in units/mg protein (Miller units) when the strains were grown in LB and HCT.

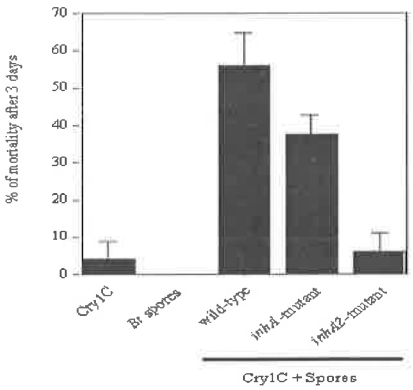

FIG. 2. Results from three pooled independent experiments. Last instar *G. mellonella* larvae were force-fed with spores alone, crystals alone or spore/crystal mixtures. In all experiments, the doses of spores and crystals were 1 ´ 10^6 and 3.2 µg *larva*$^{-1}$ respectively. For all the strains, spores alone caused no mortality. Vertical bars indicate the standard errors of the mean.

Two distinct *clpP* genes control specific adaptive responses in *B. thuringiensis*.

ClpP is the proteolytic subunit of the Clp ATP-dependent proteases, which is ubiquitous among prokaryotic and eukaryotic organisms. The role of the ClpP protein in stress tolerance, stationary phase adaptive responses and virulence has been demonstrated in many bacterial species. Based on the amino acid sequence of the *Bacillus subtilis clpP* gene, we identified two *clpP* genes (designated as *clpP1* and *clpP2*) in *B. thuringiensis* strain 407 Cry⁻. The ClpP1 and ClpP2 predicted proteins share 88% and 67% amino acid sequence identity with ClpP of *B. subtilis*, respectively. The two genes were deleted by allelic exchange in *B. thuringiensis* 407 Cry⁻. The Δ*clpP1* and Δ*clpP2* mutants were both slightly susceptible to salt stress whereas disruption of *clpP2* negatively affected sporulation and abolished motility (data not shown). The virulence of the mutant strains was assessed by injecting bacteria into the hemocoel of *Bombyx mori* larvae. The *clpP1* mutant displayed attenuated virulence (table 1). The analysis of the bacterial growth at various temperature suggests that the loss of virulence is related to the inability of the *clpP1* mutant to grow at 28°C (Fig. 3). This indicates an essential role for ClpP1 in low temperature tolerance.

TABLE 1. Role of ClpP1 and ClpP2 in the virulence of *B. thuringiensis* against 4th instar *B. mori* larvae.

Strains	LD50[a] (CFU/injected *larva*)
407 Cry⁻	4,79 (3,28-6,12)[b]
407 Cry⁻ Δ*clpP1*	>100[c]
407 Cry⁻ Δ*clpP2*	7,094 (3,56-9,91)

[a] LD50 were calculated 1 day post infection by probit analysis.
[b] 95% confidence interval.
[c] Injection of 100 cells of *clpP* deficient mutant causes 0% mortality.

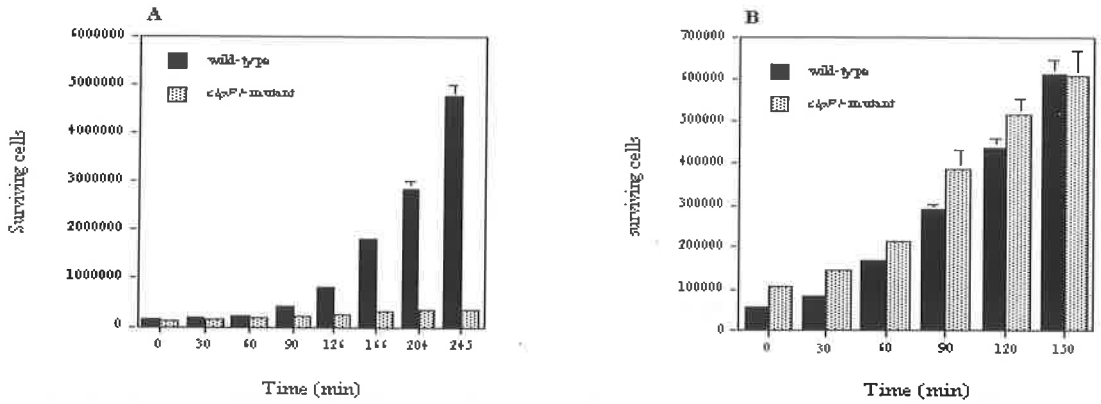

FIG. 3. Surviving *B. thuringiensis* wild-type and Δ*clpP1* cells after incubation with hemolymph from *B. mori* larvae. 200 µl of hemolymph and 20 µl of a dilution containing about 10^5 viable cells were mixed. The reaction mixture was incubated without shaking at 28°C (A) or at 37°C (B). Aliquots were withdrawn at the time points indicated and the surviving bacteria were counted. Experiments were repeated 3 times. Vertical bars indicate the standard error of the mean.

Expression of *clpP1'-lacZ* and *clpP2'-lacZ* transcriptional fusions showed that *clpP1* was transcribed at 25°C whereas *clpP2* was not, suggesting that ClpP2 cannot compensate for the absence of ClpP1 in the *clpP1* mutant cells at low temperature. Our study demonstrates that ClpP1 and ClpP2 control distinct cellular regulatory pathways in *B. thuringiensis*.

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Xenorhabdus and *Photorhabdus* virulence factors and their impacts on insect cellular immunity

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Introduction

The genus *Xenorhabdus* consists of the specific bacterial symbionts of the entomopathogenic nematodes *Steinernematidae* and was separated from the genus *Photorhabdus*, symbionts of the entomopathogenic nematodes *Heterorhabditidae* (Boemare et al., 1993). Both genera are entomopathogenic gram-negative bacteria belonging to the *Enterobacteriaceae.* The nematode hosts transport their bacteria into the haemocoel of the insect prey and the larva is killed, probably via a combination of toxin action and septicaemia. The bacterial symbionts also contribute to the symbiotic relationship by establishing and maintaining suitable conditions for the nematode reproduction. Recently, a novel toxin complex with both oral and injectable activities against a wide range of insects was identified in *Photorhabdus luminescens* (Bowen et al., 1998).

Following invasion of the insect host by the nematodes, bacteria produce potential virulence factors in the haemocoel including lipase and protease. It was shown that purified LPS or *Photorhabdus* protease fractions and lecithinase like-isomers had no toxic effect following injection into insect haemocoel (Dowds and Peters, 2002). The insect immune response consists of interactive cellular and humoral actions including phagocytosis, nodule production, encapsulation and antimicrobial peptides production. The interaction between both genera and the insect immune system has been mainly studied for Lepidoptera. Few informations are available about bacterial factors involved in overcoming cellular reactions. However, Park and Kim (2000) have recently shown that *X. nematophila* inhibited the eicosanoid pathways involved in cellular response such as phagocytosis and nodule formation.

Numerous advances were recently done in understanding of mechanisms and strategies by which mammalian pathogenic bacteria subvert their host cell processes to cause disease. Most gram- negative pathogens produce exotoxins such as cholera toxins or cytolysins (*e.g.* pore-forming proteins) and many enteric pathogens possess a specialized system for delivering toxins and modulins directly to host cells known as type III secretion system.

The objectives of the work reported here is to identify genes that encode virulence factors involved in cellular damage as a prelude to understand the strategy used by these entomopathogenic bacteria to colonize the insect host. Preliminary functional analysis using targeted knockout and gene fusion with the green fluorescent protein (GFP) is also described. Proteins that are important in pathogenesis must be first exported from the bacterial cytoplasm to the extracellular space to be active on the host cells. Our data provides informations about secretion systems present in *Xenorhabdus* and *Photorhabdus.*

1. Coupling of flagellar gene expression to cytolysin activity in *Xenorhabdus nematophila*

A series of data illustrates relationships between flagellar mediated motility and bacterial virulence in mammal hosts (Ottemann and Miller, 1997). *X. nematophila* is highly pathogenic

to insects, the LD50 is less than 20 bacteria to kill *Galleria mellonella* or *Spodoptera littoralis*. The transposition mutants are often useful to identify virulence-associated genes. However in *X. nematophila* multiple Tn5 insertions were found. Even if the Tn5 mutants were pleiotropic, all 5 avirulent mutants were non-motile and partially impaired in blood haemolysis (Hurlbert, 1994). This data prompts us to study flagellar regulon in *X. nematophila*. Over 40 genes required for the flagellar structure, assembly and chemiotaxis of *Escherichia coli* are categorised into three classes temporally expressed in a transcriptional cascade (Macnab, 1992). At the top of the hierarchy is the class I operon, *flhDC* whose products are required for expression of all other flagellar genes. The *E .coli* FlhD and FlhC proteins act as an activator for class II operons including most of the structural genes for the flagellar hook-basal body complexes plus the alternative sigma factor *fliA*, □28. The product of *fliA* gene directs the transcription of class III genes, which encode the filament protein, the hook-associated proteins, the motor proteins and various chemotaxis proteins. We have previously shown that the inactivation of the master operon of flagellar regulon, *flhDC* in *Xenorhadus*, led to the loss of flagellin expression, but also to the lack of blood agar haemolysin activity and an attenuated virulence phenotype in the insects (Givaudan and Lanois, 2000).

In this study, we constructed a *fliAZ* mutant and compared its phenotype to that of *flhD* mutant. Both mutants have in fact similar phenotypes showing that FlhDC via FliAZ controls haemolysin and virulence. In order to know if *fliA* or *fliZ* alone, or both genes are involved in these effects, a transcriptional analysis was undertaken. In contrast to several class II genes from *E. coli*, no sigma 28 consensus sequence was found in the upstream region of *Xenorhabdus fliA* operon. When *fliA* alone is over-expressed, no increase of the P_{fliA} was reported using P_{fliA}::gus fusions as reporter system. However, expression of the *fliAZ* operon showed a positive autoregulation of the FlhDC-dependent promoter via *fliZ*, the second gene of the operon. FliZ may be responsible for the control of FlhDC regulators at the posttranscriptional or posttranslational levels. Moreover, the introduction of plasmid containing *fliZ* alone under control of P_{lac} promoter in *fliA* mutant restored haemolysin production and full virulence. Altogether these data suggest that the flagellar gene *fliZ* is the checkpoint between haemolysis and virulence in *Xenorhabdus*.

In order to characterise the haemolysin active on blood agar plate, we also conducted in vitro cytolytic assay using insect haemocytes monolayers. Two different cytolytic activities on immunocompetent cells from the haemolymph of *Spodoptera littoralis* (Lepidoptera, Noctuidae) were detected in the supernatant of *X. nematophila* F1 broth-growth (see Table 1). When bacterial cells reached the stationary phase, the first extracellular cytolytic activity occurred. This haemolytic activity is heat labile and specific for sheep red blood cells. Among insect haemocyte types, granulocytes were the preferred insect target cells and lysis was preceded by a dramatic vacuolisation of the cells. The second peak of cytolytic activity occurred late during stationary phase, caused specific haemolysis of rabbit red blood cells and insect plasmatocytes were the preferred target cells. This second activity is heat resistant and produced shrinkage and necrosis of plasmatocytes (Brillard et al., 2001). These data revealed that *X. nematophila* wild type produced two independent cytolytic activities specific of different insect cell targets known for their major role in cellular immunity. As the cellular targets were different from each other, the immune depression could be achieved by decreasing the number of cells available for two major immune reactions, capsule and nodule formation involving the plasmatocytes, and phagocytosis mediated by granulocytes. We are currently validating this hypothesis in vivo.

Haemolysin FliZ-dependent fit to the early cytolytic activity that explains that the *flhD* or *fliA*mutant only produced the second haemolysin. This data may explain why these flagellar

regulator mutants remained virulent and why insects injected with the mutant take longer to die. All these data taken together suggest that one or more *flhD*-mediated properties are involved in infection but are not necessary to this process. It is likely that pathogenicity of *Xenorhabdus* towards insect hosts is determined by a large number factors and the loss of several of them may not substantially change virulence.

Recently, Young et al. (1999) have shown that *flhD* operon controls phospholipase expression and secretion in *Yersinia enterocolitica*. It was proposed that type III protein secretion by the flagellar apparatus might be a general mechanism for transport of proteins involved in virulence. We are actually examining the secretory pathway involved in haemolysin FliZ-dependent exportation.

2. Toxins and secretion system of *Photorhabdus*

We also detected the appearance of haemolysis zones on sheep blood agar using several strains of *Photorhabdus*. However, no extracellular cytolytic activity against insect haemocytes was detected using trypan blue assay in *Photorhabdus* supernatants (Table 1).

This data clearly indicates that *Photorhabdus* factors responsible for haemolysis observed on blood agar medium are quite different from *Xenorhabdus* haemolysins. It could be that (i) others insect cellular types different from haemocytes are targets of *Photorhabdus* extracellular components or (ii) that the contact between bacteria and target cells are necessary for cytolysis.

The genome of the strain TT01 of *P. luminescens* subsp. *laumondii* has been sequenced using the whole-genome shotgun strategy (E. Duchaud and F. Kunst, unpublished). Coding sequences (CDS) defined by Genemark predictions were analysed using BLASTP similarity searches. The presence of numerous genes with similarities to various haemolysins and a locus that potentially encodes type III secretion system were detected.

We first characterised a haemolysin locus (*phlBA* operon) homologous to the loci of the pore-forming, calcium-independent haemolysins from *Serratia marcescens*, *Proteus mirabilis*, *Edwardsiella tarda* and *Haemophilus ducreyi*. It belongs to the two partner secretion (TPS) family of proteins. Our experiments indicate that bacteria and horse erythrocytes need to be co-incubated to detect the hemolytic activity caused by PhlA whereas liquid hemolytic assay did not reveal any hemolytic activity in bacterial supernatants. The *phlA* mutant constructed by allelic exchange remained highly pathogenic after injection in the lepidoptera *Spodoptera littoralis*, indicating that PhlA haemolysin is not a major virulence determinant. Using the gene encoding green fluorescent protein as a reporter, *phlBA* transcription was observed in haemolymph before insect death. We propose that PhlA hemolytic activity might be involved in cells disruption that, in turn, play a role in nematode multiplication in insect haemocoele.

Of particular interest was the identification of a genomic region of 30 kb homologous to the plasmid encoded type III secretion system of virulent *Yersina ssp.* This region includes gene clusters encoding for (i) a protein type III secretion/translocation apparatus (ii) at least one effector protein, which is translocated through a "translocation needle" from the cell-adhering bacterium into the cytoplasm of the host cell (iii) regulators for gene expression and effector-secretion/translocation. The general organization of the open reading frames encoding this secretion machinery is similar to the one found on the pYV plasmid of yersiniae described elsewhere (Cornelis et al., 1998). The virulence genes of yersiniae are coordinately regulated by a network, which can be triggered by environmental stimuli. Upon ingestion of yersiniae, the bacteria reach the intestinal lumen where gene like *inv* is upregulated but the majority of the virulence genes of pYV are downregulated. After translocation into the subepithelium of

TABLE 1. Haemolysis and insect haemocyte cytolysis observed in *Xenorhabdus* and *Photorhabdus* strains

Taxon	Strain	Source (Host nematode /hospital strain)	blood agar plate haemolysis[a]	Cytolytic activity[b] of supernatants on insect hemocytes	
				Early[c]	Late[d]
Xenorhabdus nematophila	F1	*Steinernema carpocapsae*	T+	+	+
X. japonica	JP02	*S. kushidai*	T+	++	++
X. bovienii	F3	*S. affine*	T+^{w}	++	++
X. poinarii	G6	*S. glaseri*	T+^{w}	-	-
X. beddingii	Q58	*Steinernema* sp.	T+^{w}	-	+
Xenorhabdus sp.	SaV	**S. arenarium**	T+^{w}	-	+
Photorhabdus luminescens subsp. *Luminescens*	Hb (ATCC 29999^{T})	*Heterorhabditis bacteriophora*	P	-	-
P. luminescens subsp. *akhurstii*	FRG04 (CIP 105564 ^{T})	*H. indica*	V	-	-
P. luminescens subsp. *laumondii*	TT01 (CIP 105565 ^{T})	*H. bacteriophora*	T+^{w}	-	-
P. temperata	Meg	*H. megidis*	V	-	-
P. temperata subsp. *Temperata*	XINach (CIP 105563 ^{T})	**H. megidis**	P	-	-
P. asymbiotica	3265-86 (ATCC 43950 ^{T})	CDC Atlanta	AH	-	-
	1216-79 (ATCC 43948)	CDC Atlanta	AH	-	-

[a] All blood agar plates were cultured for 2 days at 28¡C before assays were interpreted. T: total haemolysis; P: partial haemolysis; AH: annular haemolysis. +: clearing halo up to 5 mm; ^{w}+: perceptible halo with sizes less than 5 mm; -: haemolysis not detected; V: Variable = annular haemolysis or total haemolysis depending on the plates

[b] insect haemocyte cytolysis were expressed as cytolytic units and percent of dead haemocytes using blue trypan counting. ++: value up to 0.75; +: value between 0.25 and 0.75; ^{w}+: value less than 0.25 ; - cytolytic activity not detected.

[c] supernatants were collected when bacterial cultures reached an OD_{620} of about 2.5 (20-h old cultures)

[d] supernatants were collected when bacterial cultures reached an OD_{620} up to 4 (48-h old cultures)

Peyer's patches and only within this environment the virulence genes on the pYV plasmid are upregulated which allows yersiniae to grow extracellularly by impeding the primary defense of the host: resistance to complement lysis and defensins, inhibition of oxidative burst of phagocytes, inhibition of release of proinflammatory cytokines and phagocytosis, induction of apoptosis in macrophages. Most of those effects are under the control of the translocated effector proteins. The only yersinae homologous effector protein found in *Photorhabdus* is similar to the newest member of the yersiniae effectors family proteins, the YopT cytotoxin.

In order to study the *Photorhabdus* type III secretion system, we constructed a translational fusion between the homologous cytotoxin found in *Photorhabdus* and the GFP reporter gene. To trigger secretion in vitro, *Yersinia* is generally grown at 28°C in a medium depleted in Ca^{++} and then transferred to 37°C. Ca^{++} depletion and temperature both control transcription of the *yop* genes. Preliminary results rule out both signals as responsible of the release of the *Photorhabdus* type III secretion system. During *in vivo* pathogenicity assays with the caterpillar *Spodoptera littoralis*, TTO1 strain carrying this fusion were only highly fluorescent in the nodules.

In *Yersinia*, YopT similar to YopE disrupts the actin cytoskeleton even if it has been described that the cell shape changes produced by YopT and YopE differ from the phenotypes ascribed for the other Yops activity (Iriarte and Cornelis, 1998). Zumbihl et al. (1999) recently demonstrated specific modification and inactivation of the GTP-binding protein RhoA by YopT, a finding that shows for the first time that an intracellularly translocated bacterial effector can induce covalent modification of a Rho GTPase. This toxin does not show any homology with other bacterial toxins. Furthermore, *Clostridium* and CNF toxins modified RhoA is still substrate of YopT, suggesting that the amino acid target of YopT is different from those known up to now to be modified by the other bacterial toxins.

Using in vitro cell culture infection experiment, we are actually examining whether the *Photorhabdus* YopT homolog is functionally secreted and translocated in mammal cells

Altogether these data suggest that mechanisms of depression of the innate immune system by pathogenic bacteria, especially those involving transport of cytotoxins (*i.e.* the type III secretion system in *Photorhabdus* and the flagellar apparatus in *Xenorhabdus*) can certainly be extended to host-pathogen interaction in invertebrates.

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Insect/Serratia interactions: the question of virulence

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Abstract

Bacteria of the genus *Serratia* are often isolated as insect pathogens. Some strains can be characterised as invasive, facultative pathogens. Once in the haemocoel, these strains will multiply rapidly bringing about death of the host through generalised septicaemia. Pathogenicity *per os* by invasive *Serratia* strains is characteristically variable, suggesting interaction with intrinsic and host related virulence factors. In contrast, strains of *Serratia entomophila* and *S. proteamaculans* consistently cause chronic amber disease in the New Zealand grass grub. Amber disease is encoded by genes in a 17kb region of a conjugational plasmid. The three major genes involved have strong protein homology to insecticidal proteins from other Gram-negative bacteria such as *Photorhabdus* and *Xenorhadbus.* Laboratory and field tests have indicated that amber disease occurs in a consistent manner with a predicable level of disease following treatment of the susceptible host insect with pathogenic cultures and strains of bacteria. Our results suggest that there are few variable virulence or virulence inducing factors associated with this interaction.

Introduction

Bacteria of the genus *Serratia* (Enterobacteriaceae) can be found in soils and organic matter in most parts of the world. Ten species have been identified within the genus (Dauga *et al.* 1990) and several species including *S. marcescens*, *S. liquefaciens*, *S. proteamaculans* and *S. entomophila* are frequently isolated from dead insects. However, whether these are true pathogens or contaminants is often unclear. Virulence is defined as the disease producing power of a microorganism (Tanada and Kaya, 1993). The term can be used synonymously with pathogenicity to mean ability to cause damage and disease in the host (Mims *et al.* 1995), but virulence is also frequently used to describe the relative pathogenicity of isolates or cultures. According to the second definition, virulence factors, or virulence enhancing factors, contribute to the degree of pathogenicity and the microbe's ability to survival within the body of the host (eg. Pettersson *et al.* 1996). Thus, in considering the virulence of *Serratia* spp. to insects we will discuss the interrelationship of disease and insects, describe the pathogenicity process, define the genetic determinants of disease in some species and examine variation in the relationship between pathogenic *Serratia* and their hosts.

Serratia as insect pathogens

Serratia spp. have been recorded as pathogens from a wide range of insects including species from the orders Orthoptera, Coleoptera, Diptera, Hymenoptera and Lepidoptera. However, most records relate to laboratory tests or insect deaths in laboratory cultures. Epizootics in the field are unusual. For most species associated with insects, mortality is caused by bacterial septicaemia flowing penetration of the haemocoel. Most *Serratia* species have limited ability to penetrate the insect cuticle and were defined as facultative pathogens by Bucher (1960). For these bacteria , pathogenicity often is dependent on secondary factors to allow entry of the host. Once delivered through the cuticle *Serratia* spp. are usually highly pathogenic, as shown by Jackson and McNeill (1998) using injection or natural oviposition by parasitoids for delivery of the bacteria directly to the haemocoel. Thus, most invasive *Serratia* spp. appear to

be opportunistic pathogens capable of growth once in the insect, but with poor and/or variable ability to penetrate the haemocoel. A different, and possibly unique, strategy for pathogenicity is shown by strains of *S. entomophila* and *S. proteamaculans* infecting the New Zealand grass grub (*Costelytra zealandica*). Once ingested, these bacteria colonise the insect's gut inducing a chronic disease state terminating in death of the infected insect (Jackson *et al.* 2001)

Pathogenicity

Once delivered to the haemocoel, insects are usually highly sensitive to *Serratia* spp. Slatten and Larsen (1967) found that as few as 5 bacteria injected per boll weevil could cause death, while 25 bacteria per insect were sufficient to kill grasshoppers (Bucher 1959). Similar numbers of bacteria caused death when injected into gypsy moth larvae (Podgwaite and Cosenza 1976). Far higher doses are required to kill insects following after *per os* inoculation or feeding and even massive doses do not always kill the treated insects (eg. Bucher 1959; Podgwaite and Cosenza 1976). Bacteria grow rapidly in the haemocoel of infected insects resulting in death from septicaemia. Among flies treated *per os*, death can occur within 3-7 days (Benoit *et al.* 1990; O'Callaghan *et al.* 1996). Large May beetle larvae (*Melolontha hippocastani*) ceased feeding within 3 days of treatment with specific strains of *Serratia* spp. and died within one to two months (Jackson and Zimmermann 1996). While variation is common in response to *Serratia* strains inoculated *per os*, there seems to be a consistent, invasive pathogenic response by some species of insects over multiple assays to specific strains of *Serratia* (eg. O'Callaghan *et al.* 1996), suggesting that host/pathogen strain matching could be important in determining virulence.

Amber disease has only been produced in the New Zealand grass grub by strains of *S. entomophila* and *S. proteamaculans* despite testing of >200 isolates of *Serratia* spp. and other soil bacteria (Jackson *et al.* 1991). Characteristics of pathogenicity of amber disease-causing *Serratia* strains have been described by Jackson *et al.* (1993) and Jackson *et al.* (2001). In summary, grass grub larvae become infected after ingesting an acute dose of $>10^3$-10^4 bacteria/larva. Infected larvae rapidly cease feeding and clear the midgut, resulting in the amber colouration characteristic of the disease. The infective bacteria colonise the gut without obvious damage to the midgut epithelial cells or invasion of cells or organs. There is some attachment to cuticular surfaces in the fore- and hindgut but most bacteria remain free floating within the gut lumen (Hurst and Jackson 2002). The chronic, non-feeding phase of disease can last up to four months depending on the size and fat reserves of the larvae at the time of infection. When the larvae are severely weakened after a long period without food, bacteria break through from the gut into the haemocoel bringing about death of the host insect through septicaemia. Unlike the variability characteristic of the "invasive" *Serratia* strains, pathogenic amber disease producing *Serratia* consistently cause amber disease in the host larvae. Little variability has been detected in the pathogenicity of the disease-causing strains. Neither amber nor invasive disease characteristics have been produced in other species despite testing on >20 other species of scarabs and a wide range of other non-target insects (Jackson *et al.* 1991; Jackson unpublished).

Genetic determinants of pathogenicity

The genetic determinants of invasive *Serratia* spp. are generally unknown. For invasive species, proteases and chitinases are thought to be involved in the disease process. There has been research on genetic determinants of these enzymes (e.g. Suzuki et al. 2001), particularly chitinases, as *Serratia* spp. are strong producers of this enzyme. The genetics of the pathogenicity genes of amber disease have begun to be unravelled. The disease is largely

encoded by plasmid borne genes (Glare et al. 1993). More recently Hurst *et al.* (2000) defined a 17 kb region of pathogenicity encoding genes including three major genes termed *sepA, sepB* and *sepC*. Transposon insertions in any of these genes prevented amber disease occurrence. While the actual proteins encoded by these genes have been predicted, the only similarities found have been with the so called "tc" family of insecticidal proteins, first described from *Photorhabdus luminescens* (Bowen *et al.* 1998). There are now several species known to be able to produce these tc proteins (*Xenorhabdus, Yersinia, Pseudomonas*) but as the disease phenology of each of the species with tc-type genes is different, this similarity to amber disease encoding genes has not yet shed any light of the possible mechanisms of disease.

SepB and its homologues show strong amino-terminal similarity to the amino terminus of the *Salmonella* virulence gene product SpvB (Gulig *et al.* 1992). The region of similarity in relation to SpvB terminates ten amino-acid residues upstream of the proline-rich region postulated to divide SpvB into separate domains (Roudier *et al.* 1992)). The carboxal terminus domain of SpvB has recently been identified as a mono (ADP-ribosyl) transferase, causing ADP-ribosylation of host cell protein (Otto *et al.* 2000). Though the function of the amino domain has yet to be defined the conserved nature of the SepB-tc type amino terminus may indicate a vital role in its interacting with an evolutionarily-conserved eukaryotic protein.

Factors affecting pathogenicity

Factors influencing pathogenicity of invasive *Serratia* strains are poorly understood, despite the characteristic variability of these interactions. Conversely, amber disease producing strains are characterised by consistency of effect. In laboratory assays, the rate of amber disease processes, infection and death, is directly proportional to temperature (Jackson *et al.* 2001). In the field, low infection rates at low soil temperatures may be due to reduced movement by the target pest.

Host factors appear to have little effect on the pathogenicity of amber disease causing *Serratia* spp. All larval stages of *C. zealandica* are susceptible to amber disease causing strains until the non-feeding prepupal state. Pupae and adult grass grubs are unaffected by these strains. Disease can, however, be prevented by the preapplication of bacteriophages prior to treatment with phage sensitive strains (O'Callaghan and Jackson 1993).

Quality control steps in the production of *S. entomophila* as the biocontrol agent Invade show consistency in pathogenicity between batches of bacteria. The unique exception in culturing has been when plasmid loss has resulted in complete loss of pathogenicity.

Virulence of *Serratia* spp. as insect pathogens

Invasive *Serratia* spp. that are frequently isolated as insect pathogens seem to be associated with an abundance of variable "virulence factors" that suggest that there is a potential for these organisms in microbial control, but that the complexity of their interactions with the host will make virulence factors difficult to elucidate. The interaction of host sensitivity factors and apparent microbial instability make study of these organisms very difficult. In contrast, amber disease causing strains are marked by consistency in pathogenicity and show little sign of variability-inducing "virulence factors". The presence of the plasmid containing the pathogenicity encoding region is a consistent indicator of the disease producing ability of these strains. Bacteria containing the plasmid with these genes show characteristics of pathogenicity with little evidence of either "disease inhibiting" or "virulence" factors in their interaction with grass grub.

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Workshop
Bioinsecticide Production Issues, with a Focus on Latin America

Bacteria production and use in some Latin American countries

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The overuse or misuse of chemical pesticides and their negative impacts are increasingly becoming cause for concern underlining the need for development of alternative pest control methods. This situation has created an awareness even in the most under-developed countries for more sound control methods and to develop integrated pest management (IPM) programs. The use of microorganisms as selective pesticides has had some notable successes. At present there are a number of bacteria, fungi and viruses which have been introduced as commercial pesticides, frequently after skilful production and formulation efforts.

Bacteria are the most promising biological control agent. Over a hundred species of bacteria which infect insects have been described. A few of them have been produced commercially including *Bacillus thuringiensis (Bt)*, *B. sphaericus (Bs)* and *B. moritai*.

The case of the insecticidal bacteria belonging to the *Bt* species has been known since 1915. Nowadays *Bt* is the principal biopesticide produced and used worldwide. This organism proved to be a highly successful weapon for fighting some agricultural pests and some vectors of diseases. But *Bt* use is still limited in developing countries because of its high costs.

The high cost of the *Bt* product in Latin America is due to production being located in the developed countries where productions costs are higher and also due to expenses paid in transportation to the operational sites. Thus local (cottage industry) production should significantly reduce costs of pest control and also could help development of local fermentation industries and improvement in utilization of regional agro-industrial by-products. This paper will present a short overview about the efforts developed by some of these groups to obtain a mass production of some bacteria, and its bio-products, either by liquid or semi-solid fermentation. The intention is not to stress out all of them, but to present some of them in order to compare the difficulties and discuss future perspectives.

General comments

The production of many bacteria considered potential bio-agents could be done by semisolid or submerged fermentation. In the former process semisolid substrates, usually moist bran, are used. Submerged fermentation is also an efficient method where the microorganism is put in contact with the substrate in a liquid phase. The reactors could be similar to those used for drugs and pharmaceutics, but it has to be pointed out that they are expensive and sophisticated. Simpler equipments could be developed to lower the costs; in many cases plastic bags and/or trays are suggested.

Careful controlling and monitoring are necessary for both processes in order to get reproducible results. Some physical parameters have to be followed during the process: pH, temperature, aeration; as well as agitation and sporulation rate. Changes in medium components can lead to increased quantities of biomass and endotoxins which in turn will lead to a cheaper product.

Local production for developing countries

As mentioned before, local production reduces expenses paid in transportation to the operational sites, reducing the costs of pest control and also could help development of local fermentation industries. Local production seems especially adequated for controlling pests in crops with high-cost production and small cultivated area, for specific local pests, and/or for the initial steps in an IPM program.

In local production some care must be especially followed by technical supervisors: quality control (including standardization and prevention of contaminants); formulation (efficiency and efficacy); fate in the environment (possible activity against non target organisms; persistence; dissemination); legislation acts (guidelines for registration).

Bacterial Uses in Brazil

The first use of this type of biopesticide was registered in Brazil in the 60´s, but studies on mass production of *Bt* started in 1970 by the development of a MSc thesis (Moraes, 1973). Since then, many researches developed submerged (batch and continuous) and solid state fermentation. The engineering parameters, agitation and aeration, separation process of the culture broth, thermo-bacteriological indexes, the drying process by conventional and spray dryer means were determined for *Bt* varieties *kurstaki*, *israelensis* and *thuringiensis* .

Several different residues and wastewater, mainly from agro-industries were studied. Both the endo and the exotoxins were produced and bioassayed. The sterilization step for some semi-solid processes required an innovation, using microwaves application to pasteurize substrates in alternative bioreactors - plastic bags of polyethylene material.

In the last few years, studies produced by Brazilian research groups have led to the development of formulations with *Bt* variety *israelensis* (*Bti)* and also *Bacillus sphaericus* (*Bs*), for disease vector control (Melo Santos, 2000). There is a group located in Far-Manguinhos/ Fiocruz (Rio de Janeiro) that got some patents for the process and for their formulation; another one from the Laboratorio de Antibioticos/ Universidade Federal de Pernambuco, that produces *Bti* and *Bs* by submerged liquid fermentation in local-built 500L fermentors. Some of these products are being presented in the SIP 2002, by Dr. Leda Regis, showing high toxicity to mosquito larvae.

Some years ago an industry named Geratec produced, registered and commercialized products based on *Bti* and *Bs,* as part of a technical cooperation with research groups. This company, established in the South of Brazil, was sold 4 years ago, and discontinued this production. Knowledge developed by Instituto Oswaldo Cruz was transfered to an industry named INPAL S.A. - Indústrias Químicas that is in charge to scale up the production of *Bti* and *Bs*.

A cottage production of *Bti* was developed by Dr Olivia Arantes with her collaborators, in the 90´s, based on previous experience of a group from Espirito Santo. They produced it in semi-solid process, but nowadays they produce the bioinsecticide in 30-40L jars, in liquid broth containing soybean flour with forced aeration. After approximately 72 hours, the whole beer is conveniently diluted and applied to mosquitoes breeding sites *(Culex* sp.). The dosage needs and the calibration of the costal application apparatus is established by the entomologist

of the staff. The whole program is developed in cooperation with the Londrina City Hall and the University. Efforts for scaling-up the process are under development.

In all mentioned cases, there was a quality control that included product purity, concentration of infective units; and biological efficacy, but no details were mentioned in publications.

Bt production in Cuba

Commercial *Bt* products from the USA, France and the former Soviet Union were used in Cuba in the 1960s against serious pests for tobacco plantation (Fernandez-Larea, 1999). As mentioned for Brazil, the high price of the products led Cuban researchers to study and develop simple and cost-effective, cottage-industry level production methods for *Bt*.

Biopesticides were first used on a massive scale in Cuba from the late 1980s, when availability of chemical pesticides was drastically reduced. *Bt* biopesticides are now produced in Cuba, both by a cottage-industry method (using static liquid culture based on waste products from the sugar industry or other crop production) and by a fermentation process in production units designed and constructed entirely in Cuba, including the fermentation equipment. Fermentation plants produce *Bt* in liquid concentrate form, which can be stored for six months.

Cottage-industry *Bt* production is carried out through a network of 220 Centers for Reproduction of Entomophages and Entomopathogens, which are distributed throughout the country. The process involves bacterial culture in liquid media consisting of agricultural or industrial by-products or waste. After 10-15 days of fermentation, preservative is added to the harvested product and it is stored for up to three months at temperatures up to 25°C. Production costs per liter are approximately US$ 0.02.

Bt large-scale production is obtained by fermentation in three Biopesticide Production Plants: two in Havana Province and a third in the central region. These products contain higher concentrations of infective units and stability can be maintained for up to six months at room temperature. Production time is about 72-96 hours. *Bt* production efficiency in these plants exceeds 90% and production costs are in the range US$ 0.50-0.60.

Cuban *Bt* products are used mainly in the control of lepidopteran defoliators in vegetables. The acaricide product is also used for mite control in citrus, potato and plantain. Strains of *Bt* var. *israelensis* are used for control of mosquito disease vectors. There are records showing that during 1997, over 1000 tons of *Bt* were produced in Cuba, 24% by industrial fermentation and 76% via solid substrate culture. The three *Bt* strains for Lepidoptera control and the one for mites are multiplied by means of static liquid culture. The *Bt* industrial plants produce four different products via submerged fermentation.

Production quality is controlled in by means of a State Standard for Quality Control, that includes product purity; concentration of infective units; and biological efficacy. All products are registered in the Pesticide Registration Office.

Production strategies in Peru

The best known production of *Bt* in Peru is developed by a group of researchers at the Universidad Peruana Cayetano Heredia / Instituto de Medicina Tropical Alexander von Humboldt. Some presentations in international conferences and were done by Dr. Palmira Ventosilla and her collaborators (H. Guerra, J. Merello, B. Infante and E. Reyes). They produce *Bt* variety *israelensis* using coconut, yucca and asparagus for culture preparation, and liquid fermentation techniques in cottage-community-scale process. The whole coconut shell is used as a bioreactor,

and its water is the main component of the medium. This product was used against some vectors of tropical diseases (malaria) that live in artificial ponds. As in other Latin American countries, a special care is taken with quality control of the products obtained. The key for their success in controlling the disease, is the community participation program. Some practical courses are also offered by the group.

Notices from Bt Product in Colombia

At least two groups work on *Bt* production in Colombia. One of them is coordinated by Dr. Sergio Orduz at the Corporación Colombiana de Investigaciones Biológicas – CIB. Another one is coordinated by Dr. Graciela Chalela Alvarez working on Bt *medellin* production at the Universidad Industrial de Santander. Unfortunately it was impossible to get any of their publications or web in formations till the time of this review preparation.

Perspectives

A biocontrol agent can be produced in an easy way to be used by the farmers. There is a need to overcome some difficulties such as spreading potential, propagation rate, virulence and stability in the field, cost of production as well as biosafety measures. For these difficulties, the application of fermentation technology to develop new promising tool based on *Bt* or other biological agents seems to be helpful.

There are a number of advantages in promoting development of local production facilities for microbial insecticides in developing countries of the Latin America: *stability* - the variation of toxicity as a result of the lengthy shipping periods and variable storage temperatures is avoided by local production; *formulations* - the local produced biocontrol product will feet better to the local habitat where the target insect is, because it could be developed for the specific local environmental conditions; biodiversity – local production explores the opportunity of the local natural entomopathogenic microorganisms disseminated all over the country; environment correct - they use local agro-industrial residues as substrates, resulting also in a low-cost product. Further outstanding achievements are the savings for the regional economy in terms of no longer importing pesticides, lowering the risks for environment contamination.

Registration requirements are still pointed out as being very expensive, so efforts must be put on establishing normative decisions concerning registration, based on simpler evaluation for such environment-friendly products.

Final comments

To meet the challenge of using biocontrol production strategies that are effective and ecologically sound, a highly multidisciplinary approach is being required.

There are multiple possibilities for Latin America cooperation programs and it could be pointed the principal ones: alternative fermentation processes, especially those convenient for local production; intensive studies on environmental impacts of biopesticides in order to promote confidence on these organisms and also to improve its utilization; development of methodologies and studies to support decisions for public policies.

There are better ways of producing, using and applying these pathogens and toxins, if we seek and develop close cooperation between entomologists, microbiologists, chemists and engineers to find them.

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Mass production of nucleopolyhedrovirus for the control of the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, in soybeans

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The Agricultural Cooperatives of Paraná State started to develop program towards the mass production of a nucleopolyhedrovirus (AgMNPV) as a bioinsecticide for the control of the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, in soybeans, in 1987. The program is located in Cascavel, Paraná, and is based on technology developed by Soybean Research Centre of Embrapa (Brazilian Agricultural Research Corporation) in Londrina, PR. Production of the virus is entirely accomplished using field populations of caterpillars during the main growing season in Western Paraná State from December to January. The virus is sprayed when larval populations reach 40 to 60 caterpillars per 2m of soybean row. Collection of dead caterpillars is done manually and starts about eight days after virus application. Over a period of 16 years, an average of 206 workers (ranging 20 to 548) were used per day during the seasons and 1.75 kg of caterpillars/person/day were collected. The system of field production offers as main advantage a huge production potential at low cost in a short period of time. However, this method requires excellent logistics and is subjected to great fluctuations in the annual production volume. Thus, the annual average production was 4,146 kg of infected caterpillars, ranging from 295 to 12,347 kg, sufficient to treat on average 228,000 hectares of soybeans. However, in the last 7 years the annual average production was 7,415 kg of infected caterpillars, ranging from 6,955 to 12,347 kg, sufficient to treat on average 408,000 hectares of soybeans. The variation is due to several factors which act on the population of *A. gemmatalis*, the most important being weather conditions. Commercially viable production of virus would require a system of constant production of caterpillars and virus to facilitate constant supply and stable revenues. Therefore, it is necessary to improve laboratory rearing techniques for the host in order to enable continuous industrialized production. Much progress has been achieved in virus extraction and formulation. Major advances are still required in the reduction of the cost of caterpillar production, especially cost of diet and labor requirements.

Technical aspects of the industrial production of entomopathogenic fungi in Brazil

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Entomopathogenic fungi have been intensively studied in Brazil, becoming more attractive as biological control agents after the beginning of the 70's, with the implementation of the program for the using of *Metarhizium anisopliae* to control of spittlebugs pests of pasture and sugarcane. Following this program, many others were implemented to explore other fungi besides *M. anisopliae*, including *Beauveria bassiana* and *Sporothrix insectorum*. It has opened a great market for the use of entomopathogenic fungi, so that nowadays there are at least 6 companies commercializing these organisms, and many laboratories being setting up to supply specific Agriculture Companies. In order to produce fungi, the companies have kept the known process of growing on solid medium, using cooked rice as substrate. For *S. insectorum*, liquid medium has also been used by fermentation process, besides solid medium. The most common formulation found in the market is dry powder, obtained by the milling of the fungus plus substrate, added with some adjuvant. Meanwhile, liquid formulation has also been offered. The final cost of production is practically the same for the fungus produced as on solid medium as in liquid one, being around 6 to 8 dollars the kilogram or liter. About 60% of the cost refers to labor and fixed expenses (rent, electricity, telephone etc.), 6% to the media components, 23 % to tax and 10% to the profit margin. Most of the companies have kept agreements with research institutions in order to maintain a good quality control of their products, besides searching for new targets and improving many aspects such as the production system, formulation and strain virulence. *M. anisopliae* has been sold for spittlebugs pests of sugarcane and pasture, robber tree lace bug, termites, grasshoppers and others, while *B. bassiana* has been sold for coffee borer, banana borer, mites, termites and others, and *S. insectorum*, for only robber tree lace bug. The total area treated annually with entomopathogenic fungi surpass 200,000 ha, with more than 70% being treated with *M. anizopliae*. The potential market for entomopathogenic fungi in Brazil is big and has increased more and more especially due to the expansion of organic agriculture.

Fungi for coffee berry borer control - Colombia

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Cenicafe's scientific advances on *Beauveria bassiana* (Balsamo) Vuillemin biology, mass production, formulation and field application, and the spread of the coffee berry borer (*Hypothenemus hampei* (Ferrari); Coleoptera: Scolytidae) on more than 715,000 hectares (comprising nearly 82% of the total Colombian coffee plantation area), have encouraged private companies to install *B. bassiana* mass production facilities. In Colombia, about 11 companies offer more than 16 products based on entomopathogenic fungi. These products are used not only in the coffee crop but also in other crops such as cabbage, corn, bean, tomato, flowers, citrus, avocado, apple, cotton, oil palm, pasture, sugarcane, plantain, banana, cassava and potato. They are also used to treat public health vectors (e.g., flies and mosquitoes), livestock pests such ticks, and for the control of plant pathogens. *B. bassiana* spores have been produced using different methods and efforts to obtain formulations that maintain shelf-life have been undertaken. *B. bassiana* producers supply sufficient quantities of spores to the farmer as fresh inoculum or as formulated products. The quality of the biological products has been enhanced due to Cenicafe's participation in testing products marketed for coffee and in the training of government officers in charge of product registration and of private companies staff. Still, there is concern among growers concerning the efficacy of these products. The main reasons are the low speed of kill, the methodology to assess efficacy in the field that often underestimates the product's control capacity, and the comparison with chemical pesticide mode of action. Challenges for entomopathogenic fungi producers to overcome these weak points must be focussed on grower education using field demonstrations, improvement of spore quantity in mass production, the sale of formulations with high spore concentrations, and the design of IPM strategies that allow for crop management with economical and ecological benefits when compared with other coffee berry borer control practices.

Thirty years of massproduction and extensive application of entomogenous fungi in China

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From the middle 1950's, some Chinese entomologists started their trial of application of *Beauveria bassiana* against the sweet potato weevil, *Cylas formicarius*, the soybean moth, *Grapholitha glycinivorella*, and the Masson's pine caterpillar, *Dendrolimus punctatus*. The progress had been slow until the early 1970's when the central government encouraged the use of the fungus against the pine caterpillar. Hundreds of tons of primary products (milled or unmilled culture, with or without fillers) were massproduced by low techniques of open solid culture in local factories or forest farms. Various application methods were used in pine plantations, such as dust or mist spray through various machines, launching by firecrackers, self-made mortars or even by explosion. Application area was roughly estimated at 30000-60000ha although field efficacies varied. The fungal application was adopted against quite a lot crop or forest pests, such as the corn borer, *Ostrinia furnacalis,* and the common rice leafhopper, *Nephotettix cincticeps*. During the last 2 decades, researches focused mainly on improvement of massproduction techniques. Various modification of biophasic culture techniques and equipments were developed and application scale was kept at about the same level as before. Strain variation problem was studied intensively and approaches against strain degeneration were sought. Registration of fungal insecticides did not become a problem for massproduction until the Ministry of Agriculture issued a regulation on massproduction with necessary license in the mid 1990's. The efforts are being paid in some companies and the first registered product will come out in this year or next year, but the large scale application is still ongoing.

Symposium (Cross-Division 2) Microbial Germplasm Repositories: The Legacy, the Problem, the Future

Global perspectives on the discovery, isolation, preservation, and exploitation of entomopathogenic fungal germplasm

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The world's culture collections include a remarkably high proportion of the known biodiversity of fungal pathogens affecting insects. This is notable not only because of the inherent importance of and interest in the possible use of these fungi as biocontrol agents, but also because of the potential of these fungi as sources of biologically active metabolites. Entomopathogens comprise only about 1000 of the total of more than 1.5 million species of fungi estimated to exist (Hawksworth, 1991). Nonetheless, within this highly specialized group it is possible to culture the great majority of these fungi even though in the ecological sense they may be considered to be obligatory pathogens. The major service culture collections holding substantial numbers of fungal entomopathogens include germplasm representing approaching 50% of the described taxa, a very high proportion of total biodiversity within a specialized fungal ecotype. It is difficult to know how much more of the overall diversity of entomopathogenic fungi might be included if the holdings of private research culture collections were included in this total.

Balancing off the good news about the total taxonomic diversity of insect fungi that can be cultured in vitro and which are available from culture collections is the necessity to recognize that the great majority of these fungi—the conidial and sexual states of ascomycetes in the family Clavicipitaceae (order Hypocre-ales)—represent a much smaller number of organisms than of described names. Despite the huge number of clavicipitaceous taxa from insects, Fig. 1 represents a clue to the understanding that this huge constellation of named taxa does, in fact, represent a comparatively smaller set of distinct organisms in which the sexual stages (species mostly of *Cordyceps*, *Torrubiella*, or *Hypocrella*) routinely have one or more conidial states, each of which are also entitled to bear nomenclaturally valid names.

Culture collections have historically played essential roles in the effort to catalog and to preserve global biodiversity and have also traditionally been some of the most important centers for research on the systematics and taxonomy of the microbes they accumulate. They also represent some of the most important centers for research on the systematics and taxonomy of the microbes they accumulate and are key collaborators for other laboratories' studies in systematics and taxonomy. More recently, culture collections have become essential partners in bioprospecting for compounds produced by fungi or other microbes, and for the newer science of genomics by means of which the astonishing range of genetic information that makes an organism is becoming known and, more importantly, understood.

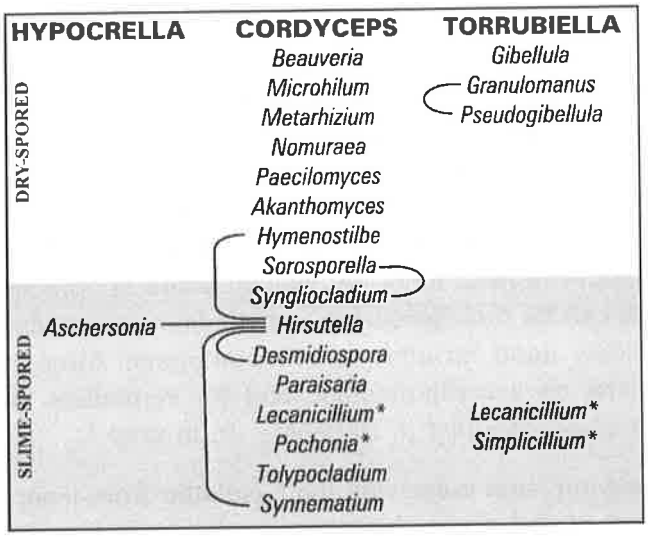

FIG. 1. Connections between entomopathogenic ascomycetes (BOLD CAPS) in the Clavicipitaceae (Hypocreales, Sordariomycetes = Pyrenomycetes) and, below, their corresponding conidial states (*italics*). Genera marked by an asterisk (*) derive from a revision of *Verticillium* section *Prostrata* (Gams and Zare, 2001; Zare and Gams, 2001). Lines indicate synanamorphic connections between alternate conidial states for individual fungi. Some amount of slime covers or suspends conidia of genera in the gray area. The Clavicipitaceae also includes other genera (not shown) pathogenic primarily for nematodes or rotifers.

Germplasm resource acquisition and management — More work is needed world-wide to survey the flora of microbial pathogens of insects and other vertebrates. Information on host spectra and geographical distributions of insect fungi in the ARSEF catalog (*www.ppru.cornell.edu/mycology/insect_mycology.htm*), confirms the presence of these fungi on a huge range of hosts and throughout the world. However, there are few places that have actually been well explored or characterized for their insect fungi. Tom Petch worked on insect fungi in Sri Lanka as a hobby group during the first 30 years of the last century; while all of his extensive work was done before the introduction of chemical pesticides, the intensive use of pesticides since their introduction and extensive habitat destruction or degradation in many parts of that island nation has apparently led to a marked loss of biodiversity in both the insect fauna and their fungal pathogens. The flora of *Cordyceps* species has proven to be very rich in Japan (Kobayasi and Shimizu, 1983; Shimizu, 1994), Korea (Sung, 1996), and also in southeastern Russian (Koval, 1977); extensive collecting for these same fungi in Thailand by N. Hywel-Jones and colleagues is expanding our knowledge of the rich entomofungal flora there. Still, the overall biodiversity of fungal entomopathogens remains generally poorly known (even in the US where many scientists are interested in these fungi). Apart from the chronic need to revise and to improve the taxonomy of known species, the potential for discovering new taxa of entomopathogenic fungi is a special challenge for the systematists who will have to do the descriptive taxonomy and for the culture collections that will play a central role in the isolation and preservation of these fungi. The discovery of high rates of endemism during decades of floristic studies on *Conidiobolus* species (Entomophthorales) mostly from soil litter in the southeastern United States by C. Drechsler and in southern India by Srinivasan, Narasimhan, and Thirumalachar (all collated by King, 1977) suggests that many species of this genus await discovery and description.

Among the greatest problems facing all fungal culture collections, whether or not they specialize in entomopathogens, is to obtain pure, axenic cultures. The more fastidious the relationship is between a fungal pathogen and its host, then it is likely that the discovery of suitable media and conditions for isolating, maintaining, and successfully preserving a culture will be increasingly more difficult (Humber, 1994). Some individual species are particularly difficult to culture. For example, *Neozygites fresenii* (Entomophthorales), a very fastidious pathogen of aphids, has resisted hundreds of attempts in several different laboratories over many years to isolate it with standard media and isolation techniques or with nutritionally complex liquid media and vegetative inoculum.

In a similar manner, many of the fastidious fungal pathogens that are difficult to isolate or to maintain in culture also present special challenges for long-term preservation. While it remains true that the most stable approach for preserving the germplasm of fastidious fungal pathogens is with cryogenic technology, solving the challenges of any particular fungus may require much experimentation to find the right combination of medium, cryoprotectant, and freezing conditions to allow the maintenance of fungi in a viable state. The *Neozygites* species affecting cassava green mite in South America and Africa has proven to be a vivid illustration of this need, in comparison to more 'routine' entomopathogenic fungi, for individualized approaches to preservation (Delalibera, 2002); this fungus can be maintained only in a medium dramatically different from other tissue culture media widely used for other entomopathogenic fungi, by using DMSO and trehalose rather than glycerol as a cryoprotectant, and for immediate and relatively rapid freezing after addition of the cryoprotectant (I. Delalibera Jr, in prep.).

The organism-related issues of obtaining, growing, and preserving fungi isolated from insects and other invertebrates constitute only a part of the continuing concerns for fungal genetic resources repositories. Recordkeeping represents another major set of issues. Most culture collections now depend on some sort of computerized database system to maintain their records of accession information, requests for and distributions of fungi, and to publicize the nature and availability of their accessions. Specialized programming skills are required to build custom database applications; nonetheless, such an approach can be much more eficient for a large collection than depending on a suite of separate software applications to meet the overall needs. The preferred way to provide information about germplasm collections, their holdings, and associated services is through the internet and electronic mail. The value of a well implemented underlying database may increase dramatically when that database can also be used as the foundation for an effective website as well as for continued research. It can also be appreciated that the traditional role of printed catalogs of collection holdings has largely been replaced by the much less expensive electronic distribution of such information, and depending on the internet for information dissemination also allows the continuous updating of that information.

The maximization of a collection's ability to maintain and to work with an increasing number and diversity of cultures depends on acquiring suitable levels of physical, human, and financial resources to meet immediate needs (or, as every curator hopes, to expand the collection to become more comprehensive and more potentially useful to its clients). Most curators of large culture collections are anxious to train personnel and to help build the capacities and overall expertise of smaller collections, particularly when those collections are located where the existing biodiversity remains underexplored but subject to loss unless the threatened microbes are collected, isolated, and preserved.

Increasingly regulated interactions between collections and their clients — The adoption in 1992 of the International Convention on Biological Diversity (ICBD) in Rio de Janeiro was not intended by the participating nations to impose restrictions on the progress of scientific research. The ambiguities and differences among individual nation's responses to the ICBD (which remains the subject of ongoing negotiations among the participating nations) are a source of endless confusion. Worse still, microbes have not yet been considered officially by the Convention, and this neglect has left the world's microbial repositories in a very difficult and uncomfortable position of having to continue operating without guidance about what sorts of practices (especially the international movement of cultures) may become rigidly encumbered in the future and curators held potentially responsible for actions for which no official policies have yet been formulated. The issues raised by the ICBD and the inseparable consideration of intellectual property rights (IPR) involved with the ownership and use of cultures retained and

distributed by culture collections are intimidatingly complex. One of the increasingly common responses to ICBD and IPR is the adoption of Material Transfer Agreements to clarify some of the rights and responsibilities about the use of cultures whose origins are so remarkably diverse.

In addition to all of the legal issues involving ICBD and IPR, collection curators must also deal with a daunting range of national and international postal, quarantine, and other regulatory considerations such as:

- periodic inspections to assure compliance with standards for handling microbes,
- obtaining required permits for the import/export of cultures or to assure that any needed permits are obtained by those receiving cultures,
- compliance with packaging standards for biohazardous materials (as most fungal pathogens of invertebrates are treated) handled by postal or courier services,
- attempting collection compliance with constantly changing regulatory policies,
- attempting compliance with restrictions on international shipments of live cultures under existing postal requirements and the developing constraints of the ICBD, and
- when seeking new material through field collection, complying with applicable local, national, and international law and treaty restrictions and requirements for obtaining appropriate permits to collect and/or to move live organisms across political borders.

There is no way to predict how much any of these collateral isues will impede the historical functions of many culture collections nor any way to predict how or when such difficulties might resolve themselves or their negative effects decrease.

Saving threatened genetic resources collections - There is an ongoing need and responsibility within the scientific community to identify other culture collections whose accessions are being threatened or endangered from any of many possible directions (financial pressures, staffing changes such as retirements or deaths, shifting politics, changing research thrusts, etc.). Such collections are often research collections that usually include isolates that have been used in significant research or have historical importance. In some instances, the curator of a collection may contact the curator of a collection that might become the new repository site; in other instances, news or even rumors about the endangerment of a collection may circulate among scientific colleagues familiar with the collection or the scientist responsible for it, and the rescuing collection may be identified or become involved only late in the process. The rescue of such collections usually involves its incorporation into a larger collection. Such transfers may require collections to be moved across national borders and, thus, force confrontation with such complex issues as the International Convention on Biological Diversity and intellectual property rights in an uncomfortably direct manner but most collection curators also feel that it is their duty to try to overcome these problems in order to rescue the threatened germplasm from extinction by autoclave or neglect.

Daunting challenges and obstacles face all microbial genetic resource collections. Nonetheless, these culture collections are now and will remain some of the most important facilities to insure the long-term survival and availability of the world's microbial biodiversity. The innumerable diverse uses of our microbial resources, whether already recognized or still undiscovered, make all such collections an invaluable resource that needs to be utilized, appreciated, and protected by the global scientific community.

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Managing microsporidian germplasm

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Studies of microsporidian taxonomy and biology are complicated by production and storage of these relatively fastidious obligate pathogens. Many research laboratories house collections of stained slides that voucher microsporidia from experimental work and field collections, mostly as Giemsa stained material. These collections are valuable but microsporidia often lack morphological characteristics that can be used to positively identify species and biotypes, or to compare them with type species that were also vouchered as stained slides. The need for production and storage of working stock for experimentation, as well as for DNA sequencing, make efforts aimed at preserving microsporidia as viable spores an important issue.

Preservation of microsporidia

Attempts were made by a number of researchers in the 1970's to develop methods for preserving viable microsporidian spores. Lyophilization and vacuum storage appeared to be successful for short periods, from 1-3 year periods, but no follow-up studies recorded viability over long time periods (Bailey, 1972; Lewis & Lynch, 1974; Pilley, 1976; Teetor-Barsch & Kramer, 1979). Frozen water suspensions of Nosema locustae showed decreased spore viability at 8 months - 3 years in water, or 1 year in cadavers at -40 address.

> >I can, if yÚ rpaon species (Undeen and Vavra, 1997). Refrigeration of microsporidia from aquatic hosts is necessary because the spores cannot be frozen or desiccated. Problems with refrigeration, however, are several. Spores continue to metabolize, eventually lose energy reserves and die; bacterial and fungal contaminants can multiply and destroy microsporidian spores; and loosely sealed vials can become contaminated or desiccated. Long-term refrigeration requires that spores are highly purified by separating them from host tissues and environmental contaminants on a density gradient (usually Ludox HS-40® produced by Dupont or Percoll®, both distributed by Sigma), but fungal contamination may still occur (Undeen, 1997, http://www.agweb.okstate.edu/pearl/scsb387/storage.htm)

Liquid nitrogen storage of microsporidia (Vavra & Maddox, 1976) is generally acknowledged to be the best choice for lvsisolate, the microsporidia can be stored in filtered or unfiltered homogenate or in cadavers. Microsporidia need no preconditioning to be stored in liquid nitrogen; they can be immersed directly. Upon thawing, however, bacteria and saprophytic fungi in unpurified isolates will proliferate, causing death of spores unless the spores are cleaned immediately.

There are several problems associated with liquid nitrogen storage. Use of electricity-dependent storage units risks power outages or failure of the units, and repeated freezing and thawing of spores cause rapid loss of viability (Maddox and Solter, 1996). Use of liquid nitrogen tanks that are not dependent on an energy source is also risky if liquid nitrogen levels are not checked on a regular basis and liquid nitrogen is allowed to evaporate. Microsporidia, however, can persist in either the liquid or gaseous phase, so levels can drop quite low before tanks need refilling. In addition to issues associated with filling the tanks, most tanks require considerable laboratory space.

Microsporidian spores are viable immediately after removal from liquid nitrogen and thawing but we have noticed some relatively quick reduction in viability when we ship spores or hold them under refrigeration for some time after removal. It is not known whether this is a direct effect of the storage on spores or if viability loss results from the addition of streptomycin/ fungizone, which we add to the samples to retard bacterial and fungal growth after thawing. Jiri Vavra of Charles University, Czech Republic recently experimented with sodium azide as an antibiotic and found that 0.01% sodium azide works well for short periods of time, but long term storage was not successful (personal communication). It appears that shipping freshly isolated and purified spores (isolated in Ludox gradients in sterile deionized water) with the addition of a small amount of streptomycin and fungizone is probably the best method.

The INHS liquid nitrogen microsporidian collection

In 1966, Joseph Maddox began storing microsporidia in liquid nitrogen tanks at INHS. We now have five 43-liter tanks holding approximately 1800 1-ml samples of entomopathogenic microsporidia, a limited number of viruses, a few Helicosporidia, and bacteria. The collection consists of approximately 100 species or biotypes of microsporidia. Some samples are original isolates from different host species and some are conspecific isolates from different host populations. In addition, many vials contain working stocks for active research. Most of the collection consists of microsporidia isolated from hosts in the U.S., but we hold permits for several species from overseas sources.

The INHS viable microsporidia collection is currently databased in Microsoft FileMaker Pro. The database accurately represents the collection, but location discrepancies that occur due to entries and withdrawals by multiple users need to be rectified. Older isolates are lacking information that we now record such as whether the isolates were obtained from laboratory colonies or field collections. Some of this information is retrievable from the Maddox hard copy files.

Issues concerning the INHS collection and future collections

The original microsporidian isolates were placed in glass vials that were either heat-sealed or sealed with screw caps. These isolates are dangerous to handle and may not be thawed without direct supervision. For the past 20 years, only cryovials expressly manufactured for liquid nitrogen storage have been used. We currently use vials (Vangaard®) that are manufactured to tolerate both the liquid and gaseous phase of liquid nitrogen.

Our preferred cryoprotectant is 50% glycerol (Maddox and Solter, 1996). Glycerol is less dense than sucrose and less biologically active than dimethyl sulfoxide, and it is relatively easy to rinse out of thawed spores using centrifugation. Little research has been conducted, however, on the comparative viability of microsporidia stored in different cryoprotectants or combinations of cryoprotectants, and this issue should be explored. Likewise, as mentioned above, there are problems with the use of antibiotics (penicillin, streptomycin, fungizone, sodium azide) that need to be examined further. Refrigerated spores or spores frozen at higher temperatures may have different responses to both cryoprotectants and to antibiotics.

Aquatic microsporidia are not stored at INHS; most aquatic species stored in the U.S. are held in the USDA/ARS laboratory of J.J. Becnel (Gainesville, Florida) and at the Connecticut Agricultural Experiment Station laboratory of T. Andreadis (New Haven, Connecticut). These isolates are mostly stored in sterile water under refrigeration, as spores of most aquatic microsporidia cannot survive freezing (Undeen and Solter, 1996). While, as mentioned above, some aquatic spores can be stored for relatively long periods in this manner, proline in infectivity after 5 months of storage and loss all infectivity after 17 months of storage (Andreadis, 1991)

In February of 2002, a blanket permit was requested by L. Solter for collection and storage at INHS of entomopathogenic terrestrial microsporidia from all sources worldwide. The INHS laboratory was inspected, standard operating procedures were developed, and the permit was approved by USDA APHIS in April of 2002. This permit offers the potential for a wide variety of terrestrial microsporidia to be stored for future scientific study, but there will be problems and issues associated with such a permit and with the ability to collect and voucher living specimens. Some of the concerns include the following.

1. Isolates from laboratory colonies are problematic because it is often not known whether a microsporidium obtained from a colony is a natural disease organism or is an opportunistic pathogen transmitted from other host species in a laboratory setting. Care should be taken to correctly identify the source of microsporidian infection before assuming that an infection is naturally occurring in a particular host. Less likely, perhaps, but possible, is that a field-collected host from which a microsporidium is isolated is not the natural host.

2. Microsporidian spores that are shipped between laboratories sometimes arrive in putrefying solutions, which strongly impact spore viability. We have been successful in rescuing some species by producing infections in laboratory hosts to provide a clean, viable isolate for storage, but some microsporidia are very host specific and we may not be able to produce new spores if we do not have a suitable host or if the spores have degraded excessively. We need to better communicate with researchers regarding the best methods to transport spores.

4. Multiple samples of microsporidian isolates need to be housed in two or more collections so that one harmful event will not destroy all collected germplasm, but this is a long and labor intensive process and may not be possible with isolates from hosts that are unusual or difficult to rear. We don't yet have an agreement with another laboratory to store duplicate isolates. Liquid nitrogen tanks are bulky, cost approximately $1300 each for the 43-liter size and must be maintained by filling on a regular schedule. Our oldest tanks probably need to be replaced because evaporation rates are increasing. Other types of liquid nitrogen storage are available, however, that are not as demanding of space or attention, but may be more expensive.

5. Distribution of holdings has not been a problem in the past, although we have had to refuse requests to produce spores on demand. We have been able to supply isolates, usually on a one-time production basis or if we have multiple vials of an isolate (but refer to the shipping problems with previously frozen spores). We have no funds for maintenance of the collection, nor for production and harvest of spores. Agreements will need to be made and recorded regarding distribution of microsporidia collected and sent by researchers for vouchering in liquid nitrogen. No details concerning 'ownership' have yet been addressed. We currently do not release microsporidia for research without permission of the original submitting scientist unless isolates were sent with 'no strings attached' (the preferred status).

6. Isolates received for storage that are identified as described species should be propagated for ATCC as an additional repository and source of working stock for research. ATCC usually charges for storage by setting a fee for isolates. An important issue is that all isolates sent to ATCC should be positively identified with a known original source of field-collected hosts to avoid mistakes of species identity by researchers who request spores. A suggestion for discussion is whether all named isolates should be sequenced before submission to ATCC in the future.

7. Does Germplasm = DNA? Microsporidia stored in ethanol and even on stained slides can be sequenced. It is not known how long these materials retain DNA integrity or under what conditions they should be stored. The DNA alone, however, cannot currently be used to generate a microsporidian infection in order to study a species.

8. The INHS permit does not cover aquatic microsporidia, simply for the reason that INHS does not have storage facilities for this group and so no blanket permit was requested. There may be some possibilities for storage of aquatic forms, such as freezing in tissue culture (10 % DMSO) for up to 18 months as was achieved for Brachiola [Nosema] algerae (Moura et al., 1999). The extent to which B. algerae, found in mosquitoes but also occurring rarely in corneal infections of humans, is aquatic, however, is questionable. In addition, few species of microsporidia tested thus far have been amenable to tissue culture.

Germplasm collections of both aquatic and terrestrial microsporidia are needed for studies of biodiversity, biological systems, taxonomy, and biological control. There are many issues that must be explored and addressed concerning a coordinated effort to preserve microsporidium germplasm. We are currently taking steps in the direction of developing a repository that will be available for future research programs.

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Entomopatogenic bacteria repositories

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The conservation and the research of the microorganisms, animals and vegetals genetic resources constitute a vital practice in the technological and scientific development. The microbial genetic resources are extremely important to pharmaceutical, industrial and agriculture areas as well as the environmental preservation. Although the major diversity of the living system is linked to microorganisms that are estimated to be around 500.000 to 6 million species, only 3.500 bacteria, 90.000 fungus, 10.000 protists and 4.000 virus have been described until now. The microorganisms play a vital role in the agriculture sustainability and the tropical regions are probably those, which constitute the richest source of new species. However, in Brazil as well as in all the other Latin America countries, the exploration of microbial genetic resources is incipient. There is an enormous economical potential in the exploration of these resources and in order to be explored it requires a prospecting, adequate preservation of strains ex situ in collections, and the evaluation of the biotechnological and agronomic potential of the material. The preservation of the natural reserves can ensure the preservation of the microorganisms in situ. However, there are still many implications related to the use of this approach for the preservation of microorganisms. The classical form of preservation of microorganisms, mainly bacteria, has been the preservation/conservation ex situ through collections. The preservation of living cultures represents an available genetic resource for research, teaching and exploration. The pattern strains contribute to the knowledgement of the biodiversity of microrganisms and to the quality control of the developed products based on microorganisms. Besides that, the collection may serve as deposit of biological material aiming patent purposes. Countries such as the United States, England, Japan and many others keep collections with specific objectives. In most of the cases, these great foreign materials charge a high price for the strains. More than 1500 microorganisms found in nature are known for their capacity to control agricultural pests of economical interest. More than 100 bacteria have been isolated from insects and recognized as causing them diseases (alves 1998). Besides that, these microorganisms represents source of genes that can be used in genetically modified organisms, as in the case of over 100 different genes of *Bacillus thuringiensis*, which codify proteins against insects (Crickmore et al. 1998). The collection of new strains of bacteria can provide a great potential in the exploration of pests biological control agents. For example, the bioinsecticides based on *Bacillus sphaericus* and *B. thuringiensis* bacteria that have been used in the control of many species of mosquitoes and coleopterans (Tigano & Monnerat, 1999). The classical way of bacteria preservation has been the preservation/conservation ex situ, through collections. Nowadays there are efficient conservation procedures in vitro. Mainly in Europe and in the United States there are important collections of strains more specialized. However, most of the cases, the collections do not give emphasis to the tropical region microorganisms and, in addition, in many of these collections the strains are not available freely. The bacteria are preserved in their sporulated form, in paper strips in ampoules kept in room temperature. This technique allows the storage of strains for many years and makes easy the exchange among institutions. All over the world, many strains of Bt and Bs have been isolated and currently many laboratories are still searching for new ones. There are collections spread all over the world and it's supposed that there are 40000 known strains. Among them, there are some that are effective against many agricultural pests, members of lepdoptera, diptera and coleoptera, and against other groups of invertebrate

(Edwards ET. Al. 1988; Feiltelson et al. 1992; Crickmore, et al. 1995). In 1999, some Latin America groups interested in Bt and Bs collections made up a project called "Development of bioinsecticides produced from entomopatogenic bacteria to control insects". This project has 11 research groups from different Latin America countries and from France that work in the bioinsecticid area. This project has allowed a great academic exchange, as well as the formation of specialized human resources and the conveyancing of technology among the participant groups. It's expected that the small farmers and the population in general are beneficiary as long as they can count on a strategy of biological control of insects and vectors of diseases from their own countries and for keeping a healthier environment. The Bt and Bs collections that are currently available and that will increase during the project have been obtained from samples collected from a great variety of ecosystems. This constitutes a great advantage as we have in the tropical region a great biodiversity that will make possible to find and select new highly effective strains to pest control. As for example, in this region the *B. thuringiensis* subsp. *Medellin* strain was isolated (Orduz ET. Al. 1998) which has been broadly characterized and which contains the Cry11Bb toxin which is one of the most powerful against mosquitoes (Thiery et al., 1998). On the other hand, the characterization of the Mexicans strains has shown that the major part of the potentially new strains is in the tropical region (Bravo ET al., 1998). This project aims to generate products, which contain different active proteins in order to develop new products that may be used in the resistant populations to the known toxins.

Perspectives and challenges facing insect viral germplasm repositories

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Insect viruses can be applied as agents for the control of pests and disease vectors. The knowledge about the biology of these viruses and their application has increased in the last decades. The identification and characterization of insect viruses is essential to find new potential bioinsectides, for registration of commercial products as well as to get more information about their relatedness and phylogeny.

The following families of invertebrate viruses are currently classified on the Seventh Report of the International Committee on Taxonomy of Viruses (Academic Press, 2000): *Poxviridae, Iridoviridae, Baculoviridae, Polydnaviridae, Ascoviridae, Circoviridae, Parvoviridae, Reoviridae, Birnaviridae, Rhabdoviridae, Bunyaviridae, Picornaviridae, "CrPv-like viruses", Tetraviridae, Nodaviridae, Togaviridae, Flaviviridae* and *Metaviridae*. The *baculoviridae* is the most important family for biological control purposes. Baculoviruses are efficient, highly specific safe to vertebrate, plants and microorganisms and can be integrated with other control tactics in a pest management program.

There is almost no literature available about existing virus repositories. In order to obtain some information about the current status of the virus collections we decided to make a survey among insect virologists using the SIP web site. We are presenting the information derived from thirty five answers obtained from the survey. The main points chosen were about how the virologists are collecting, storing and characterising their viruses. We also got information about the current limitations and suggestions concerning the establishment of a central insect viruses repository. Finally, a list of viruses available in different collections is presented.

The data came from insect virologists from different countries such as from USA, Canada, Mexico, Brazil, Argentina, England, France, Portugal, Spain, Egypt, South Africa, Australia and Japan. The scientists are involved in different research fields such as biological control, insect pathology and epizootiology, microbiol pesticides, molecular biology of insect viruses, insect virus ecology, insect cell culture, production of baculovirus as bioinsecticides, production via fermentation, increasing efficacy of baculovirus by sinergistic additives and UV light protectants, baculovirus gene expression, entomopoxvirus genetic and application, mosquito pathology, small RNA insect viruses, epidemiology of pathogens of bees, studies on cricket paralysis-like virus, etc.

Collecting insect viruses

In general virus are collected from dead insects with signs of a possible viral infection. Live insects can also be systematically collected in the field. Usually this systematic collection is made when viruses have not been reported for a given insect species in a certain region or there is another purpose such as finding new isolates (geographical) of a given virus.

After collection, viruses have been stored by different procedures. In case of baculovirus, occlusion bodies are stored at - 20 °C , - 80 °C or lyophilyzed, and Budded Virus samples are stored at + 4 °C and - 80 °C. When there is a cell line available for virus replication the stocks

are maintained by cell culture growth and clonal isolation. It is very important to keep passage numbers low. Those viruses that have been plaque purified are approximately 4-5 passages away from the original plaque isolate.

In some laboratories purified or semi-purified preparations of insect virus are made 50% to glycerol and stored at - 20 °C or freeze-dried with cryoprotectant.

Characterization of insect viruses

Several techniques have been routinely used for characterization of insect virus. Transmission electron microscopy and light microscopy are commonly applied as a first step to virus identification. The most widely technique used to characterize the viruses the restriction enzymes analysis of viral DNA (REN). This is also important to distinguishing between viral isolates. Protein analysis by polyacrylamide gel electrophoresis (SDS-PAGE) and also serological identity/relatedness using imunodiffusion, Elisa and Western blots are very useful techniques. Finally several molecular techniques like cloning, sequencing and PCR analysis have been used more and more in many laboratories. Bioassays are also used as a way to evaluate the virulence of virus isolates.

Limitations on the establishment of a insect viruses repository

Currently there are very few collections and usually upon the effort of a single or small group of scientists. There is no collection available for teaching or research purposes. Multiplying the viruses requires expertise, quality control and availability of host-species. To have a virus repository it is needed to question acquisition time, cost, purity and passage number. Besides it must be considered the authentication of the virus identity and some regulations (APHIS regulations).

A important point is that usually the virus is very specific to one insect species. Not all insects are routinely maintained in the institutes. It is not always possible to rear the insect on artificial diet in laboratory conditions. In general, it requires research to establish the right conditions, it may very laborious and very time consuming.

Another possibility is to grow the virus isolates in cell culture. However, there are few established cell lines available. Some baculoviruses exhibit a high degree of genetic instability when propagated *in vitro* and variants identified in serial passage may not represent the constituents of the original isolate. While most molecular genetic studies of baculoviruses have been performed with plaque-purified strains propagated in cell culture, field isolates often show considerable genetic heterogeneity.

The serial passage of nucleopolyhedrovirus (NPVs) in cell culture can generate a variety of mutations or defective viral populations. Defectives Interfering Particles (DIPs) and FP (few polyhedra) mutants are the most common mutations caused by the passage effect. As a consequence, there is loss of virulence for target insects since in most cases the number of virions within the polyhedra or total polyhedra count in the nuclei is reduced.

Actually there is a lack of easily resourced inventory of insect viruses and contacts to obtain characterized viruses. A major concern is the loss of virus isolates from individual collections when its owner retires or programs are shut down. Also, the availability of resources to maintain a virus collection is another concern.

List of insect viruses in individual collections (based on survey data)

TABLE 1. *Baculovirus / Family* baculoviridae

1. *Adoxophyes orana* NPV
2. *Adoxophyes orana* GV
3. *Agraulis sp NPV*
4. *Agrotis. segetum* NPV
5. *Alabama argillacea* NPV
6. *Amathes C-nigrum* NPV
7. *Anagrapha falcifera* NPV
8. *Anticarsia gemmatalis* NPV
9. *Archips argyrospila* GV
10. *Archips argyrospila* NPV
11. *Artogeia rapae crucivora* GV
12. *Autographa californica* NPV
13. *Bombyx mori* NPV
14. *Buzura supressaria* NPV
15. *Cadra cautella* GV
16. *Cadra cautella* NPV
17. *Cadra figulilella* NPV
18. *Chhoristoneura conflicta GV*
19. *Choristoneura fumiferana* GV
20. *Choristoneura fumiferana* NPV
21. *Cryptophlebia leucotreta* GV
22. *Culex nigripalpus* NPV
23. *Cydia pomonella* GV- CpGV
24. *Diatraea saccharalis* GV
25. *Dione Juno juno* NPV
26. *Epinotia aporema* GV
27. *Erinnyis ello* GV
28. *Euproctis chrysorrhoea* NPV
29. *Euproctis subflava* NPV
30. *Galleria mellonela NPV*
31. *Helicoverpa armigera* GV
32. *Helicoverpa armigera* NPV
33. *Helicoverpa pura* NPV
34. *Helicoverpa zea* NPV
35. *Hemileuca maia* NPV
36. *Homona magnanima* GV
37. *Hyphantria cunea* NPV
38. *Lacanobia oleracea* GV
39. *Lymantria disparNPV*
40. *Malacosoma disstria NPV- MadoNPV*
41. *Mamestra brassicae NPV- MbNPV*
42. *Mamestra configurata NPV – MacoNPV*
43. *Megalopyge albicolis NPV*
44. *Mocis sp. GV*
45. *Neodiprion sertifer NPV*
46. *Ochlerotatus sollicitansNPV*
47. *Orgyia leucostigma NPV*
48. *Orgyia pseudosugata NPV*
49. *Orgyia thyellina NPV*
50. *Perigonia lusca NPV*
51. *Pieris rapae GV*
52. *Platynota idaeusalis NPV*
53. *Plodia interpunctella GV*
54. *Plutela operculela NPV*
55. *Plutela xylostella GV*
56. *Porthetria dispar NPV*
57. *Pseudaletia loreyi NPV*
58. *Pseudaletia separata NPV*
59. *Pseudaletia sequax NPV*
60. *Pseudaletia unipuncta GV*
61. *Pseudoplusia includens NPV*
62. *Rachiplusia nu NPV*
63. *Rachiplusia ou NPV*
64. *Spodoptera eridanea NPV*
65. *Spodoptera exigua NPV*
66. *Spodoptera frugiperda GV*
67. *Spodoptera frugiperda NPV*
68. *Spodoptera latifascia NPV*
69. *Spodoptera littoralis NPV*
70. *Spodoptera litura NPV*
71. *Spodoptera mauritia NPV*
72. *Spodoptera ornithogalli NPV*
73. *Trichoplusia ni SNPV*
74. *Urbanus proteus NPV*
75. *Xestia c-nigrum GV*
76. *Xestia c-nigrum NPV*

TABLE 2. Other insect viruses / Family

Amsacta moorei Entomopoxvirus -*Entomopoxviridae*
Amyelosis Chronic stunt vírus - *Caliciviridae*
Bee acute paralysis- *Picornaviridae*
Bee slow paralysis- *Picornaviridae*
Black beetle vírus (BBV)- *Nodaviridae*
Bombyx mori densonucleosis - *Parvoviridae*
Choristoneur fumiferana Entomopoxvirus- *Entomopoxviridae*
Choristoneura biennis Entomopoxvirus- *Entomopoxviridae*
Cricket paralysis virus (CrPV)- *Picornaviridae*
Cypovirus sp -*Reoviridae*
Drosophila C virus - *Picornaviridae*
Drosophila small RNA virus - *Picornaviridae*
Flock house vírus (FHV)- *Nodaviridae*
Helicoverpa armigera stund virus -*Tetraviridae*
Helicoverpa zea tetravirus- *Tetraviridae*
Heliothis virescens ascovirus 1a- *Ascoviridae*
Limantria dispar cypovirus 11- *Reoviridae*
Mosquito iridescent viruses - *Iridoviridae*
Nodamura vírus (NoV)- *Nodaviridae*
Nudaurelia beta virus- *Tetraviridae*
Nudaurelia omega virus- *Tetraviridae*
Orgyia pseudosugata cypovirus 5- *Reoviridae*
Pariacoto vírus (PaV)- *Nodaviridae*
Spodoptera fugiperda ascovirus 1a - *Ascoviridae*
Thosea asigna vírus (TaV)-*Tetraviridae*
Trichoplusia ni cypovirus 5- *Reoviridae*

Improvement of the current situation on insect viral germplasm repositories

The establishment of a central insect viruses repository is a general demand by those responding to the survey. However, it was suggested to keep a back up held elsewhere. Another main concern is the long-term maintenance of the collection. The need to establish a link to the SIP web site with a list of persons who have which viruses and how to contact them was a general recommendation to be taken as an immediate goal.

Another suggestion was to utilize existing culture collections to deposit samples, as for example the ATCC (American Type Culture Collection). Currently, Dr. Suzanne Thiem and Dr. Gary Blissard are serving on the American Society for Virology ATCC collections advisory board. In this capacity they have been soliciting deposits from various insect virologists. Presently they are focusing on obtaining deposits of AcMNPV strains that are widely used and sequenced viruses, due to the current capacity of the ATCC to process a large influx of viruses. In addition to ATCC there may be other repositories that could bee utilized for archiving insect virus species. In Brazil, Embrapa Genetic Resources and Biotechnology (Brasília, DF) is starting

a program to organize a central virus repository in collaboration with other institutes of Embrapa and some Brazilian universities.

Although there are several limitations when working with viruses, a major agreement is that the insect virology community should make a strong effort to have reference stocks and references laboratories, like the ones for other microorganisms.

Symposium (Nematodes 2) Entomopathogenic Nematodes: Research Trends

Ecological genetics of entomopathogenic nematodes: Are there Metopopulations?

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A metapopulation is a population of subpopulations. If dispersal is low, then subpopulations remain genetically distinct and weakly selected deleterious alleles can reach high frequencies in local populations. This can lead to inbreeding depression and extinction of local populations. The semi-isolation of sub-populations means that they are likely to differ with respect to the deleterious alleles they harbor. Therefore, benefits accrue among the hybrid offspring of residents and immigrants, as the bad effects of any recessive alleles they receive from one parent are likely to be masked by the alleles from the other parent. One of the hallmarks of metapopulations is the appearance and disappearance of subpopulations from habitat patches as a result of frequent extinction and recolonization. The disappearance of entomopathogenic nematodes soon after their application to the soil is well documented. However, the nematodes do perpetuate at certain locations naturally. Therefore, elucidating the factors/processes that prevent the extinction of nematode populations at certain sites is important to develop novel conservation approaches for the use of entomopathogenic nematodes. We explored the possibility of the existence of a metapopulation dynamics in natural populations of the entomopathogenic nematode, *Heterorhabditis bacteriophora* on a golf course. We found that the infective juvenile longevity (at 25°C in autoclaved tap water) and tolerance to major environmental stresses including heat (survival 40°C for 2 h), ultraviolet (UV) radiation (original virulence remaining after exposure to 302 nm UV for 5 min), hypoxia (survival at approximately 0% dissolved O_2 at 25°C for 96 h), and desiccation (survival in 25% glycerol at 25°C for 72 h) differed significantly among populations. Intrinsic dauer juvenile longevity, defined as the number of weeks to 90% mortality (LT_{90}) estimated using probit analysis of nematode survival data at 25°C varied between 11 to 16 weeks among populations and survival after exposure to different stresses varied between 25-100%. Furthermore, these nematode populations showed differences in the isozyme patterns for several metabolic enzymes when analyzed through a cellulose acetate gel electrophoresis technique. Thus, the differences in the isozyme patterns, infective juvenile longevity, and tolerance to ubiquitous environmental stresses in populations of *H. bacteriophora* isolated from a single 200 m^2 grassland locality strongly support the hypothesis that population structure of heterorhabditid nematodes is highly fragmented. Further research is needed on the extent of gene flow and mechanisms of migration among the subpopulations of *H. bacteriophora* to unequivocally confirm the existence of a metapopulation dynamics.

Evaluating nontarget effects on below ground invertebrates

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Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae together with their symbiotic bacteria, *Xenorhabdus* and *Photorhabdus,* respectively, represent a unique biological control agent. The nematodes vector the entomopathogenic bacteria within their gut into the insect host, where the bacteria multiply and produce a wide range of toxins and hydrolytic exoenzymes that are responsible for the death and bioconversion of the insect larva into a nutrient soup, that is ideal for nematode growth and reproduction. The nematodes reproduce until the nutrient supply becomes limiting at which time they develop into infective juveniles, which are recolonized by the symbiotic bacterium (Forst & Clarke, 2002). The nematode-bacterium complex kills insects so rapidly (48hr) that they do not form intimate, highly adapted, host-parasite relationship that could be found in the majority of other parasites used as biological control and therefore can exploit a wide host range of insect species (Grewal & Georgis, 1998). They can be easily mass-produced using conventional fermentation technology, are exempt from registration requirements in many countries and therefore several formulations of entomopathogenic nematodes (EPNs) are available on the market for the control of soil and cryptic pests in North America, Europe, Asia, and Australia (Grewal & Georgis, 1998).

Entomopathogenic nematodes are mostly used as inundative application and the infective juveniles are released in massive numbers with the aim of obtaining immediate pest suppression. Entomopathogenic nematodes and their associated symbiotic bacteria have been considered a safe approach to pest control. They have been proved to be not harmful to the humans and others warm-blooded vertebrates (See Ehlers & Hokkanen 1996 and references therein). The immune system of warm-blooded animals seems to be able to eliminate EPNs (encapsulation by macrophages). The lack of impact on warm- blooded animals has also been attributed to the fact that EPNs cannot survive at the temperatures greater than 37°C . However, high temperature adapted species of nematodes from tropical and subtropical environments possibly require attention because their possible capacity to grow and to infect at human body temperature (Ehlers & Hokkanen 1996). Possible allergic reactions against specific proteins of EPNs for production and application personnel could occurs, as is the case with any foreign proteins (Ehlers & Hokkanen 1996). The symbiotic bacteria *Xenorhabdus* and *Photorhabdus* are known to excrete several metabolites into the culture broth but so far no toxicity to humans has been documented (Boemare et al. 1996).

Due to the fast decline of EPNs to low levels after application, low persistence in the field, low survival on the foliage, and low mobility, the potential environmental impacts of EPNs are more likely to occur in the soil of treated fields (Ehlers & Hokkanen, 1996). Several short-term laboratory and few field tests were performed especially with mobile species above ground and Akhurs & Smith (2002) compiled examples of reported tests performed since 1970.

Among the vertebrates cold-blooded species, toads (Kermarrec & Mauleon, 1985, Kermarrec et al. 1991) and frogs (Poinar & Thomas 1988), were demonstrated to be affected under laboratory conditions with very high dosages of *S.carpocapsae* and *Heterorhabditis*

bacteriophora. However, under field conditions the negative results could not be reproduced (Bathon 1996). Among the invertebrate species representatives of the Symphyla, Collembola, Arachnida, Crustacea, Diplopoda, Hymenoptera, Coleoptera, Gastropoda, and Tardigrada showed susceptibility to the entomopathogenic nematodes (See Akhurst & Smith 2002 and references therein). However, as pointed out by Akhurst & Smith (2002), these studies are sometimes dismissed because they are conducted under controlled conditions with high levels of constant exposure and some stress on the experimental animals. Also many of the species tested, especially the mobile above ground, may have little chance to be exposed to the EPNs. Field studies with more realistic doses and appropriate nontarget species, in the correct life stage, should be performed in order to clarify some of the results and to make a proper evaluation of the impact of EPNs on nontargets.

According to Barbercheck & Miller (2000), the risk of adverse effects on nontarget arthropods is probably the greatest in insects with at least one soil dwelling stage that are the same order or family as the natural host. Results of some studies conducted in field conditions show that only small reductions in field populations of nontarget species have been associated with application of EPNs. No significant adverse effects of entomopathogenic nematodes were observed on populations of collembolans and mites (Ishibashi *et al*. 1987; Georgis et al , 1991) or on nontarget insects in the families Carabidae, Histiridae, Staphylinidae and Gryllidae (Georgis et al., 1991). Rethmeyer and Bathon (1992) investigated the impact of *S.feltiae* and *Heterorhabditis* spp. on different coleopteran families in a beech- oak forest, a pine forest margin, a field crop and an orchard. They found few reductions in population size of five coleoptera families. However, they recovered higher number of some coleopteran species from nematode-treated plots than from untreated plots.

Little is known about the influence of entomopathogenic nematodes on diversity of native fauna/flora in below-ground food webs. In one study, Ishibashi & Kondo 1986, reported that single applications of EPNs to bark compost resulted in decrease of fungivorous, predatory, and plant-parasitic nematodes with effects lasting up to 5-7 weeks, and suggested that EPNs could have significant impact on soil food webs and their ecological roles.

In the past ten years, strong evidence has emerged that commercial applications of EPNs significantly reduce populations of plant-parasitic nematodes (Grewal & Georgis, 1998) This phenomenon was first reported by Bird & Bird (1986) in a greenhouse trial, in which they found that root-knot nematode *Meloidogyne javanica* populations were suppressed by application of the *Steinernema glaseri*. Since this first report, this phenomenon has been confirmed in the laboratory or greenhouse studies by several researchers (Ishibashi & Kondo, 1987; Ishibashi & Choi, 1991; Smitley, 1992; Grewal *et al*. 1997; Perry *et al*. 1998; Lewis et al., 2000). In an intensive search for the mechanism of this suppression, allelochemical effects of entomopathogenic nematodes/bacteria complex were found on several facets of *M. incognita* life cycle (Grewal et al., 1999). The studies indicated that *Xenorhabdus* and *Photorhabdus* bacteria produce allelopathic effects on root penetration, development, egg production, egg hatch, and survival of plant-parasitic nematodes. While these effects are desirable to reduce plant damage by plant-parasitic nematodes, fundamental questions arise about the safety of entomopathogenic nematodes/bacteria complex to other nontarget soil invertebrates and on the functioning of below ground food webs. Impact on the nematode soil community can play an important effect on important soil process since they have a significant role in regulating primary production, predation, decomposition of organic matter, and nutrient cycling (Griffiths, 1990).

In order to clarify this aspect field trials were conducted by Somasekhar *et al.* (2002) in a turfgrass ecosystem, where two native species of EPNs *Heterorhabditis bacteriophora* strain GPS11 and *H. bacteriophora* strain HP88 and one exotic species *H. indica* strain LN2 were

applied. The results showed significant reduction of the abundance, species richness, diversity, and maturity of the nematode community by reducing the number of genera and abundance of plant-parasitic, but not free-living nematodes, up to 60days of experiment. Total nematode abundance significantly decreased in all the treatments relative to the untreated control, reflecting the differences that could be attributed to the disturbance induced by pest control treatments. These results were the first to indicate selective suppression of plant parasitic nematodes by entomopathogenic nematodes, *H. bacteriophora* and *H. indica,* with no adverse effect on free-living nematodes. Among the EPNs, the exotic species (*H. indica* strain LN2) showed more effect compared to the native ones. The results from those studies confirmed the hypothesis that EPNs have the potential to affect the diversity of native fauna in soil ecosystems even though they do not have any direct parasite/host or predator/prey relationship. In this study, the impact of entomopathogenic nematodes on the soil nematode community can be interpreted as a beneficial nontarget effect based on the importance of plant parasitic nematodes as agricultural pests. However, the antagonism between plant parasitic and entomopathogenic nematodes is an unexpected nontarget effect. The metabolic products of symbiotic bacteria of EPNs were reported to possess broad spectrum of biological activity including insecticidal, nematicidal, antimycotic, anticancer and antibiotic properties (Webster et al., 1998). Maxwell *et al.* (1994), state that the broad antimicrobial activity of *X. nematophilus* has raised concerns about the impact of such agents released from the fragmenting host cadavers on soil microorganisms. At the present time there are no records of the range of activities of these metabolites and the exact mechanisms that result in the observed effects need to be solved in order to ascertain the safety of EPNs to other organisms in the soil food webs. Although the impact of EPNs on soil nematode community structure observed in that study could be a short-term effect confined to the application sites, it is significant because EPNs are applied often as inundative strategy and repeated applications of these nematodes to control recurring pest populations may sustain the impact. Also, the fact the exotic EPN species used in that test resulted in a more significant reduction of plant parasitic nematodes, suggest a potential competition /displacement with the native species, indicating the importance of the use of native species of biological control agents as much as possible.

The displacement of native EPNs specie by a non-endemic species is another kind of potential effect to nontarget species and in general is not easy to demonstrate. Barberchek & Millar (2000) conducted field experiments to determine the effects of an introduced EPN (*S. riobrave*) on two native EPNs (*H. bacteriophora* and *S.carpocapsae*). Different combinations of native and introduced nematodes were applied to field plots in no-till and conventionally tilled corn, and the soil was sampled periodically throughout the season to determine the relative success of each nematode in terms of their ability to cause infections of test insects. The application of the non-endemic EPN had a negative impact on the two endemic species of EPNs. However did not occur complete displacement of the native species. Application of introduced *S.riobrave* resulted in highest levels of insect mortality associated with a reduction of proportion of infection by both of the native nematodes. Based on the results showed by the three species studied, the authors suggested that some behavioral or other mechanism may exist that reduces spatial overlap and could allow an introduced species to establish without leading to the complete displacement of the native species. Patchiness of EPN distributions probably favorable for co-existence of multiple species at a site, reduce the risk of displacement of native by non-native nematodes. Long-term monitoring is necessary to elucidate this hypothesis.

Future research should evaluate long-term effects of entomopathogenic nematodes and their symbiotic bacteria on below ground food webs in microcosms and in agriculture ecosystems with experiments designed to evaluate alterations in functional diversity, decomposition rates, and nutrient cycling and long-term monitoring.

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Virulence mechanism of a slug-parasitic nematode and its associated bacterium

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Introduction

Slugs are important pests of a wide range of agricultural and horticultural crops worldwide (Godan, 1983; South, 1992). The grey garden slug *Deroceras reticulatum* is the most common species and often regarded as the most serious pest (Wilson et al., 1993). *Phasmarhabditis hermaphrodita*, a bacteria-feeding nematode, has potential for the biological control of mollusk pests. The nematode is a lethal parasite of various slugs and snails including *D. reticulatum* and is harmless to other beneficial invertebrates (Glen and Wilson, 1997). Wilson et al. (1995b) recovered over 150 bacterial isolates associated with the nematode. However, nematode yield in *in vitro* cultures and pathogenicity to slugs differs with different species of the associated bacteria (Wilson et al., 1995a, b). *Moraxella osloensis* was finally selected as the preferred associated bacterium for rearing *P. hermaphrodita* in monoxenic culture (Wilson et al., 1995b). A commercial product, NemaSlug™, based on *P. hermaphrodita* has been developed in England. However, a high dose of the nematodes (3 ´ 10^9 infective juveniles/ha) is required for effective plant protection in the field (Grewal et al., 2001). Pathogenicity of the mass-produced nematodes varies among different batches and aged *P. hermaphrodita* are less virulent (Tan and Grewal, 2001b). All these factors restrict further development of the product. Therefore, information on the virulence mechanism of the nematode/bacterium complex is necessary to develop a high quality biological control product for managing slug pests in landscapes, nurseries, and field crops, worldwide.

Infection behavior of *P. hermaphrodita*

The parasitic cycle of *P. hermaphrodita* is initiated by the third-stage dauer (enduring or non-aging) juveniles. The dauer nematodes can invade *D. reticulatum* through the dorsal integumental pouch immediately posterior to the mantle, and enter the shell cavity via a short canal, then develop into self-fertilizing hermaphrodites that produce progeny, resulting in host death (Wilson et al., 1993). When the food source is depleted, the nematodes form new dauer juveniles to leave the cadaver in search of new slug hosts. Tan and Grewal (2001a) found that the dauer juveniles of *P. hermaphrodita* invade *D. reticulatum* within 8-16 hr following external exposure, with the posterior mantle region containing the shell cavity serving as the main portal of entry (Fig. 1). Moreover, the dauer juveniles can recover, multiply, and produce new generation of dauer juveniles in the slug and slug feces homogenates thus demonstrate that *P. hermaphrodita* is a facultative parasite of *D.*

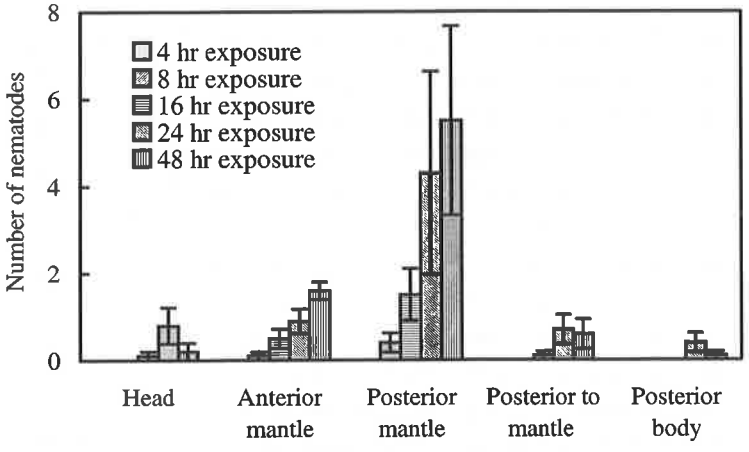

FIG. 1. Time and route of invasion of *P. hermaphrodita* into *D. reticulatum*.

reticulatum and can complete its life cycle under non-parasitic conditions associated with the host. Furthermore, although the non-dauer (juvenile and adult) nematodes can kill the slug after injection into the shell cavity of the slug, only the dauer juvenile can serve as an infective stage through external exposure (Fig. 2).

Pathogenicity of *M. osloensis*

M. osloensis is a gram-negative aerobic bacterium, which is coccal or rod-shaped, but tends to be pleomorphous (Bovre, 1984). The bacterium is an opportunistic human pathogen and has been found to cause several human diseases (Buchman et al., 1993; Fijen et al., 1994; Stryker et al., 1982; Sugarman and Clarridge, 1982). Wilson et al. (1995b) reported that a 24-h culture of *M. osloensis* that was injected into *D. reticulatum* hemocoel was not pathogenic. However, Tan and Grewal (2001b) discovered that the aged cultures of *M. osloensis* were pathogenic to *D. reticulatum* after injection into the shell cavity and hemocoel (Fig. 3). Moreover, coinjection of penicillin and streptomycin with the aged bacterial cultures reduced their pathogenicity to the slug. Further research suggested that reduction and loss of pathogenicity of the aged dauer juveniles of *P. hermaphrodita* to *D. reticulatum* results from the loss of *M. osloensis* from the aged nematodes (Fig. 4). Furthermore, axenic J1/J2 *P. hermaphrodita* were non-pathogenic after injection into the shell cavity (Fig. 5) and the pathogenicity of the dauer juveniles depended on the number of viable *M. osloensis* carried by the nematodes. Therefore, it was concluded that *P. hermaphrodita* acts as a vector to transport the associated bacterium *M. osloensis* into the shell cavity of *D. reticulatum* and the bacterium is the main killing agent in the nematode/bacterium complex (Tan and Grewal, 2001b).

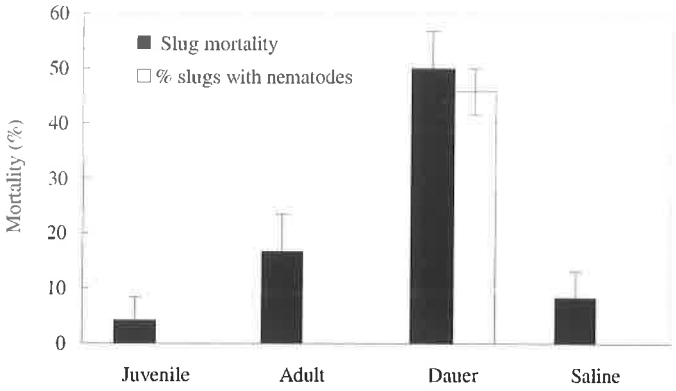

FIG. 2. Infectivity of different life stages of *P. hermaphrodita* to *D. reticulatum* at 15 days after external exposure to the slugs (50 juvenile, 50 dauer, or 80 adult nematodes per slug).

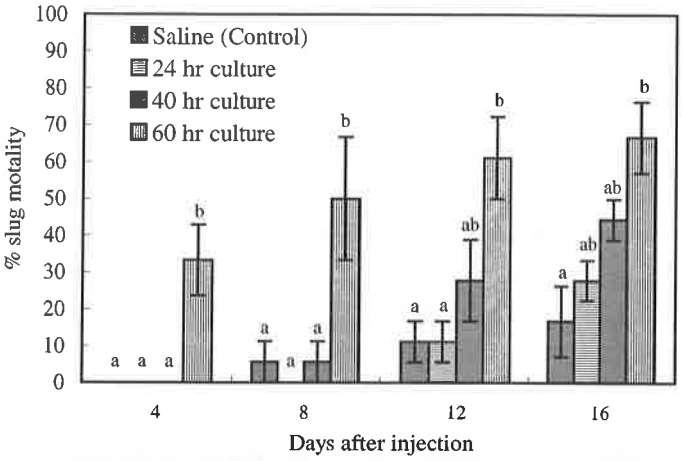

FIG. 3. Percentage mortality of *D. reticulatum* following injection of different aged cultures of *M. osloensis* into hemocoel. Values differ significantly at P < 0,05 as indicated by different letters.

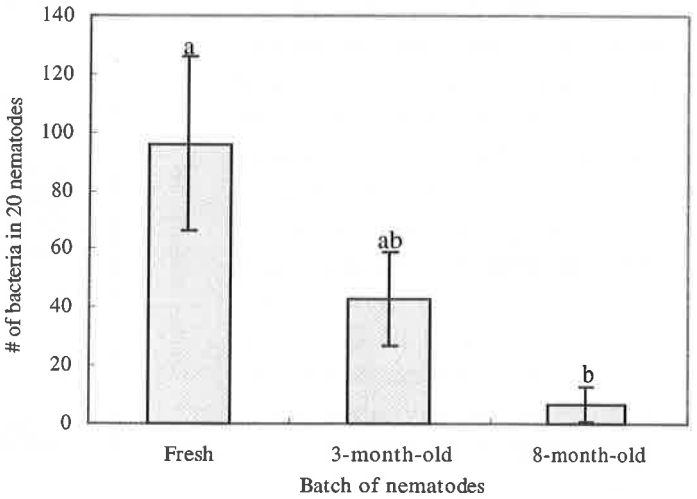

FIG. 4. Number of virable *M. osloensis* in 20 dauer juveniles of *P. hermaphrodita* from a fresh, a 3-month-old, and an 8-month-old batch. Different letters indicate significant at *P < 0.05*.

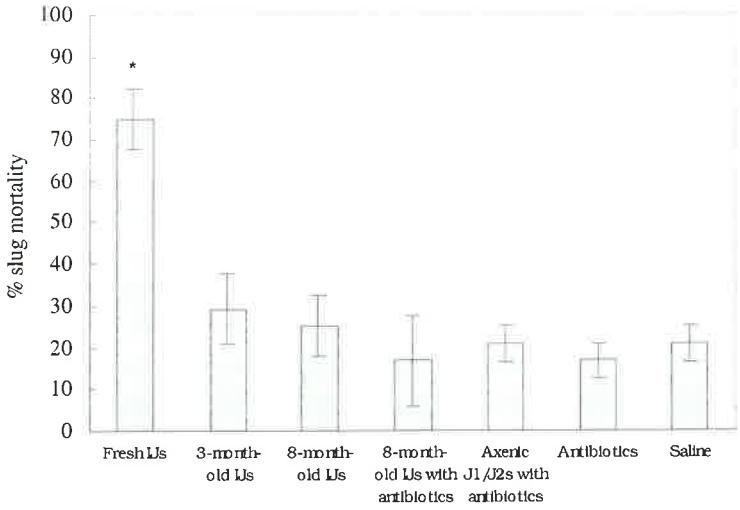

FIG. 5. Percentage mortality of *D. reticulatum* following injection of axenic and aged *P. hermaphrodita* into the shell cavity. (*) significant at $P < 0,05$.

Endotoxin activity of *M. osloensis* lipopolysaccharide

Lopipolysaccharide (LPS) usually plays a major role in the pathogenesis of gram-negative infection (Enright and McKenzie, 1997). A related bacterium, *Moraxella catarrhalis*, the third commonest pathogen of the respiratory tract of humans, is thought to liberate LPS for its pathogenicity (Cullmann, 1997; Storm et al., 1991). We discovered that *M. osloensis* also produces an endotoxin(s) to kill *D. reticulatum* when injected into the shell cavity (Fig. 6). Injection of purified lipopolysaccharide (LPS) from *M. osloensis* cultures into the shell cavity caused slug death with an estimated LD_{50} of 48 mg per slug. No contact or oral toxicity of the LPS to the slug was detected. Isolated lipid A portion from the LPS was toxic to the slug after injection into the shell cavity, but the polysaccharide portion was not. Moreover, we semiquantitated the LPS as 6 ´ 10^7 endotoxin units per milligram and detected endotoxin activity in the lipid A portion but not in the polysaccharide portion by *limulus* amebocyte lysate assays. Furthermore, the analysis of the LPS by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by visualization with silver staining revealed that the LPS from *M. osloensis* is a rough-type LPS with an estimated molecular weight of 5,300.

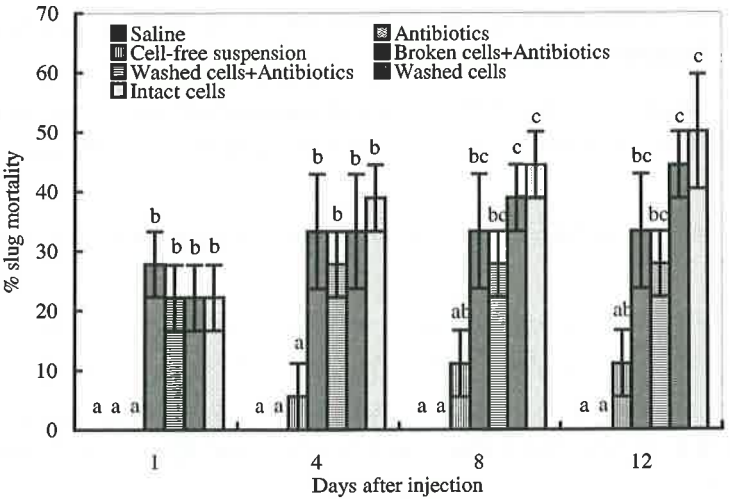

FIG. 6. Percentage mortality of *D. reticulatum* following injection of different components of three-day *M. osloensis* cultures into the shell cavity. Values differ significantly at $P < 0,05$ as indicated by different letters.

Expression of *M. osloensis* virulence factors during culture

Surface exposed antigens have been considered as virulence factors of *M. catarrhalis* such as LPS (Enright and McKenzie, 1997; Storm et al., 1991), outer membrane proteins (Murphy, 1990), and pili (Ahmed et al., 1992; Marrs and Wier, 1990; Riktomi et al., 1991). Tan and Grewal (2001b) indicated that intact cells from 60 hr *M. osloensis* cultures were more pathogenic than those from 40 hr bacterial cultures against *D. reticulatum*. Moreover, washed (unbroken) or broken *M. osloensis* cells treated with penicillin and streptomycin from 3-day cultures also were more pathogenic than those from 2-day cultures after injection into the shell cavity of the slug (Tan and Grewal, 2002). Further, Tan and Grewal (2002) discovered that heat and protease treatment, and 2-day storage at 22°C increased the endotoxin activity of the 2-day broken cells but not of the 2-day washed cells treated with the antibiotics thus suggest that a proteinaceous substance(s), which probably is an outer membrane protein(s) structurally associated with LPS, may mask the LPS toxicity in the 2-day bacterial cells. We also found that 1- or 2-day *M. osloensis* cultures were either not or less pathogenic to the slug whereas 3-, 4-, or 5-day *M. osloensis* cultures were highly pathogenic. A series of light micrographs of Gram-stained bacteria from 1 to 5-day *M. osloensis* cultures shows that the bacteria tended to adhere gradually together in the first three days, and *M. osloensis* cells from 3-day cultures formed a special net-like structure, however, the bacteria began to separate or loose after 3 days. Further, their corresponding transmission electron micrographs demonstrate that pili-like projections were rarely present on the surface of *M. osloensis* cells from 1-day cultures, but reached maximal density on the cellular surface of the bacteria from 3-day cultures. The amount of LPS produced by 1×10^{10} *M. osloensis* cells was not obviously different over cultural time by SDS-PAGE of proteinase K-digested lysates of the bacteria following silver staining. It is suggested that the LPS, outer membrane protein(s), and pili-like projections are virulence factors for *M. osloensis*. The change of the endotoxin activity of *M. osloensis* cells over cultural time may be related to the structural change of the bacterial cell wall.

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Symposium (Bacteria 2) Bt Transgenic Plants and Insect Resistance to Bt Toxins

Current status of *B. thuringiensis* resistance and *B. thuringiensis* resistance-management in Bt cotton in the U.S.

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Bt cotton has been commercially grown in the U.S. since 1996. In 2000, 1.4 million hectares of Bollgard cotton were grown representing 25-30% of the total market. However, in some regions within the cotton belt such as Alabama, statewide use has consistently exceeded 60% Bollgard cotton since 1996, and some regions within the state have exceeded 90% since 1996. Field observations and laboratory bioassays conducted since 1996 have not consistently shown any field tolerance or resistance development by tobacco budworm, *Heliothis virescens*, cotton bollworm, *Helicoverpa zea*, and pink bollworm, *Pectinophora gossypiella* anywhere in the US. In 2001, EPA renewed a 5 year conditional registration for Bollgard cotton, probably partly because no resistance events were reported. Besides requiring Bt resistance management practices such as "High Dose plus Refugia", U.S. EPA further requires registrants of transgenic insecticidal crops to develop and implement a program for monitoring toxin resistance to their registered product(s) and directing mitigation actions against resistance, if and when it occurs. The Heliothine Cry1Ac resistance monitoring and mitigation committee was established in 2001 to prepare a document for such a plan for Bollgard cotton. This presentation will discuss the current proposed draft document from the Heliothine Cry1Ac resistance monitoring and mitigation committee, current status of laboratory selection of *H. zea* to Cry1Ac, and other on-going research topics regarding Bt resistance in Bt cotton.

Bacillus thuringiensis toxin and nematodes: mechanisms of resistance and toxicity

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Our laboratory is studying the mechanism of action of *Bt* toxins in the nematode *Caenorhabditis elegans*. *C. elegans* is a hermaphroditic nematode species that naturally feeds on bacteria in the soil. It has a 3.5 day generation time and each hermaphrodite gives rise to ~300 progeny. The genome has been completely sequences, forward and reverse genetics are readily achievable, and there is an extensive genetic and molecular map for cloning genes. These characteristics make *C. elegans* a good system for studying *Bt* toxicity and resistance. By studying *Bt* toxins in *C. elegans*, we can complement insect studies to broader our understanding of these important toxins, elucidate conserved toxicity mechanisms, and investigate the usefulness of *Bt* toxins as nematode control agents.

To date, the most studied nematicidal *Bt* toxin is Cry5B. We chose this toxin because of its overall 24% amino acid identity in the toxin domain with Cry1 family toxins, with the highest identity being in subdomain 3 (33%). Furthermore, it has recently been shown by Garcia-Robles et al that Cry5A, a member of the same *Bt* toxin subfamily as Cry5B, has a mode of action similar to the Cry1 toxins. As such, we predict that knowledge gained by studying Cry5B will be relevant towards insecticidal toxins as well.

We have demonstrated that Cry5B damages the nematode intestine (Marroquin et al., 2000). The gut of *C. elegans* L4 (fourth larval staged) hermaphrodites shows constriction, vacuolization, and pitting when fed toxin. The animals are sick and die in several days. At the ultrastructural level, we can see major changes in gut morphology in as little as 3 hours after initiation of feeding.

We have screened for *C. elegans* mutants resistant to Cry5B. One of our major approaches has been to treat large populations of worms with the mutagen EMS and plate the second generation first staged larvae on plates containing toxin-expressing *E. coli*. After a few days, we select for resistant nematodes that have developed to the adult stage. Using this screen and others, we have isolated more than 40 resistant alleles in five complementation groups that we call the *bre* or *Bt* toxin resistance genes. We have verified using several criteria that these mutants are highly resistant. For instance, when fed even high doses of Cry5B toxin, *bre* animals show normal, undamaged intestines.

To date we have cloned two resistance genes, *bre-5* and *bre-3*. *bre-5* was cloned using a standard mapping/complementation approach and encodes a putative ?-1,3-galactosyltransferase (Griffitts et al., 2001). This enzyme transfers galactose via a ?-1,3 linkage to growing carbohydrate chains found on proteins and lipids. Our canonical null allele, *ye17*, results in a premature stop codon about 70% of the way through the protein. We hypothesize that *bre-5* is involved in adding a carbohydrate to a toxin receptor and that this carbohydrate modification is required for toxin to recognize and bind to the target gut.

To test this hypothesis, we first demonstrated where BRE-5 function is required for toxin action. Mosaic animals were made in a *bre-5* mutant background using a *bre-5* transgene (coupled to a nuclear GFP marker expressed in most somatic cells) that could be lost during somatic divisions. Resistant animals were selected from these mosaic animals. The results of

this experiment were dramatic—animals that had lost BRE-5 function in the gut were resistant to toxin. Thus, *bre-5* normally functions in the gut to confer toxin susceptibility. Preliminary expression results confirm that BRE-5 protein is expressed in the gut in a punctate cytoplasmic (Golgi?) pattern.

To test whether *bre-5* is required for toxin to bind to the gut, we used an indirect in vivo assay. Cry5B toxin was labeled directly with rhodamine and fed to L4 staged hermaphrodites. Within 1-2 hours, toxin can be seen to colocalize with autofluorescent lysosomal gut granules inside of intestinal cells. In *bre-5* mutant hermaphrodites, however, uptake of toxin inside of the gut cells is not seen, although there is significant toxin present in the gut lumen. These data suggest that *bre-5*, and by extension the carbohydrate modification made by *bre-5*, is needed for toxin to bind the *C. elegans* gut.

To address whether *bre-5* mediated resistance might be restricted to Cry5B or whether might be relevant to other toxins, we test the effects of Cry14A toxin on wild-type and bre-5 mutant worms. Cry14A is ~34% identical to Cry5B in the toxin domain and is reported to be toxic to both nematodes and coleoptera. Indeed, we verified that ingestion of Cry14A by *C. elegans* causes a toxic response very similar to that of Cry5B. Furthermore, *bre-5* mutant animals are resistant to Cry14A, although not to the same extent as Cry5B. These data indicate that *bre-5* mediated resistance is relevant to multiple *Bt* toxins and raises the possibility that it could occur with other toxins and other organisms as well, including insects.

More recently, we have cloned and characterized a second resistance gene, *bre-3,* which encodes a transmembrane-domain containing protein. We are undertaking localization and mosaic analysis to better understand its function. Genetic data suggest that *bre-5* and *bre-3* function in the same resistance pathway. We are making progress towards cloning the *bre-2* and *bre-4* genes and towards identification of the receptor molecule modified by *bre-5*.

In a separate set of experiments, we are testing whether *Bt* toxins might be effective against nematodes other than *C. elegans*. We have characterized the response of five nematode species to most of the crystal toxin proteins found in the Cry5-Cry6 family of toxins. We have found that some *Bt* toxins are toxic to multiple nematodes, including one parasitic nematode, and that these toxins affect the health, development, and fecundity of nematodes. We have also undertaken structure function studies of one of these toxins and have narrowed down the active toxin to a 42 kDa core.

Managing resistance to Bt plants through use of gene and promoter strategies and field tactics

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Transgenic plants expressing insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) are revolutionizing agriculture (Roush and Shelton 1997). *Bacillus thuringiensis*, which had limited use as a foliar insecticide, has become a major insecticide because genes encoding Bt toxins have been engineered into major crops grown on 11.4 million ha worldwide in 2000. These transgenic crops represent a paradigm shift for insect control (Caprio et al. 2000) because the expression or synthesis of toxic proteins can be custom-tailored to specific agroecosystems, and the expression can be varied according to the pest control need.

While transgenic plants offer many unique opportunities for the management of pest populations, they also present new challenges. Perhaps the greatest of these is the potential for resistance to occur. There are at least five possible ways to slow selection in favor of resistance by transgenic plants. Of these, four utilize plants with constitutive expression of Bt toxins: (1) express toxin genes only moderately strongly, so that not all susceptible individuals are killed; (2) provide refuges for susceptible insects while expressing the genes as high as possible within acceptable limits to avoid deleterious effects on yield, health or the environment; (3) deploy different toxins individually in different varieties; (4) deploy plants expressing a mixture of toxins. Among these options, the refuge-high dose (#2) and pyramiding (#4) strategies seem most promising (Gould 1998; Roush 1997; Roush 1998). Currently the only commercially available strategy in the U.S. is use of a high dose with a refuge, and U.S. growers of Bt cotton must chose one of three structural refuge options: 95:5 external-structured unsprayed refuge, 80:20 external-sprayed refuge, or 95:5 embedded refuge (US EPA 2000a).

A fifth general option, much less studied, is to control the expression of the Bt genes in each plant so that they are expressed only when or where needed. This could be done through tissue-specific, temporal-specific, or inducible gene promoters. In principle, specific promoters could be used to express genes only in (a) the most important tissues such as fruit, other reproductive tissues, etc. ("tissue- or structure-specific" expression), or (b) critical growth periods ("temporal-specific" expression; Gould 1988). Alternatively, (c), the genes could be environmentally induced, perhaps by the spraying of an environmentally benign chemical (Williams et al. 1992). These tactics are not necessarily exclusive; a temporally-specific promoter may be effectively structure-specific if it is turned on only when needed (e.g., late in the season and affecting only the top of a plant).

A major issue for the high dose-refuge strategy (although less so for pyramiding) has been that non-transgenic crops are the most practical and reliable option for refuges. Care must be taken in managing the insect population within the refuge to insure that sufficient susceptible alleles will exist, while at the same time ensuring that damage to the refuge plants is minimized (Shelton et al. 2000). Inducible expression, timed only for those periods in which the crop is most sensitive to damage and only when pest populations exceed a damage threshold, could help to overcome this problem by using the entire crop as a refuge for some period of time.

To explore resistance management options for several of these tactics we have used a unique model system. This system is composed of broccoli plants transformed to express different Cry toxins combined with the use of different populations of the diamondback moth (DBM), *Plutella xylostella* (L.), a major pest of crucifer crops worldwide and the first insect to have developed resistance to Bt in the field (Tabashnik et al. 1990; Shelton et al. 1993).

Broccoli plants expressing cry1A genes

We cultured Cry1A-resistant DBM, developed Cry1A expressing plants, and tested resistance management strategies in the greenhouse and the field with plants expressing Cry1A toxins.

Our greenhouse studies provided the first empirical evidence that an insect population with an initially low frequency of Bt resistance alleles can, within a few generations, develop high populations of resistant individuals on Bt transgenic crops (Tang et al. 2001). Pure stands of Bt plants (0% refuge) resulted in rapid evolution of highly resistant DBM populations; increasing the size of the refuge delayed resistance.

Our first field experiment examined the effect of refuge size and refuge placement (mixed vs. separate refuges) on the distribution of the larvae within the plots as well as the level of resistance in DBM at the end of the season (Shelton et al. 2000). Our results demonstrated that the cumulative number of larvae per plant on refuge plants through the season in the 20% mixed refuge was significantly lower (6.4 vs. 14.6) than in the 20% separate refuge. This finding indicates that, as in our previous greenhouse experiments, a separate refuge is more effective at conserving the number of susceptible alleles for DBM because larvae on these refuge plants are more likely to survive to adults (either SS or RS) that can mate with RR individuals and thereby reduce the number of RR offspring.

Our second field experiment examined the results of spraying the plants in the 20% separate refuge, a practice allowed by EPA. Our results from these tests demonstrated that despite high rates of susceptible immigration, when resistance allele frequencies in the plot were high, spraying the 20% separate refuge resulted in progressively and significantly higher levels of resistance over the course of the season than when the 20% separate refuge was not sprayed. The important point demonstrated was that spraying the refuge may reduce its potential to dilute resistance.

These studies pointed out the value of the refuge and the importance of managing susceptible alleles in the refuge. Most importantly they also point out the need to move forward with the next generation of resistance management strategies, including pyramiding multiple insecticidal genes.

Pyramided plants expressing both cry1A and Cry1C genes

Theoretical models suggest that varieties that pyramid two dissimilar toxin genes in the same plant have the potential to delay resistance much more effectively than single toxin plants singly or in mosaics or seed mixtures (Roush 1997, 1998), and industry is working to develop such varieties.

In addition to developing the Cry1A plants noted above, we also have developed plants that express high levels of Cry1C toxin that could control Cry1A-resistant DBM (Cao et al. 1999). Additional work allowed us to develop populations of DBM that were resistant to either Cry1A or Cry1C (Zhao et al. 2000), or both toxins. A final step was to produce plants that expressed

both toxins and this was done by sexual crosses and characterized for Bt protein production and control of susceptible, Cry1A-resistant (Cry1A^R) and Cry1C^R DBM (Cao et al. 2002). ELISA analysis showed that Cry1Ac and Cry1C proteins were produced in the hybrids and in their F_1 progeny at levels comparable to the original single gene parental lines. Silencing of the *cry1Ac* and *cry1C* genes was not observed even though both genes were controlled by a 35S CaMV promoter.

Using these unique insect and plant populations we conducted greenhouse tests evaluating different management strategies for deploying plants that express Cry1A and Cry1C. The objective of the greenhouse study was to determine whether an insect population that contains a moderate frequency of genes for resistance to Cry1A and Cry1C develops resistance to both toxins faster or slower when subjected to plants that express both toxins simultaneously, in mosaics, or sequentially. There were three treatments:

1. 80% plants with pyramided expression of Cry1Ac and Cry1C + 20% refuge;
   80% Cry1Ac plants + 20% refuge until control failures occur due to resistance followed by 80% Cry1C plants + 20% refuge;
   40% of the plants express Cry1Ac + 40% express Cry1C + 20% refuge;

A synthetic population of DBM containing genes for resistance to Cry1A (0.03) and Cry1C (0.10) Bt toxins was used in the cage study. In each of the 4 replicates for each treatment, 20% non-transgenic broccoli plants were used as the refuge. This study is on-going as this paper is submitted and more recent results will be presented at the conference. What is presented here are the results to date.

After 18 generations of selection, the allele frequencies of Cry1Ac resistance were over 0.5 in all replicates of the mosaic treatment and the sequential treatment (Cry1Ac plants first), resulting in complete control failure for Cry1Ac plants. The allele frequency of Cry1C resistance was also over 0.5 in one of the four replicates of the mosaic treatment (mean = 0.39 in 4 replicates). The mean allele frequency was 0.22 for Cry1Ac- and 0.07 for Cry1C-resistance in the cages with pyramided two-gene plants. These results indicate that pyramided two-gene plants could significantly delay resistance development to each or both toxins.

Inducible promoters

Another approach for managing resistance, frequently mentioned but rarely studied in detail, is inducible (rather than constitutive) expression of Bt genes. Induction triggered by application of a benign chemical may provide the greatest flexibility to the grower, by permitting an in-crop refuge for part of the growing season and by causing Bt protein production only when an economic threshold is reached. Thus, it more closely follows IPM principles by using insecticidal treatments only when required.

We identified a transgenic broccoli line (T73) carrying one copy of the *PR1a/cry1Ab* expression cassette which produces Cry1A protein and controls DBM only after application of a chemical inducer related to salicylic acid (BTH) (Cao et al. 2001). Western blots and ELISA analyses of T_1 plants showed that the Cry1Ab protein was detectable as early as 12 hours after induction. The protein continued to be present for at least one month. Segregation ratios of T_2 seeds from line T73 showed that the T_1 plants were heterozygous. We now have 12 inducible transgenic T_2 plants of line T73 growing in soil. T_3 seeds from them will be used to identify homozygous T_2 inducible plants for further use.

Using these plants we will test the hypothesis that using Bt transgenic plants in which a chemically inducible promoter is used to express a Bt toxin at a specific time during the plant's development will effectively control insects while lengthening the time for resistance development, thereby improving the current EPA-endorsed resistance management strategy which is based on plants with a high dose of a single constitutively expressed Bt protein combined with a refuge.

The future

The development and implementation of engineered insecticidal plants is currently in its infancy and the only available technology is that of Bt-transgenic plants. Using the DBM/Bt-broccoli system as a model we have investigated aspects important to the long-term deployment of this novel technology. While the Bt broccoli/DBM system may not exactly duplicate the currently available insect/Bt crop systems such as cotton, corn and potatoes, it can help identify areas for further work. At the same time as more field studies need to be conducted to refine the presently utilized recommendations, industry, public-sector scientists and farmers must work together to develop a second generation of technology and implementation strategies to ensure the even longer term durability of Bt-transgenic plants.

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Transgenic *Bt* rice expressing a synthetic *cry1B* gene: expression strategies and field protection against the striped stem borer

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Abstract

We investigated whether the conditional, wound-inducible production of a *Bacillus thuringiensis* (*Bt*) Cry endotoxin may provide a hopeful and efficient alternative of protection from stem borer damage to the high dosage, constitutive or tissue-specific toxin delivery approach generally used in transgenic *Bt* crops. A synthetic *cry1B* gene controlled by the promoter region of either the constitutive ubiquitin (*ubi*) gene or of a wound-inducible proteinase inhibitor (*mpi*) gene from maize was introduced in rice by microprojectile bombardment. Accumulation of Cry1B from undetectable level to 0.2% of total soluble protein, 8-12 hours after mechanical wounding, was demonstrated in leaf and pith tissues of lines harbouring the pmpi-*cry1B* construct. In these plants, Cry1B was not detected in pollen and seed endosperm and embryo, unlike in pUbi-*cry1B* plants. Two out of 43 pmpi-*cry1B* plants were found to exhibit full protection against SSB damage. Field evaluation with artificial and natural infestations of the most promising ubi-cry1B and mpi-cry1B lines in Delta Del Ebre, Spain during the summer crop season 2001 confirmed the level of protection observed in greenhouse assays.

Introduction

Protection of transgenic rice plants through expression of *Bacillus thuringiensis* (*B.t.*) endotoxin genes has been extensively documented since 1993. Most of the published studies aimed at obtaining a high level expression of genes encoding insecticidal Cry proteins in all the tissues and through the cycle of the rice plant in using strong constitutive promoters -such as the CaMV35S, the rice Actin-1 or the maize Ubiquitin-1. Full protection against major insect pests such as the Striped Stem Borer (SSB; *Chilo suppressalis*) the Yellow Stem Borer (YSB; *Scirpophaga incertulas*) and the leaffolder (*Cnaphalocrocis medinalis*) has been obtained both in *japonica* (*cry1Ab* : Fujimoto *et al.* 1993, Cheng *et al.* 1998 ; *cry1Ac* : Cheng *et al.* 1998 and *cry1B* : Breitler *et al* 2000) and *indica* (*cry1Ab*:Wünn *et al.* 1996, Datta *et al.* 1998 ; *cry1Ac*: Nayak *et al.* 1997 and *cry2a* Bano-Maqbool *et al.* 1998) rice. High level of field protection of an elite Chinese hybrid cultivar, from leaffolder and YSB attacks through constitutive production a composite Cry1A(b):Cry1A(c) protein has also been reported recently (Tu *et al.* 2000). Another strategy of expression of insecticidal proteins is to direct it specifically to the physiologically and economically most important parts of the plant. Expression of synthetic *cry1Ab* coding sequence under the control of the PEP carboxylase (Koziel *et al.* 1993) or of a pith specific (Novartis, unpublished) promoters has thus proven effective in fully controlling SSB and YSB in transgenic *indica* rice (Ghareyazie *et al.* 1997, Datta *et al.* 1998). An alternative to continuous expression of the insecticidal protein at the whole plant or tissue level is a conditional expression directed by a wound-inducible promoter, which could allow its production both only where and when it is needed, i.e at the attack site. This study aimed at determining

whether the use of the promoter region of a wound-inducible gene allows sufficient expression of a *cry* gene in transgenic rice to afford a full protection against SSB attacks both in greenhouse and field infestation assays. We first prepared direct gene transfer constructs bearing a synthetic *cry1B* coding sequence (Bohorova *et al* 2001) controlled by the promoter region of either the constitutive maize ubiquitin gene (Christensen and quail 1996) (pUbi-cry1B) or the −689/+197 (C1) region of the wound-inducible maize gene -the maize proteinase inhibitor gene (*mpi*) (Cordero *et al.* 1994) (pC1mpi-cry1B) in pBKS+ vectors and introduced in the elite mediterranean japonica rice cultivar Ariete through microprojectile bombardment (Breitler *et al.* 2000, 2001). We conducted a comparative evaluation of the accumulation of the protein in response to wounding in various tissues and organs of transgenic lines harboring these constructs. We further compared the protection afforded by wound inducible and constitutive expression of the Bt gene through T1 to T6 generations of selected lines, under greenhouse then field conditions.

Results and discussion

Cry1B accumulates upon wounding in vegetative tissues of pC1-cry1B plants Expression of the *cry1B* gene was first examined in unwounded and wounded tissues of blade and sheath parts of both old and young leaves and in basal and apical pith internodes of pC1mpi-*cry1B* plants. We used as constitutive control comparable tissues of pUbi-*cry1B* plants. Wound-induced accumulation from undetectable steady state levels of Cry1B was observed in all the tissues, irrespective of their origin and maturity. By comparing band intensities with those of the positive control we estimate that accumulation of Cry1B reached up to 0.2% of total soluble proteins in the two highest expressers (lines 9 and 53), 24 hours after the wound treatment. The time-course of wound induction was investigated by collecting mechanically wounded blade segments of the antepenultimate emerged leaf of several plants per event, at regular time intervals over 24 hours. A rapid increase of the amount of Cry1B was detectable as early as 1 hour after mechanical injury and found to attain a maximal level 8-12 hours after the stimuli. Cry1B is not detectable in pollen and mature seed endosperm of pC1mpi-cry1B plants Expression of the pC1mpi-*cry1B* gene fusion was further examined in pollen of dehiscing anthers and in seed endosperm and embryo of mature seeds: the Cry1B toxin was not detected in these tissues, even when protein load was increased to up to 50μg protein per well for pollen extracts. In contrast, considerable amounts of Cry1B accumulated in both pollen and seed endosperm and embryo tissues of plants harboring the pUbi-*cry1B* construct.

pC1mpi-cry1B lines are protected from SSB attacks in greenhouse bioassays

Three selected independent events (9,12 and 53) harbouring intact copies of the pC1mpi-*cry1B* construct were found to exhibit a stable level of protection when challenged with second instar (L2) SSB larvae over T1 to T6 generation. These lines have been obtained through a rather limited effort of transformation (one single experiment involving 4 bombardment plates and yielding 43 independent primary transformants). When the 3 lines were bioassayed, a complete protection against SSB damages and 100% toxicity was observed in event 9. Events 53 and 12 exhibited limited damage resulting from early feeding of SSB larvae, since no weight increase and 100% mortality were scored on recovered larvae. Dissection of control plants at 4 days showed the presence of third instar larvae which had penetrated the stem and fed on inner pith tissues. Seven to 15 days after infestation, control plants displayed extensive feeding damages, with all tillers exhibiting dead hearts, and larvae found at the fourth to fifth instar stage. Contrastingly, larvae recovered at 4 or 7 days on plants of the best pC1mpi-*cry1B* events were still in L2 (no head capsules found) and had neither gained weight nor penetrated the pith. Damage were limited to 1 cm of tissue causing restricted browning.

Seven or 15 days after infestation, all the larvae recovered on plants of the best protected events 9 and 53 were found dead. No feeding damage was observed in leaf sheaths. Larvae occasionally bored the stem (small holes resulting from an early feeding) but did not feed on it, and there was no further consequence on tiller development, lodging and flowering abilities.

pC1mpi-cry1B plants are protected from SSB damage in a field infestation assay

Field evaluation with artificial and natural infestations of the most promising pUbi-cry1B (Ariete event 64 Breitler *et al* 2000) and pC1mpi-cry1B (Ariete event 9.1 Breitler *et al* 2001) was conducted in Delta Del Ebre, Spain during the summer crop season 2001. Two additional T_3 lines obtained later through *Agrobacterium*-mediated transformation and harboring a single T-DNA copy of the ubi-cry1B construct (Ariete events 33.1 and 3.4, unpublished results) were also included in the trial. Experimental design consisted in Fisher Blocks (8 treatments and 3 repeats under natural infestation, one repeat under both natural and two repeated artificial infestations during the months of June and July). Two collections of samples were performed in the plots, mid-august (flowering stage) and early october (harvest stage). The second collection of samples which consisted in the dissection of 6 plants per elementary plot, allowed the overall recovery of 867 larvae from 192 plants, representing an average of 4.5 larvae per plant. The level of infestation was very high and almost all the larvae were recovered within the piths of plants of control plots (Figure 1A). Level of protection (mean number and average weight of the larvae recovered per plant) of biolistic events 9.1 and 64 was comparable though being slightly lower than that observed among *Agrobacterium* transformation events 3.4 and 33.1constitutively expressing cry1B (Figure 1B). The design of the trial also allowed a preliminary evaluation of agronomic performance of the lines. No significant difference was observed between transgenic plants and their conventional control counterparts treated with

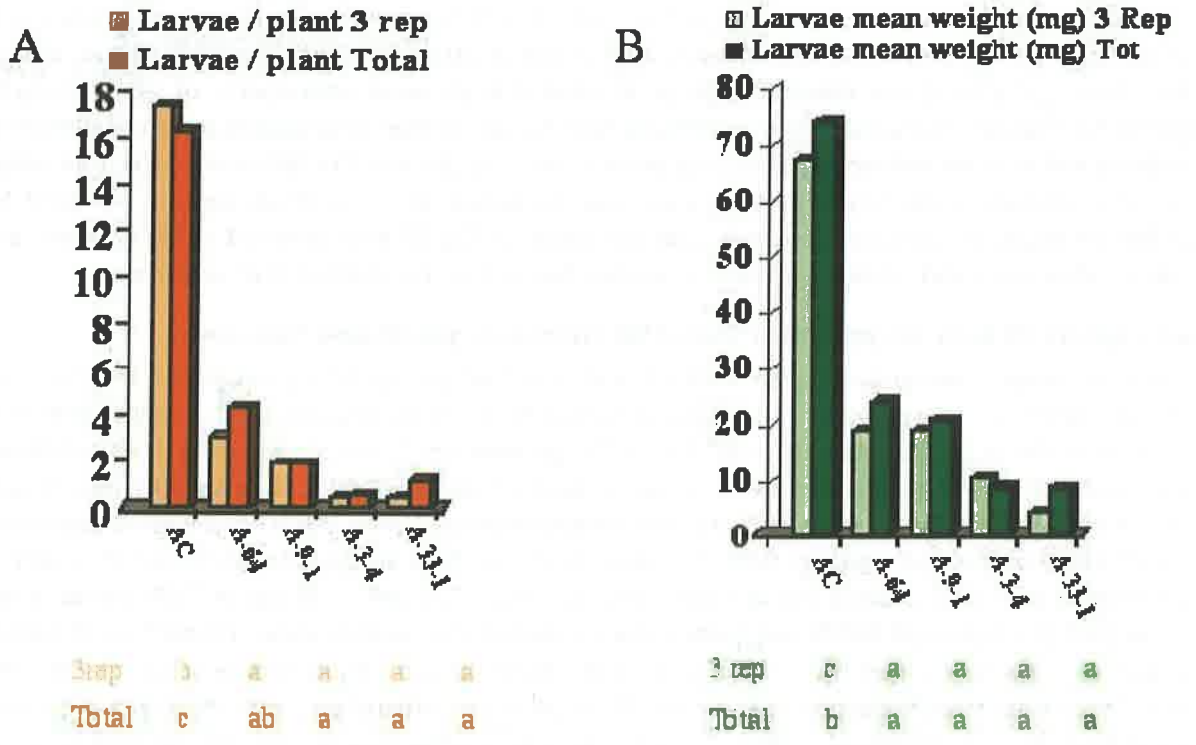

FIG. 1. Number (A) and mean weight (B) of striped stem borer larvae recovered after dissection of randomly collected control plants (AC) and transgenic plants of lines A64, A9-1, A3-4 and A33-1, including (Total) or not (3 rep) the fourth replicate which has been artificially infested with L2 SSB larvae.

insecticide, in terms of tiller number, heading date and seed harvest, suggesting that neither the transformation/regeneration process nor the expression of the *Bt* gene induced a yield penalty.

Conclusion

Our results present the first evidence that wound inducible expression of a synthetic *Bt* endotoxin gene is a valuable strategy for protecting a transgenic crop. The use of conditional promoters for driving genes encoding insecticidal proteins appears a more "natural" strategy mimicking endogenous defense gene of the plant which are generally shut off or expressed at a very low steady state level when biotic and abiotic stresses are not prevalent .Advantages in using inducible vs. constitutive promoter can be summarized as follows: i. magnitude of the wound response appears independent of the nature and age of the vegetative tissue, ii. toxin is produced in response to attack of phytophagous insects and reaches levels affording full protection iii. toxin does not accumulate over plant development both saving energy building blocks and possibly minimizing potential for transgene silencing and resistance build-up in the insect population, and iv. toxin does not accumulate in pollen, seed embryo and endosperm and crop residues, minimizing food and environmental concerns. In our project, current efforts are aiming at generating through *Agrobacterium*-mediated transformation additional C1mpi-cry1B transformation events representing a wider range of cry1B expression upon wounding and even more complete protection. To comply with biosafety regulations, these new lines will be devoid of selectable marker gene through the cloning of the gene cassette in a second T-DNA unit different from that containing the selectable gene (Breitler *et al* in prep), or between junctions of the *Ac* transposable element (Cotsaftis *et al* in press) for producing "clean" transformants. Further research should be devoted to compare the stability of expression and protection, host-insect interactions and impact on non-target organisms in transgenic rice lines constitutively or conditionally expressing Bt *cry* genes.

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Symposium (Microbial Control 1) Solar Irradiation of Fungal Pathogens: Deleterious Effects, and Mitigation through Genetics and Formulation

Tools of the solar-UV trade: Light sources, filtering, measuring irradiance and selecting biological weighting factors (action spectra)

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Adverse effects of solar UV irradiation on fungal spores, including those of entomopathogenic fungi, are well recognized. The half-life of conidia positioned where they will receive direct sunlight, such as the upper surface of a leaf, has been reported as less than two hours. As a result, solar UV radiation (UVB = 290-320 nm, UVA = 320-400 nm) is deemed a potentially serious impediment to successful field implementation of entomopathogenic fungi for insect and mite control. Sunlight, like most natural phenomena, includes an extremely variable set of conditions. The most important of these are intensity and spectral content. Both of these parameters vary due to season, time of day, location, altitude and atmospheric conditions. It is difficult to carry out year-round solar experiments in temperate areas because of the extreme variation in intensity between summer and winter. For example, in Logan, Utah, the mid-day intensity of sunlight UV peaks shortly after the summer solstice and is sufficiently high for meaningful experiments only during the approximately 120 days surrounding that period. This eliminates outdoor experiments for most of each year. Also, even in extremely sunny locations there are frequent days of at least patchy cloudiness. Since the timing of sun blockage by clouds is irregular, cloudy days also are not amenable to outside irradiation experiments. A third problem at our location in Utah two years ago was smoke from numerous local forest and grass fires. This seriously reduced the solar radiation reaching our experiments.

Due to the uncontrollable circumstances associated with working out of doors, most studies on effects of solar radiation on biological systems are done in the laboratory. This leads to the central questions of solar UV research; namely, how do we obtain realistic doses of the adverse wavelengths in solar radiation, and how do we decide the dosages (intensity) and spectra to utilize in the laboratory? The first issue is based on analysis of the sunlight at the location of interest. There are general spectra and intensities published and these can be used as a basis, as can UV models (Bjorn and Murphy 1985) or data from monitoring networks (see end of this paper). Ideally, if planning to do field work in a specific area, one should take local spectral irradiance measurements at various seasons and times of day. Lamps or combinations of lamps and filters are then devised to attempt replication of the local conditions. These are basically hardware problems. The type of instrument used to analyze the output of the sun or artificial lamps is of no small importance. We have used two basic types, which range in current cost from $1,000 to $40,000 USD. The most precise analysis of light output is with a spectroradiometer, which measures spectral irradiance (intensity at one-nm intervals). We normally do this from 250 nm to at least 400 nm. Measurements from 250 to 290 nm are

taken to assure that no wavelengths below 290 nm pass through the filter (see below). Such precise measurement allows utilization of weighting formulas, as will be discussed later, to correct for the variability in biological effects of different (particularly short) wavelengths in the overall spectrum. We have used a device of this type (Optronic spectroradiometer) for all of our lamp studies. This is calibrated for each use with a National Institute of Standards and Technology traceable tungsten/halogen lamp for intensity as well as a mercury-vapor lamp for wavelength accuracy. In our outdoor experiments, we have used output from continuously operating radiometers (either broadband or multi-filter narrowband units) that were calibrated with the spectroradiometer. There have been some hardware innovations in recent years which may prove useful for some applications. An example is a very small spectroradiometer with diode array (which can provide simultaneous readings of many wavelengths) for approximately $4,000 US. We know of no one using these for insect pathogen research at the moment. The least expensive radiometers, namely those which wide-band average, will afford a general idea of output; but of course cannot be used in devising or utilizing spectral-weighting formulas and cannot be used to compare sources with different spectral outputs (e.g. Sayre and Kligman 1992).

As mentioned above, lamps are used in virtually all published reports on the effects of solar irradiation on insect pathogens. Nevertheless, lamps can only approximate the spectrum and intensity of bright sunlight. Those available were devised for a variety of uses. For example, there are fluorescent bulbs with high UVB output which were designed to enhance weathering of products exposed to sunlight, such as paint. There are other fluorescent bulbs with high UVA output, designed for material ageing through glass and one of their uses is in suntan salons. These lamps can be used separately, in combinations, or combined with Xenon lamps which have high intensity and contain UVA, UVB and visible light. Even with these sophisticated irradiation systems, UV-A levels often are considerably less than that found in nature. There are lamps on the market designed specifically as "solar simulators". In general, these are based on reasonably potent (300 to 1,600 watt) lamps. Their spectral output is somewhat similar to mid-day sunlight (although careful measurements should still be taken), and these instruments have been used in a number of research projects with good results. The device is expensive, for example our small Oriel solar simulator with a target area of 100 cm^2 costs approximately $14,000 USD. One obvious advantage of the fluorescent lamps, besides low cost, is their vastly increased target area which allows simultaneous trials of a variety of strains or different times of exposure. Over-all intensity of irradiation from lamps can be varied by changing the distance between the subject and the lamp. Alternations in spectra are dealt with by filtering. Of key importance in all experiments with lamps is the elimination of all wavelengths below 290 nm. The region of the spectrum below 280 nm is referred to as UVC and includes the wavelengths emitted by germicidal lamps. The biological activity of these short wavelengths, particularly around 254 nm, is extremely high. These shorter wavelengths, however, do not exist in sunlight due to their elimination by the earth's atmosphere; and, therefore, any adverse effects from their presence in artificial light is misleading. Their elimination can be assured with several types of filters. The most commonly used material is cellulose diacetate. It is preferred because it truncates the UV spectrum at 290 nm in an abrupt manner similar to the solar spectrum. A caveat to users of cellulose acetate for removing UVC and short wavelength UVB from lamps: the acetate becomes somewhat opaque to UVB rather rapidly. At some wavelengths it can lose over half its transmittance in less than 70 hours (Middleton and Teramura 1993). While this change in transmittance can be reduced by locating the filter closer to the sample rather than adjacent to the lamp, or "preageing" the filter, a good protocol is to change the filter for each experiment. Since the cellulose diacetate is not manufactured as an optical filter, it is important to check each roll of the film before use.

An attempt to purchase cellulose diacetate in Argentina last year failed, and we are not aware of a cellulose diacetate source in South America. Another approach to filtering out UVC and short wavelength UVB radiation is to use glass (Schott) filters with a lower cut off of approximately 290 nm (e.g., WG 320). The expense of these filters generally limits them to small target areas only, and their cutoff is not as abrupt as cellulose diacetate. There is a recent report recommending the use of Pyrex brand baking trays for removing radiation below 290 nm (Lepre et al. 1998). The UV cutoff of this glass was variable between trays, and also was less sharp than an optical filter. We also find the Pyrex UV cutoff to be less sharp than that of cellulose diacetate (unpublished). An advantage of the Pyrex is its stability under long-term UVB radiation, therefore eliminating continually replacing the filter. To remove UVC and UVB, a polyester film (Mylar) is routinely used. This material is more stable than acetate, but it also becomes more opaque to UV with long-term exposure. Therefore, it should be replaced on a regular schedule. The removal of all UV (UVA, UVB, UVC) can be accomplished with a Llumar film. This product's lower cut off is at about 400 nm. The use of this film will permit studies of visible wavelengths. A complicating factor is that the response to UV irradiation is temperature dependent. Accordingly, the experiments must be conducted in chambers with tight temperature control. There can be tremendous heat build-up in outdoor direct sunlight experiments and, therefore, temperature control systems must be used. In our case, we have floated our experiments on temperature-controlled water.

Spectral weighting functions are usually employed to permit a basis for comparison between various irradiation systems and sunlight. These functions are dimensionless factors that represent the different biological effectiveness of each wavelength. By convention, these factors are normalized to one at 300 nm. Multiplying the weighting factors by the spectral irradiance produces an integrated "biologically effective irradiance". These weighting factors have been developed for many responses: DNA damage in plants and microbes, plant growth responses, fungal development, plant pigment induction, mortality of fish larvae, and even non-biological responses such as yellowing of plastics. Several of these spectral weighting functions differ considerably from each other. Consequently, selection of the most appropriate spectral weighting function is critical, and this selection has profound effects on the outcome of the experiment (Caldwell et al. 1986, Paul 2000). Unfortunately, there is often little information available for guidance in selecting weighting functions. Most are derived from laboratory experiments with monochromatic or narrowband radiation, and are usually conducted without the wavelengths that induce repair mechanisms. Thus, if possible, the appropriateness of different weighting functions should be evaluated under realistic conditions (sunlight). For our work with the entomopathogen *Metarhizium*, there was little precedent in the recent literature for a spectral weighting function pertinent to the inhibition of conidial germination. We followed a recommendation of Paul et al. (1997): the average response of nine fungal action spectra (which had been derived decades earlier) corresponded closely with the recently developed action spectra for DNA damage in plant seedlings (Quaite et al. 1992). We used this plant DNA damage weighting function in our work with *Metarhizium* (Braga et al. 2001a); and subsequent experiments utilizing several combinations of filtered sunlight supported the selection of this weighting function, at least for some of the fungal isolates (Braga et al. 2001b). Experiments where the dose-response of the organism is not linear [e.g., with the bacterium *Pseudomonas*, Miller et al. (2001)], provide little guidance in selection of an appropriate weighting function. In selecting what type (field or laboratory) of experiment to perform, one must weigh the difficulties of simulating realistic sunlight in the laboratory against the limitations of season and weather for conducting field experiments.

Sources: For links to manufacturers of UV measurement instruments, and UV monitoring networks on all continents, see the web site of the World Meteorological Organization: http://www.srrb.noaa.gov/UV/

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Damage to fungi from solar/UV exposure, and genetic and molecular-biology approaches to mitigation

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Introduction

UV radiation is of high biological relevance since many living organisms have been fighting against its cytotoxic effects from the beginning of their evolution (Friedberg *et al.*, 1995). The UV portion of the spectrum has been pointed out as one of the major factors responsible for the control of natural fungus populations (Parnell *et al.*, 1998). If, on the one hand, UV radiation can reduce the inoculum of important pathogens and limit their dispersal in the environment, on the other hand its deleterious effects may prevent, in many situations, the use of entomopathogenic fungi for the biological control of insect pests (Moore *et al.*, 1993; Fargues *et al.*, 1996; Morley-Davis *et al.*, 1995; Braga *et al* 20001a,b,c,d; Braga *et al.*, 2002).

Biological effect of UVA and UVB radiation

The ultraviolet spectrum is conventionally divided into three wavelength intervals: UVA (400 to 320 nm), UVB (320 to 280 nm) and UVC (280 to 100 nm). The UV fraction of the solar spectrum that reaches the Earth is exclusively composed of UVA and UVB, since atmospheric ozone drastically reduces the penetration of radiation with wavelengths of less than 320 nm and excludes wavelengths below 290 nm. In quantitative terms, UVA is responsible for about 95% of the total energy of the UV spectrum that reaches the surface of the planet and UVB is responsible for the remaining 5% (Frederick and Alberts, 1991). In terms of biological effectiveness, this proportion can be drastically altered due to the greater effectiveness of UVB radiation, which has been demonstrated by action spectra developed for different biological systems (Peak and Peak, 1983; Paul *et al.*, 1997).

UVB radiation is directly absorbed by different cellular macromolecules including DNA and protein, causing DNA photodamage and mutagenesis. Pyrimidine dimers are the most prevalent DNA damage induced by UVB radiation. In contrast, UVA is weakly absorbed by most biomolecules but is oxidative by nature, generating reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) through interaction with intracellular chromophores. The ROS, in addition to being mutagenic, can damage all known biomolecules (Griffiths *et al.*, 1998). The complexity of the solar spectrum, the multiplicity of the effects of the different spectral fractions and the diverse interactions among them cause the effect of exposure to radiation on the cells to be highly complex (Moore and Morley-Davis, 1994; Paul *et al.*, 1997; Fargues *et al.*, 1997).

Cell Protection against the damage induced by UV radiation

Many microorganisms are exposed to solar radiation during part or all of their life cycle. The deleterious effects of UV radiation have led the cells to develop a series of defense systems whose basic function is to maintain the integrity of genetic material and of the cell components essential to life. The genetic basis of cellular tolerance to radiation is multifactorial and complex, involving (1) pigments localized on the surface which can block the entry of radiation into the cell; (2) proteins and enzymes capable of protecting the macromolecules and the cell components

from the harmful effects of radiation; (3) complex enzymatic systems capable of repairing the damage induced by radiation, and (4) enzymes that can inactivate the toxic substances induced by radiation. Due to their evolutionary importance, several mechanisms involved in cellular tolerance to UV radiation are highly conserved among living organisms (Friedberg *et al.*, 1995).

In metabolically active cells, exposure to UV radiation triggers a set of responses that increase protection against radiation and facilitate repair of damaged structural or enzymatically-active biomolecules. Among these responses are the temporary interruption of the cell cycle, the reduction of the rate of DNA duplication and the increase in the transcription of genes involved in protection and repair of biomolecules. These mechanisms are important for the stability of genetic material since most of the mutations induced by UV arise as the result of faulty nucleotide incorporation during the semiconservative duplication of damaged DNA fragments (Friedberg *et al.*, 1995).

Recent advancements in genomics and proteomics has permitted a better understanding of the complex cell responses of fungi to stress-inducing agents such as temperature, osmotic variations, chemical substances, and radiation. DNA microarrays currently are one of the most powerful and versatile tools of genomics. Commercial biochips developed after the complete sequencing of the *Saccharomyces cerevisiae* genome have permitted the simultaneous analysis of the expression of more than 6500 yeast genes, allowing an overall analysis of the metabolic changes suffered by the cells during exposure to stress-inducing agents (Goffeau, 2000; Tusher *et al.*, 2001). The use of biochips, in addition to permitting the analysis of the expression of hundreds of genes previously associated with the cellular response to UV radiation, will permit correlation of these genes with other important cell systems such as generation of energy, metabolism of nucleic acids and proteins, and pathogenicity. We have found that discrete members of an 840-member-EST library show differential hybridization to mRNAs prepared from *Metarhizium. anisopliae* mycelium exposed to UVA and UVB for 1 or 2 h and in comparison with RNA from non-exposed controls. We see that there is an effect of time: in 1 h samples amongst the most upregulated genes are those for DNA repair, whereas these are accumulated less in the 2 h samples where proteins for ribosomes dominate.

The proteomic approach for the identification of proteins related to the tolerance to radiation complements the genomic analysis because it measures the final expression product rather than an intermediate and may reveal post translational effects such as protein modifications (Lockhart and Winzeler, 2000). Synthesis and accumulation of specific proteins have been observed in UV-exposed cells. Among the proteins induced by radiation are stress or heat shock proteins including several members of the chaperone gene family. These proteins also are produced in large scale during conidiogenesis (Rensing *et al.*, 1998), perhaps as a mechanism to protect the conidia prior to their germination. Variations in deposition of this group of proteins may partly explain the higher tolerance to heat and UV radiation observed in conidia of certain *M. anisopliae* strains like ARSEF 324. Both heat and UV are capable of denaturing proteins, and such damage may be relieved by mechanisms involving chaperones.

Our expectation is that the analysis of metabolic and genomic changes induced by exposure to UV radiation will permit a better understanding of the cell response to radiation and the identification of new sites where it will be possible to intervene in order to increase or decrease fungal tolerance to radiation.

Like most polygenic traits, tolerance to UV radiation is of a quantitative nature. There is wide variability in the tolerance to UV radiation both among species and among strains of yeasts and filamentous fungi (Fargues *et al.*, 1996; Paul *et al.*, 1997; Braga *et al.*, 2001d). This

variability in tolerance reflects natural adaptation to different environmental conditions including intensities of UV radiation (Fargues *et al.*, 1996; Bidochka *et al.*, 2001; Braga *et al.*, 2001d). The high intraspecific variability facilitates the selection of strains with greater tolerance to UV radiation and the genetic improvement of the trait.

We have also observed that UV tolerance can be manipulated in the laboratory by growth of single strains on different media. Thus, formulation for conidial production as well as amendments to conidial preparations that would protect against UV radiation are factors for consideration in commercial applications. At the biochemical level, these phenotypical variations in UV tolerance may be a consequence of differential accumulation of protective substances and/or endogenous photosensitizers.

Photobiology and photochemistry of conidia

Obtaining conidia more tolerant to UV radiation is highly desirable for the expanded use of fungi as bioinsecticides. In contrast to cells with high metabolic activity, dormant cells or cells with limited metabolic activity such as the spores of bacteria and yeasts and the conidia of filamentous fungi have a very limited ability to react to radiation. In these cells, the damage caused by radiation accumulates over time and is only repaired during the beginning of germination. The tolerance of conidia and spores to UV radiation and to other environmental factors depends on morphologic, physiologic and biochemical properties, including protective substances, synthesized during conidiogenesis or sporulation. Among the known protective substances present in conidia and spores are pigments, proteins that can interact with DNA and enzymes involved in cell repair and detoxification. Recently, we demonstrated the importance of pigmentation of *M. anisopliae* conidia for UV tolerance. White, violet and yellow mutants were on average more sensitive to UV radiation than wild-type conidia with green pigmentation. Revertants showing the original green spore color also were restored to higher levels of tolerance to radiation.

It is well established that UVB radiation is the fraction of the spectrum that presents the highest biological effectiveness and the highest potential to damage entomopathogenic conidia within the wavelengths that reach the earth's surface. In a recent series of field experiments, we demonstrated that solar UVA also reduces the culturability and delays the germination of *M. anisopliae* conidia (Braga et al 2001c).

As opposed to bacterial spores (Nicholson *et al.*, 2000), little is known about the photochemistry of the conidia and, to our knowledge, the damage induced by UV radiation to genomic and mitochondrial DNA or to other biomolecules and important cell systems has not been precisely characterized. We believe that the identification of the genes involved in tolerance to solar radiation which are expressed during conidiogenesis and germination as well as the identification of protective substances present in the conidia will be fundamental steps for the development of fungal strains more tolerant to the physical agents of the environment.

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Mitigation of solar damage to microbial control agents through formulation and application technology

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With biopesticides, it is always useful, and sometimes essential, to consider formulation and application as an integrated "delivery system", since together with packaging and purification during mass production, they encompass a number of linked issues that can substantially influence operational success. There is quite an extensive literature on the use of both formulation and application to mitigate the effects of sunlight (in practice, the UVB wavelengths) on many classes of microbial control agents. However, this literature can sometimes give a misleading impression about implementation with practical products. As illustration, we describe some of our own investigations into the formulation of 'Green Muscle' (oil-based *Metarhizium anisopliae* var. *acridum*). A number of experiments were carried out, using both simulated and natural equatorial sunlight, to assess both the absorbance of formulating media and the effectiveness of a number of additives. Amongst others, oxybenzone was found to be efficacious in the laboratory (with formulating oils also giving some protection), and this was tested in the field. However, trial results indicated that field performance was practically unaffected by presence of oxybenzone and the significant extra expense was not warranted. Subsequent efforts in formulation development focused on improvements to the spore extraction process, in order to make flow through application equipment more reliable. This purification of the agent contrasts with the development of entomopathogenic virus formulations, where presence of ground-up host insects is amongst the most effective means of providing UV protection. Although dozens of potential sunscreens have been described, in practice it is cheap natural products (such as molasses, carbon and skimmed milk powder) that have been included most commonly in both viral and bacterial preparations. Optical brighteners are also efficacious, and their combined use as spray tracers (as an aid to implementing slow acting biopesticides) is discussed.Formulations must be compatible with the application equipment to be used, and microbial agent products are most likely to be adopted if minimal changes to normal practice are required from growers and operators. Unfortunately, equipment is often selected for habitual or pragmatic reasons, and does not necessarily maximise dose transfer to the biological target. Nevertheless many target pests occur cryptically, and the best targets for spray application often include sites such as the underside of leaves: the plant itself therefore providing physical protection from solar radiation. The efficacy of many agents (chemical as well as biological) may be improved by placement of residues on the leaf undersides, and a number of innovative nozzles have been developed over the past decades. At least three techniques were developed during the 1980s, that used electrostatic forces to coat both upper and lower leaf surfaces, but adoption to date has been disappointing. Various configurations of upwardly pointing nozzles (on drop-leg lances or boom extensions) and the use of air assistance are perhaps more cost-effective and easily implemented techniques.

Symposium (Cross-Division 3) Microsporidia within Entomophthorales

Origin and metabolic adaptation in microsporidia

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Early molecular and ultrastructural data suggested that microsporidia might be an ancient, primitive lineage of eukaryotes that arose before common features such as the mitochondrion evolved. However, this notion has now been thoroughly discredited by molecular phylogenies based on a number of protein-coding genes. These phylogenies demonstrate that microsporidia are closely related to fungi, which are neither primitive nor particularly ancient. However, due to the limited taxonomic sampling of most fungal genes, it has remained unclear whether microsporidia are a protozoan sister group to fungi, or whether they actually are *bona fide* fungi. Recently, alpha-tubulin and beta-tubulin phylogenies have been used to examine this relationship. Phylogenies based on both genes show microsporidia evolving from within the zygomycetes, and suggest a specific relationship between microsporidia, Entomophthorales and Zoopagales. The fungal origin of microsporidia puts many microsporidian characteristics in a new light. For example, the tiny genomes of microsporidia are clearly the result of severe reduction, a characteristic that permeates all levels of microsporidian biology. Similarly, the unusual metabolism of microsporidia is now emerging, and is clearly not primitive. Instead, the microsporidian metabolic machinery is an *ad hoc* mixture of enzymes left over from the fungal mitochondrion and derived from lateral transfer, in some cases from bacteria, while in others from animals, perhaps their hosts. Altogether the current image of microsporidian parasites is one of extremes in adaptation and reduction.

Characteristics of the microsporidia; reasons to ponder that microsporidia are highly evolved fungi

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Nosema bombycis, the first described species of microsporidia and etiological agent of Pebrine disease in silkworms, was originally identified as yeast and placed in the Schizomycetes (Naegeli, 1857). Balbiani (1882), after a series of studies on this organism, decided that it lacked essential characters of the Schizomycetes but had affinities to the Sporozoa Leuckart, 1879. He established the taxon "Microsporidies" for *N. bombycis* and the few known but unnamed species of microsporidia and the group has not been considered related to the Fungi since that time. Recent molecular analyses, however, have determined that the group possesses several gene sequences that suggest this group is closely related to the Fungi (Keeling, 2001; Hirt et al., 1999). Since then, other biological features of the microsporidia and fungi have been analyzed to determine common traits or distinct differences that may provide additional insight on this relationship. However, these comparisons are confounded by widespread convergence and rapid divergence of these groups. Therefore, the following discussion is an attempt, in a very general way, to examine some of the similarities and differences between microsporidia and fungi. It is hoped this will stimulate investigations on both groups to help clarify the many points where information is unclear or lacking.

The microsporidia are a large group of strictly obligate, intracellular parasites that infect most animal groups (from Protists to Man) but are not known to infect plants or fungi (Becnel and Andreadis, 1999; Vavra and Larsson, 1999). Only the spores of microsporidia are walled and spores examined thus far contain large amounts of trehalose. Vegetative growth is by non-motile amoeba-like stages (often multinucleate) with simple plasma membranes. Although variable is some respects, all microsporidian spores are definitively and uniquely characterized by containing a coiled polar filament. At germination, the polar filament is inverted to become a tube for transport of the sporoplasm into the host cell. The fungi constitute an extremely large and diverse group of heterotrophic organisms devoid of chlorophyll, have a cell wall, are non-motile (some species have motile reproductive cells) and reproduce by means of a tremendous variety of spore types (Alexopoulos et al., 1996). Fungi are usually filamentous and multicellular and glycogen is the primary carbohydrate storage product (trehalose in yeast and lichens). Obligate parasitic fungi infect plants, animals and in some cases even other fungi.

Cytological Structures

Mitochondria, perixosomes, and lysosomes have not been identified in microsporidia but are known to occur in the fungi. Both groups contain paramural bodies. The Golgi apparatus of microsporidia are said to be of a special type mainly consisting of a vesicular meshwork without stacked cisternae. While some fungi possess typical stacked cisternae, most groups have Golgi which consists of a single cisternal element (Alexopoulos et al., 1996). Centrioles are absent in all microsporidia and most fungi. In microsporidia, spindle fiber attachment is to a spindle plaque located on the nuclear envelope with small associated polar bodies often connected to the spindle plaque by filaments. In the fungi, centrioles are present only in the Chytridomycota and are composed of nine triplets. In most true fungi, centrioles are replaced

by spindle pole bodies (SPB) and multivesicular bodies. Ribosomes of the fungi are of the typical eukaryotic size of 80S while the ribosomes in the microsporidia are prokaryote-size (70S) consisting of a large (23S) and small (16S) subunits. They lack the 5.8S subunit but a homologuous region is found at beginning of the 23S subunit(Vossbrinck and Woese 1986).

Invasion

Spores in both microsporidia and fungi initiate infection in a host. Some microsporidia are characterized by only one type of spore while others form up to four types of spores during the course of the life cycle. Fungal species can also form up to four different spore types generally characterized into sexual (oospores, zygospores, ascospores, basidiospores) and asexual (conidia or sporangiospores) forms. The mechanisms for invasion are distinctly different for microsporidia and fungi. Fungal spores attach to the host surface and produce a penetration germ tube that gains access through enzymatic and mechanical activity. All microsporidian spores contain a polar filament that, on appropriate environmental stimulation, rapidly discharges the filament, which everts to become a hollow tube. The tremendous pressure within the spore at discharge (up to about 60 atm) propels the sporoplasm through the tube (which can reach lengths of up to 500μm in length!) into a host cell thus avoiding host defense systems.

Replication

Many microsporidia and most fungi have uninucleate cells and both have cells with 2 nuclei. In the microsporidia this arrangement is called a diplokaryon and is a stable arrangement of paired, haploid nuclei. In fungi, this arrangement is called a dikaryon and represents 2 unpaired haploid nuclei. Mitosis in both microsporidia and fungi is intranuclear, meaning that the nuclear envelopes do not break down during division as occurs in most eukaryotic cells. Both groups contain species with documented meiosis with comparable types of synaptonemal complexes formed in the prophase meiotic nucleus. Vegetative multiplication in microsporidia is by binary and/or multiple fission of wall-less plasmodia. In fungi, walled hyphae develop by apical growth, in yeast by budding or binary fission. Sporulation in microsporidia varies from bisporous sporogony that produces 2 spores from each sporont to polysporous sporogony producing many spores. This process can involve meiosis or nuclear dissociation of diplokarya. Sporulation in fungi is highly variable (see Alexopoulos et al., 1996).

Reproduction

Both fungi and microsporidia reproduce both sexually and asexually, although not necessarily at the same time. Asexual species of microsporidia are of two basic forms: one is uninucleate (haploid) throughout development and produces uninucleate spores while the other is diplokaryotic (diploid) throughout development and produces diplokaryotic spores. Asexual fungi are uninucleate (haploid) throughout development producing uninucleate spores. Sexual forms of microsporidia typically have an alternation of uninucleate development producing haploid spores (some sequences involve meiosis) and diplokaryotic development producing diplokaryotic spores. Some involve an obligate intermediate host. Sexual forms of fungi have an alternation of haploid and diploid cell states, which usually involves meiosis and produces only uninucleate spores. Some involve an intermediate host.

Biochemical

Trehalose is a major reserve carbohydrate in all species of microsporidia tested while glycogen is the major reserve carbohydrate in fungi. Trehalose is a reserve disaccharide of some fungi especially yeast and lichens. The cell and spore walls of fungi contain chitin while chitin is known to occur only in the endospore of microsporidian spores.

Others features

All known microsporidia are obligate, intracellular parasites while parasitic fungi can be either facultative or obligate. Microsporida infect only animals, from protests to man, while parasitic fungi infect plants, animals and other fungi. Viruses and other fungi attack fungi but there is no conclusive documentation for any pathogens of microsporidia.

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Microsporidian roots and branches within the Zygomycota? Take a number and step in line!

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The search for greater sense in trying to find where the putative point of linkage between the Microsporidia and Zygomycota might occur should be put in the greater context of changing concepts about the Zygomycota itself. This large assemblage of fungi is now being demonstrated to be phylogenetically hetero-geneous but has received comparatively little attention among phylogenetic mycologists trying to assemble the most reasonable interpretation of evolutionary patterns within the true fungi. The true fungi are now interpreted include the flagellate Chytridiomycota and the 'higher' fungi, ascomycetes (and their associated conidial states usually treated among the Deuteromycota) as well as the basidiomycetes, with these divergent groups linked together in some manner still to be confirmed through the Zygomycota.

Vastly more effort has been expended to study the molecular systematics and phylogeny of ascomycete, basidiomycete and deuteromycete fungi than has been devoted to the 'lower' zygomycete and chytrid fungi. It is understandable that the molecular systematics of chytrids has not been studied much since so few of them are easily cultured or handled in the laboratory. A very large proportion of the Zygomycetes are saprobes that can be readily grown in culture and are available from one or another culture collection; few of these, however, have been included in major molecular studies on genetic diversity or phylogenetics. Many of the more fastidious zoopathogens within the Zygomycota are also either difficult to manage in culture and are either only rarely collected and/or have never been cultured successfully; this situation does not prevent the effort to place micro-sporidia within the Zygomycota but it does increase the level of the challenge.

Indeed, the elucidation of phylogenetic relationships within the fungi has become a major thrust for many mycologists, and it is probably fair to say that mycological systematics is in the midst of one of its greatest and most productive flowerings as a direct result of the rise of molecular systematics techniques. While the dramatic resculpting of the general understanding of phylogenetic relation-ships among the fungi was by no means based mainly on PCR-based molecular techniques, these newest techniques in the systematics toolchest have had a profound effect in confirming or extending the changes being contemplated through the use of other approaches. The traditional concept of the Kingdom Mycota began disintegrating years ago when it was realized that the oomycetes, a huge and diverse group comprising very many significant pathogens of plants or animals, could no longer be retained among the true fungi; these 'pseudofungi' have been reassigned to an even more diverse Kingdom Straminipila (or Chromista) that comprises all organisms that produce a tinsel-type flagellum whose surface is invested with hair-like mastigonemes (Barr, 1992; Dick 2001).

The kingdom Zygomycota was long thought to comprise a diverse set of fungi distributed among two classes: The Zygomycetes included the mainly saprobic and common fungi of the Mucorales, the so-called merosporangiferous fungi of the Kickxellales and Dimargaritales, the mostly zoophilic or zoopathogenic fungi of the Entomophthorales and Zoopagales, the arbuscular mycorrhizal fungi in the Glomales and Endogonales; the class Trichomycetes included primarily endocommensals attached to the peritrophic membrane in the guts of a very diverse range of aquatic and marine arthropods. In the wake of advances in many aspects of our understanding of these fungi, the Zygomycota is now being reinterpreted to be a heterogeneous assemblage:

- The arbuscular mycorrhizal fungi have just been removed from the Zygomycota by Schuessler et al. (2001) and put into a new phylum Glomeromycota that is also thought to share a common ancestor with the ascomycetes and basidio-mycetes. It is unknown whether the Glomeromycota occupies a branch off the line to the higher fungi or a node through which the line of evolution passes.

- The inclusion in the Trichomycetes of the Amoebidiales, morphologically simple organisms living primarily as ectoparasites on arthropods, has been increasingly doubted; molecular data suggests that *Amoebidium parasiticum*, the type and most common species of this order, is a protozoan rather than a fungus (Benny and O'Donnell, 2000).

- There is some doubt about the monophyly of the Harpellales (the largest, most widely distributed and most commonly encountered order of Trichomycetes) (Gottlieb and Lichtwardt, 2001). The placement of the Harpellales within the Zygomycota remain unquestioned since the sexual spores of these fungi are zygospores. Lichtwardt et al. (2001) provide the most complete available summary about the Trichomycetes.

- The hypothesis by Nagahama et al. (1995) that *Basidiobolus* shows stronger genetic similarity with the Chytridiomycota than with the Entomophthorales in which this genus is historically placed remains contentious. Cavalier-Smith (1998) proposed the class Bolomycota for *Basidiobolus* because the organelle associated with nuclei during mitosis contained microtubules, but so formalized a split of the Entomophthorales has not been widely accepted or followed.

- Despite the unquestioned molecular differences that separating *Basidiobolus* from the rest of the Entomophthorales, there are many significant similarities with these fungi; whether the Entomophthorales should be split will depend on bringing many more taxa and a diverse range and substantially larger number of sequences into the analysis.

- There is molecular evidence for polyphyly on a massive scale within the recognized families and, to some extent, even among genera within the Mucorales (O'Donnell et al., 2001).

- Relative to other Zygomycota, data on sterol chemistry (Weete and Gandhi, 1997) and genetic sequences (O'Donnell et al., 2001) suggest that at least some species in the family Mucoraceae (Mucorales) are, despite their very simple morphology, derived rather than ancestral forms.

- Sterol chemistry suggests that the Entomophthorales what may be close the putative ancestral state for the Zygomycota (Weete and Gandhi, 2001) while the group of orders including the Kickxellales, Dimargaritales, Zoopagales, and Mucorales is derived and, presumably, phylogenetically more recent.

In view of the perspectives noted above, it must be conceded that the fungi traditionally placed in the Zygomycota should be recognized as an artificial assemblage. The 'old' classification must derive to some extent from convergent evolution of the morphologies of phylogenetically diverse fungi. Resolving the 'real' relationships among these fungi and determining the broader phylogenetic affinities both pointing downwards to the ancestral (presumably chytrid) fungi and upwards into the higher fungi—is the only way to gain any reasonable perspective on the overall course of fungal evolution.

And, now, after this background we finally come to the issue at hand: A broad consensus is growing that the Microsporidia are not among the oldest existing eukaryotes but are organisms derived from by some remarkable biological reductions from fungi. The major evidence indicates that the phylo-genetic roots of the Microsporidia lie within the Zygomycota.

The Microsporidia have been linked so far to least two different groups within the Zygomycota, to the Trichomycetes and the Entomophthorales. Because there is little reason to believe that these two large arthropod-associated groups within the Zygomycota are themselves very closely related, the molecular methodologies that have led to these separate conclusions deserve careful evaluation.

Apart from the merit of any biologically based analysis about the 'real' fungal affinities of the Microsporidia, the problem of which organisms have been selected for inclusion in any of the analyses of a microsporidian-fungal linkage profoundly affects the discussion. Dendrograms can link only those organisms for which data are included, and dendrograms are constructed exclusively by computer programs incapable of reasoned judgments that may, at any point during or after the construction of the trees, superimpose knowledge apart than the raw data manipulated by the algorithms. That microsporidians are being linked with fungi in every molecular analysis includes taxa from both of these groups is strong evidence for the correctness of the removal of the Microsporidia from the Protozoa. Because the most comprehensive analyses show a greater affinity of the Microsporidia with fungi in the Zygomycota, further meaningful analyses must include a much broader and deeper selection of fungi in and closely related to the putative target points for the linkage than has been the case up to now. Individual studies done to date have included only very few entomophthoralean or tricho-mycete taxa and a similarly small number of microsporidians. Clearly, a compre-hensive study to perform a single analysis of data from a large number of diverse gene sequences from a wide spectrum species of microsporidia, Trichomycetes, and Entomophthorales along with a good selection of supposed outgroup fungi from other groups within the Zygomycota and Chytridiomycota. Extensive sampling for microsporidians and trichomycetes is made difficult since obtaining useful DNA samples of many taxa in either group will usually depend on separating and purifying very small amounts of material from a host. Cultures of many Harpellales (Trichomycetes) are now available, but most are of *Smittium* species whereas this order includes two families and at least 33 genera (eight of which are in culture; see *http://www.nhm.ukans.edu/~fungi/cultures.html*). Similar problems about the lack of available cultures and difficulty of finding specimens in the field apply to species of *Neozygites* and other other Entomo[hthorales, and yet *Neozygites* species are arguably among the most important entomophthoralean taxa to include in studies to elucidate microsporidian affinities.

A comparative study of beta-tubulin sequences (Keeling et al., 2000) is one of the most intriguing to date: This study included five microsporidians, three trichomycetes, four entomophthoraleans, and a wide range of other zygomycetes and ascomycetes. Using this single structural gene, the microsporidians clustered together on a clade with the trichomycetes and one mucoralean genus and are rather distant from the included entomophthoraleans.

It is reassuring that the closest fungal candidates for association with the microsporidia are also entomogenous. The entomogenous Entomophthorales all grow inside the host body (primarily in the hemocoel); there is no strong evidence that any of these fungi become intracellular parasites during any phase of their development. However, all of the phytoparasitic taxa of the Entomophthorales show intracellular growth: *Completoria complens* (Completoriaceae) and *Ancylistes* species (Ancylistaceae) are intracellar fern gametophytes and desmid algae, respectively, and *C. complens* even appears to grow as as vegetative protoplasts (Humber 1989). Trichomycete fungi are usually gut commensals in their hosts and remain, therefore, topologically outside the host body; exceptions to this include *Smittium morbosum*, which may penetrate the host gut (and possibly even contact the host hemocoel), and the occasional presence of large numbers of harpellid cysts usurping ovogenesis in the ovaries of adult nematoceran flies (see Lichtwardt et al., 2001). Microsporidian ancestors

apparently possessed mitochondria since some mitochondrial genes have been incorporated into the genomes of these amitochondrial parasites; such a loss, however, is not altogether unique since many anaerobic organisms (including *Neocalli-mastix*, a genus of chytrid fungi found only in ungulate mammalian rumens) are also amitochondrial. The polar filaments that are so key to the infection of host cells by microsporidia are strongly echoed in the appendages formed inside trichospores and which serve in the successful transmission of trichospores to a new host; there is no functional or developmental similarity between microsporidian polar filaments and the secondary capillary conidio-phores produced by some fungal entomophthoralean pathogens. Such secondary conidiphores are even formed by *Hirsutella aphidis* (Balazy, 1985), a clavicipi-taceous aphid pathogen; these secondary conidiophores are morphologically and functionally similar to the long, hair-like necks typical of the conidiogenous cells for most *Hirsutella* species).

These and other characters and points of correspondence in the biology of the microsporidia and their closest putative fungal relatives will be discussed.

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Symposium (Microbial Control 2) Microbial Control of Insect Pests of Potato; from Tiera del Fuego to the Great White North

Insect pests of potatoes in the Western Hemisphere and the potential for their control using entomopathogens

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The exploitation of the potato, *Solanum tuberosum,* as a crop originated with the Incas in Peru over 5000 years ago (Thurston, 1994; CIP, 2002). Today a multitude of potato varieties are grown throughout the Western Hemisphere from Southern Argentina and Chile to several provinces in Canada. Despite potent allelochemical defenses, *S. tuberosum* is attacked by a panoply of invertebrate pests including insects, mites, slugs, and nematodes. Insects feed on virtually every part of the plant (leaves, stems, and tubers) and several of these pests also serve as vectors of phytopathogens. Insect pests and their relative importance vary from region to region, but the predominant means of control is through the use of broad spectrum conventional insecticides. Microbial control offers an alternative to conventional insecticides that is safe to applicators, consumers, and the environment including insect natural enemies of pest insects (Lacey et al. 2001a). In this presentation I will highlight the more important insect pests in the Western Hemisphere, their pathogens and the potential of these pathogens for microbial control.

The potato tuber moth (PTM) is considered the most serious pest of potato tubers in the field and in storage in warm, dry areas of Mexico, Central America, the inter Andean valleys of South America (Raman, 1994; International Potato Center Website [CIP, 2002]). Larvae of PTM bore into stored potatoes and along with the action of rot causing bacteria can rapidly destroy them. The most widespread of the tuber moths is *Phthorimaea operculella*. The Andean PTM, *Symmetrischema tangolias,* is active in the mid elevation Andean region and *Tecia solanivora,* a Central American species has spread to Venezuela and Colombia in recent years. The PTM granulovirus plays a key role in the management of the common potato tuber moth, *P. operculella* in stored potatoes. The developmental research for a simple technique for multiplication and formulation of the virus was conducted at CIP and will be presented in detail by Dr. Lagnaoui in the next talk in this symposium. Certain varieties of lepidopteran-active *Bt* also have potential for control of *P. operculella* and other tuber moths.

The Andean potato weevil, *Premnotrypes* spp., is another serious pest of potato in South America and the most damaging insect pest at altitudes above 2,800 meters in the Andean region of Bolivia, Peru, Ecuador, Colombia, and Venezuela (CIP, 2002). Currently, 15-40 % of tubers are commonly infested in high altitude potato by weevil larvae at harvest time despite the use of insecticides. Adult weevils add to the damage by feeding on potato foliage. The fungus, *B. brongniartii*, is currently used as a microbial control agent of this pest in parts of Peru, Colombia, and Bolivia (Raman, 1994; Ewell et al., 1994). Entomopathogenic nematodes

have demonstrated good activity against other weevil species with subterranean larvae (Booth et al., 2000). It is possible that they may have potential for control of *Premnotrypes* spp.

The leafminer fly, *Liriomyza huidobrensis*, is an insect that becomes a serious pest of potato in coastal Peru and Chile and in certain areas of Brazil, Central America, and other countries when insecticides are used intensively and where particularly susceptible cultivars are planted (Raman, 1994; Ewell et al., 1994; CIP, 2002). In certain situations *L. huidobrensis* has reduced potato yield by 45 62%. Currently microbial agents are not utilized for control of this pest. The recommended management strategy for control is based on the use of resistant cultivars, use of sticky yellow traps to capture adult flies, and habitat management to favor the increase of natural enemies. The use of insect growth regulators, when necessary, is a final option (CIP, 2002).

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, is a widespread defoliator of potato and other solanaceous vegetables and is considered a major pest in the Northern Hemisphere. It may have 1 to 3 generations per year depending on latitude and weather and overwinters in the pupal stage. When infestations are high, the crop can be completely defoliated before tubers are large enough to warrant harvest (Hare, 1980). Until the mid-1980s, the fungus, *Beauveria bassiana* was the principal microbial control agent for CPB. Control of CPB ranging from poor to excellent has been reported for the fungus (Hajek et al., 1987; Poprawski et al., 1997; Lacey et al., 1999; Wraight and Ramos, 2002). *Beauveria bassiana* offers the advantage of recycling in host cadavers and persisting in the soil beneath potato plants thereby affecting the survival of subterranean stages of the beetle. The discovery of *Bacillus thuringiensis* var. *tenebrionensis* (Langenbruch et al., 1985) and other *Bt* toxins with activity against beetles, broadened the options for microbial control of CPB. The bacterium provides excellent control of larvae, especially when applied against early instars. Timing and frequency of application, amount of inoculum, spray coverage, crop canopy, rainfall and UV inactivation can have strong influences on the efficacy of both pathogens (Bystrak et al., 1994; Wraight and Ramos, 2002). Entomopathogenic nematodes and engineered spiroplasmas have also been proposed as microbial control agents of CPB (Hackett et al., 1992; Berry et al., 1997). Recent literature reviews address the potential for biological control of CPB including the integrated use of entomopathogens, predators and parasites (Cloutier et al., 1995; Moldenke and Berry, 1999; Lacey et al. 2001b [in English and Spanish]).

Several aphid species are pests of potato throughout the Western Hemisphere. The green peach aphid (GPA), *Myzus persicae*, is especially important because of its role in transmitting potato leafcurl virus and other viruses. For example, in the potato growing areas of the Pacific Northwest of the United States, the focus of insect control efforts is primarily on GPA and many other insect pests (CPB, Lepidoptera) are coincidentally controlled by the broad spectrum insecticides that are used. The potato aphid, *Macrosiphum euphorbiae*, can also be abundant on potato, but its role as a vector is less serious. Naturally occurring fungi are important regulators of aphid populations (Latgé and Papierok, 1988), including aphids on potatoes (Soper, 1981). The use of fungicides to combat late blight of potato, *Phytophthora infestans*, has been correlated with aphid outbreaks due to the suppressing effects of the fungicides on entomophthoralean fungi that normally control the aphid (Lagnaoui and Radcliffe, 1998). Although development of fungi as mycoinsecticides of aphids in potatoes has been studied (Soper, 1981), no large scale implementation of artificially cultured fungus in natural populations of aphids in potato has yet been attempted.

The larvae of click beetles (*Agriotes, Limonius*, Coleoptera: Elateridae), also known as wireworms, can be locally important pests of potato tubers (Jansson and Seal, 1994), especially

if potatoes are rotated with crops preferred by the beetle, such as grains. Relatively little research on the microbial control of these pests has been conducted. Toba et al. (1983) attempted unsuccessfully to infect larvae of *Limonius californicus* with entomopathogenic nematodes. The fungus, *Metarhizium anisopliae,* has been reported from wireworms and is currently under development for control of species in Agassiz, British Columbia, Canada, Lethbridge, Alberta, Canada and Yakima, Washington, USA.

Several other insect species are regarded as potato pests, but their economic importance varies from region to region. These comprise: several lepidopteran species in the family Noctuidae including loopers (*Autographa californica*), army worms (*Spodoptera praefica*), cutworms (*Agrotis ipisilon, Peridroma saucia*) and the potato stemborer (*Hydraecia micacea*); potato leafhopper (*Empoasca fabae*, Homoptera: Cicadellidae); potato flea beetle (*Epitrix cucumeris*, Coleoptera: Chrysomelidae); tuber flea beetle (*Epitrix tuberis*, Coleoptera: Chrysomelidae); root worms (*Diabrotica* spp., Coleoptera: Chrysomelidae); white grubs *(Melanothus sp., Polphylla sp.*, Coleoptera: Scarabaeidae); blister beetles (*Epicauta* spp., Coleoptera: Meloidae); potato stalk borer (*Trichobaris trinotata*, Coleoptera: Curculionidae); potato scab gnat (*Pnyxia scabiei*, Diptera: Sciaridae); potato psyllid (*Paratrioza cockerelli*, Homoptera: Psyllidae); silverleaf whitefly (*Bemisia argentifolii*, Homoptera: Aleyrodidae); and grasshoppers (*Melanoplus spp.*, Orthoptera: Acrididae). Entomopathogens with potential for control of these species are presented in Table 1.

TABLE 1. Entomopathogens proposed for microbial control of miscellaneous insect pests of potato.

Loopers, army worms	*Bt,* baculoviruses
Cutworms	nematodes, *Bt*, baculoviruses
Potato leafhopper	*Zoophthora radicans*
Silverleaf whitefly	fungi (Hyphomycetes)
White grubs	nematodes, *Bt*, *Paenibacillus spp.*
Grasshoppers	*Nosema locustae*, Entomophthorales, *Metarhizium*
Root worms	nematodes

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Microbial control of potato tuber moth and Andean potato weevil in South America

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The potato production in South America is essential for food security of the inhabitants of the Andes, particularly those in rural areas. Unfortunately, many pests (insects, pathogens, nematodes and weeds) are known to affect potato production in this region, causing severe losses. In the Andes, the most damaging potato pests are the Andean potato weevils and the potato tuber moths. Most weevils are in the genus *Premnotrypes* and often referred to as the Andean potato weevil complex. Three species of moths constitute the potato tuber moth complex (P. operculella, S. tangolias and T. solanivora). Despite the heavy use of insecticide in this region, both moths and weevils are still causing severe losses to potato crops. Over the years, CIP scientists developed an integrated management strategies based on the use of locally produced biopesticides. The fungus, *Beauveria brogniartii*, is used to control weevil larvae and the Baculovirus (PoGV) is used to control potato tuber moth larvae. These naturally occurring entomopathogens are now used in Peru, Ecuador, Colombia, and Bolivia. The use of the biopesticides is low-cost, effective and particularly attractive to poor farmers, relieving them of the high costs and undesirable effects of toxic insecticides.

The discovery, development and death of *Bacillus thuringiensis* var. *tenebrionis* as a microbial control product for the Colorado potato beetle

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When it was first discovered in 1983 (Krieg et. al.), the beetle-active *Bacillus thuringiensis* var. *tenebrionis* created great excitement and hope in the world of microbial control. With its novel activity against larvae of the Colorado potato beetle – a major pest of potatoes in North America and many European countries — *B.t. tenebrionis* was rapidly developed – first, as a biopesticide in 1988, and then in the form of transgenic potatoes, in 1995. Yet what at first seemed to be a discovery that could breath new life into the small and struggling biopesticide industry, *B.t. tenebrionis* was to suffer a series of setbacks that led to the withdrawal of all but one bioinsecticide product from the market, and the complete cancellation of sales of *Bt tenebrionis* transgenic potatoes. The forces that shaped the brief, downward trajectory of this organism span the spectrum from the familiar microbial control issues of host range specificity and lack of effective delivery to the target insect, to the less familiar, but no less important issues of patent disputes, the political movement against transgenic plants, the dynamics of the agrichemical industry, and even industrial espionage. There is no single reason why *B.t. tenebrionis*, despite moderate to excellent field performance, failed so dismally as a microbial control product. But an examination of its brief but complex history is worthwhile, not only as an interesting distillation of some of the major trends in agriculture over the past 20 years, but also as an instructive exercise for microbial control researchers and developers.

Until the discovery of *B.t. tenebrionis* in 1983 (sometimes referred to as *B.t. san diego* in the 1980s literature), the possibility of controlling beetle pests with *Bacillus thuringiensis* had not been considered. With its unique, square shaped protein crystal that was composed of a single, 65 kDa delta endotoxin (currently designated cry3Aa1), *B.t. tenebrionis* produces acute toxicity in a variety of chrysomelid beetle larvae (including Colorado potato beetle, elm leaf beetle, cottonwood leaf beetle) as well as the tenebrionid yellow meal worm, *Tenebrio molitor* (Herrnstadt et. al., 1986). The insect host range is quite limited for this pathogen, with susceptibility observed only for these two beetle families. As for other commercially available varieties of *B.t.*, there is no mammalian or non-target toxicity. Activity of *B.t.t.* occurs primarily against 1st and 2nd instar CPB larvae, the *B.t.* toxin must be ingested to be infected, and the half life of the delta endotoxin crystals on leaves is brief (Zehnder and Gelernter, 1989).

With the Colorado potato beetle as the most important economic pest in the *B.t. tenebrionis* host range, several commercial products targeted against this pest and based on the spores and endotoxin crystals were introduced during the late 1980s and early 1990s. The first genetically engineered potatoes to express the *B.t.t.* toxin were introduced in 1995 (Table 1). Although most of the products in Table 1 are no longer available, several small retail companies still re-package *B.t. tenebrionis* for sale to home gardeners and small organic farmers.

The market introduction of *B.t.* tenebrionis was welcomed by potato growers in North America and Europe, particularly those who had had difficulty controlling CPBs that were resistant to a broad spectrum of conventional insecticides. However, the *B.t.t.* products were difficult to use because precise application timing, excellent spray coverage of foliage, and a high frequency of application were required for the desired level of control. In addition, larval mortality occurred 2 or more days after application, a phenomenon that growers were unused to. But the lack of

TABLE 1. Commercial products based on *Bacillus thuringiensis* var. *tenebrionis*

Commercial name	Company	Active ingredient	Product fate
M-One	Mycogen	Non-engineered *B.t.t.* spores and crystals	Discontinued
M-Trak	Mycogen	Genetically engineered, killed *Pseudomonas fluorescens* cells expressing the *B.t.t. delta endotoxin*	Discontinued
Novodor	Abbott	Non-engineered *B.t.t.* spores and crystals	Still available
Foil	Ecogen	Non-engineered *B.t.t.* spores and crystals	Discontinued
Raven	Ecogen	*B.t.t.* transconjugant	Discontinued
Trident	Sandoz	Non-engineered *B.t.t.* spores and crystals	Discontinued
NewLeaf potatoes	Monsanto/NatureMark	*B.t.t.* delta endotoxin expressed in potatoes	Discontinued

other alternatives, due to widespread CPB resistance to conventional insecticides, fueled grower interest in *B.t.t.* despite its drawbacks and the product was slowly and gradually adopted.

In the early 1990s, the introduction of imidacloprid, a highly effective, relatively low toxicity, highly persistent, new class of insecticide chemistry, changed the status of *B.t.t.* significantly. Potato growers found that unlike *B.t.t.,* imidacloprid was vastly easier to use., and was also more effective. Within two years of the introduction of imidacloprid, *B.t.t.* sales, never very large in the first place, had plummeted. By 1998, only one product was still registered for use. In addition to the fact that it was difficult to use, and to competition offered by imidacloprid, economic problems within the biopesticide industry played a role in the demise of sprayable *B.t.t* products.

These included

- Competition for biopesticide sales among several small, newly emerged companies. During the late 1980's and early 1990's, there were at least 8 small U.S. biopesticide companies in operation. In addition, large agrichemical companies such as Ciba-Geigy, Bayer and American Cyanamid were also marketing biopesticide products
- Lower than projected sales volumes of biopesticide products
- Competition from new, less toxic classes of pesticides such as imidacloprid.
- The high cost of supporting *B.t.* patents and patent disputes

The 1995 introduction of New Leaf potatoes, which had been engineered to produce the *B.t. tenebrionis* toxin in the foliage, addressed many of the performance issues associated with *B.t.t.* sprays. For example, the delta endotoxin was delivered at very high levels — roughly 10 times the LD90 for 2nd instar larvae of the CPB – and it was delivered continuously in the foliage (Whalon and Ferro, 1998). As a result, the difficulties associated with spray timing, coverage and frequency were erased, and control of CPB larvae was excellent. Despite its technical success, however, New Leaf potatoes were a commercial failure. At the "height" of their success, they were planted on only 3% of the U.S. potato acreage, and by 2001, *B.t.* potatoes were no longer for sale. Key reasons for ths product's demise include:

- Lack of activity against other key potato pests, including aphids.

- Availability of alternative products such as imidacloprid, with similar efficacy against CPB, as well as activity against aphids and other pests.
- Fear of genetic engineering: as the first *Bt* crop approved for human consumption, consumers and the companies that supplied them were wary of the safety of engineered potatoes. Companies such as McDonald's, McCain's, Burger King, Frito-Lay and Procter and Gamble declared that they would not accept engineered potatoes (Wall Street Journal), and several of the U.S.'s most important trading partners, including Japan, opted to ban genetically engineered crops.

The fall of *B.t. tenebrionis* as a biopesticide and as a transgenic crop came as a surprise to many microbial control researchers and companies. It is impossible, in hindsight, to determine whether its failure could have been predicted or avoided. But the "lessons learned" below may help future efforts to avoid some of the pitfalls that *B.t. tenebrionis* encountered.

- The role of rapidly changing markets, and the need for accurate market assessments can never be overestimated in the development of biopesticides.
- High levels of product performance are not always enough to insure success. An understanding of the entire pest complex and of the customer who will buy the product are at least of equal importance.
- The costs of supporting patents must be weighed against the market potential. In many cases, the costs of patent support far outweigh the potential profits, especially in the small markets that biopesticides typically address.
- Small companies frequently have difficulty in sustaining product development and commercialization properly due to lack of expertise and lack of cash. It may be that the small markets typically addressed by biopesticides are not commercially viable and that some other economic model may be necessary to insure the sustainable use of these products.

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Microbial control of Colorado potato beetle in potatoes in rain-fed potato agroecosystems in the Northeastern US

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Introduction

The Colorado potato beetle (CPB) is a perennial pest of potatoes throughout northern temperate regions of the world. Widespread use of chemical insecticides against this insect have lead to repeated problems with insecticide resistance (Grafius 1997), and has spurred interest in biological alternatives.

The fungal pathogen, *Beauveria bassiana,* is recognized as a potential biocontrol agent and attempts have been made to manage both the soil dwelling (Cantwell et al. 1986) and foliar feeding (Wraight and Ramos, 2002) stages of this pest with the applications of *B. bassiana* conidia. Although the level of pest control achieved with the fungus has seldom equaled that achieved with standard insecticides (Wraight and Ramos, 2002), many researchers have reported significant reductions of CPB populations following treatment.

However, to date, the most widely used microbial for manangement of CPB is *Bacillus thuringiensis tenebrionis (Btt).* Commercial formulations of *Btt* are available, and well timed applications against small larvae have resulted in successful management (Zhender et al. 1992). However, because of the extended oviposition period exhibited by this pest, the short residual activity of *Btt*, and the low virulence of *Btt* against adult beetles, sole reliance on this pathogen has required 7–10 applications per season in Massachusetts (Ferro, personal communication).

Strategy and Field Trials

Natural suppression of insect populations is seldom achieved by a single mortality factor, but by many agents acting in concert. Such an approach to biological control may result in more stable pest suppression. Combining *B. bassiana* and *Btt* for CPB management may provide quicker kill and suppression of pest damage than with *B. bassiana* alone, and provide more long-term pest suppression than with *Btt* alone. *Btt* rapidly arrests feeding by larvae, whereas, *B. bassiana*-treated larvae continue to feed for some time. However, *B. bassiana*-infected cadavers can produce inoculum for subsequent infections (Fernandez 2001), whereas *Btt* does not replicate extensively in infected larvae, and natural epizootics of this pathogen are unknown in CPB populations.

Four field trials were conducted in Maine from 1993 to 1995 to compare pest suppression and defoliation with each of these microbials alone and in combination. *Btt* (Foil®, Ecogen, Langshorn, PA) treatments consistently resulted in lower densities of large larvae and less defoliation compared with controls and *B. bassiana* (Mycotrol® provided by Mycotech Corp., Butte, MT) alone treatments. Although *B. bassiana* treatments provided significant control of large larvae in only one of four trials, they did consistently reduce defoliation, and similar to *Btt*, significantly reduced the number of first-generation adults (Groden and Drummond, unpublished). No interactions were observed between *Btt* and *B. bassiana* in the Maine trials, and the effects of these two agents when applied in combination were generally additive.

Combination treatments consistently resulted in lower densities of large larvae in all trials, less defoliation in two of three trials, and fewer new adults produced in three of four trials.

Field tests comprising multiple treatments in replicated small plots were conducted in NY from 1997 to 2000 with objectives similar to those of the Maine trials. Three to four applications of *B. bassiana* strain GHA alone produced no more than moderate reductions (20–65%) of first generation larval populations relative to controls (Wraight and Ramos, 2002). Tests in which applications were initiated against third-instars provided no significant control. Recommended label rates of *Btt* alone applied on a weekly schedule produced significantly greater levels of control (44–88%) (Wraight and Ramos, unpublished). As was also observed in Maine, however, treatments with *B. bassiana* and *Btt* in combination provided the highest and most consistent levels of population reduction (79–95% control when tank-mixed and applied weekly), and results from NY indicated a significant though low level of synergism between treatments (Wraight and Ramos, unpublished). This underscores the excellent compatibility and potential for highly beneficial joint action of these two well known microbial control agents.

Issues related to efficacy

Field applications of live pathogens, such as *B. bassiana* conidia can be particularly sensitive to improvements in application technology, timing of applications, and residual activity. We have been investigating how efficacy of this pathogen against CPB larvae can be maximized.

Application technology: The effects of hydraulic sprayer configuration on efficacy of *B. bassiana* foliar applications were evaluated during three field seasons in NY (Wraight and Ramos, 2002). Treatments were applied using a backpack sprayer fitted with two differently configured booms, viz, one with nozzles affixed to lateral drop tubes and directed upward to target ventral leaf surfaces and another configured with nozzles directed to spray downward from 30 cm above the crop canopy. The sprayer with nozzles attached to drop tubes deposited an average of 752 conidia/mm^2 on the upper surfaces of the potato leaves and 482 conidia/mm^2 (39%) on the lower surfaces, whereas the alternative sprayer deposited 1,062 vs 50 conidia/mm^2 on the respective leaf surfaces. Applications targeting small larvae from below canopy produced substantially greater population reductions than applications from above canopy (43-65% vs 0-18%). On the other hand, sprayer configuration had no effect on efficacy of applications targeting large larvae. Sprays from both above and below canopy caused no significant control of late-instar larval populations, but equivalent high levels of control (75-78%) of first generation adults (Wraight and Ramos, 2002).

Fungal formulation

Formulation in oil carriers has received considerable attention in recent years as a means of increasing fungal efficacy for ultra low volume applications, especially against rangeland pests (see Wraight et al. 2000). Few studies have assessed effects of formulation in field crops such as potatoes. The potential for increasing efficacy of *B. bassiana* in emulsifiable oil formulations designed for high-volume applications was investigated over four field seasons in NY. In the first season, during which heavy rain fell immediately following two of the three initial applications, an emusifiable oil formulation (Mycotrol ES) provided substantially greater control than a wettable powder (Mycotrol WP) (65 vs 8%), suggesting greater rainfastness of the oil formulation (Wraight and Ramos 2002). In the three subsequent tests, however, no significant differences in population reductions or defoliation were recorded between the two formulations (Wraight and Ramos, 2002; Wraight and Ramos, unpublished).

Timing of applications

To evaluate optimal timing of *B. bassiana* applications against CPB larvae in potatoes in the Northeast U.S., a computer model was built to simulate primary infection following the application of conidia onto a susceptible CPB larval population (Joergensen, 2001). The model, a dynamic computer simulation model, consisting of a time-varying distributed delay algorithm (Long et al. 2000b), was validated (8 field data sets from 6 field seasons and 2 locations) and demonstrated to be a good predictor of field infection rates. Runs of the model were conducted to assess optimal timing for foliar conidial applications targeted at spring and early summer larvae. Results suggest that for a single application, timing during peak second instar is the most effective, resulting in the greatest reduction of defoliation and summer adult emergence. Two applications yield better control than a single application and the optimal timing of applications are at peak occurrence of second instars and peak occurrence of third instars. Results from NY tests (Wraight and Ramos, 2002) were in accord with these results, as applications of *B. bassiana* made at 3–4 day intervals were significantly more effective than applications applied weekly.

Persistence on foliage

Studies conducted in Maine (Fernandez et al. 2001) have demonstrated that most primary infection of CPB larvae in *B. bassiana* treated fields probably occurs as a result of direct exposure to conidial sprays. However, larvae moving across treated foliage can pick up lethal dosages of conidia, and residual conidia on foliage may be particularly important as a source of inoculum for hatching eggs and larvae which avoid exposure to direct spray or escape infection by molting shortly after sprays. Evaluation of foliar persistence of the Mycotrol® ES formulation of *B. bassiana* conidia was conducted in Maine in 1999 on three dates between 7 July and 30 August (Joergensen 2001). Residual activity of treatments applied at 6 AM and 6 PM were compared by assaying foliage against CPB larvae. Mortality was greater for PM treatments at 6 and 12 hours post spray than AM treatments and did not differ significantly from that observed immediately post spray (0 h). Residual activity declined in both treatments from 12 to 96 h when there were no differences in mortality of larvae fed foliage with either AM or PM sprays and untreated control foliage. Accumulated solar radiation (W per m^2) explained 73% of the variation in residual activity of conidia. The CPB/Bb primary infection model was modified to incorporate additional infection of newly hatched larvae and molted larvae due to residual activity of conidia. The model demonstrated that the additional residual activity achieved by applying *B. bassiana* in the evening versus the morning increases resulting larval mortality by 18%. If improved formulations of *B. bassiana* with uV protectants could extend the half life of residual activity from 24 to 96 h post treatment, larval mortality would be increased by 55%.

Studies of persistence of conidia on dorsal vs ventral surfaces of potato foliage in NY over several field seasons have produced both expected and unexpected results (Wraight and Ramos, unpublished). Based on viability of recovered spores, conidial half-life on dorsal surfaces exposed to sun was, as expected, less than 0.5 days, whereas, half-life on ventral surfaces was longer (in most cases, e ≥1 week). Unexpectedly, however, direct quantification of conidia in leaf washes, viz., counts of total viable and inviable conida (by hemacytometer) revealed that more than half of the conidia were physically removed from both the dorsal and ventral surfaces of the foliage within 1 day after application. The mechanism of removal is unknown but presumed to be weathering by wind, as rain was not a factor. The tests were conducted on small plants and on upper-canopy leaves fully exposed to the environment, nevertheless, this result was not anticipated and warrants additional study. Also surprising were results of

a field test in which foliar sprays of *B. bassiana* were applied against CPB in the morning versus late evening. Control was poor in both cases and, contrary to the anticipated result, numerically higher in the morning-treated plots than in the evening-treated plots (44 vs 23%). The difference, however, was not significant ($F_{[1,4]} = 4.7$; $P = 0.096$).

Persistence in the population

Continued impacts of *B. bassiana* on CPB pest suppression following the initial effect of foliar treatments is dependent on the fungus persisting in the host population and the environment. Trials conducted in both Maine and New York have resulted in significant reductions in emergence of summer adults following treatments directed against CPB larvae, and significant incidence of infection in those summer adults that do emerge from the soil.

Studies conducted in Maine to assess the potential for horizontal transmission of *B. bassiana* from targeted larvae to subsequent life stages of the host population have demonstrated that although no transmission occurs between larvae on the foliage (Fernandez 2001), up to 24% of surviving prepupae can become infected from encounters with sporulating cadavers on the soil surface (Long et al. 2001a,b).

Conclusions

Small plot field studies in both NY and ME demonstrate that the combination of the two microbial agents, *B. bassiana* and *Btt,* may provide an efficacious alternative to chemical insecticides for management of Colorado potato beetles in rain-fed potato agroecosystems the Northeastern U.S. Although not always consistent, laboratory, field and modeling evidence suggests that efficacy of foliar applications may be enhanced with oil formulations applied in the evening against peak small larval populations using drop nozzle spray booms. The combination of agents does significantly reduce defoliation and subsequent beetle populations. Horizontal transmission from primary infected cadavers to soil dwelling stages of the beetle results in secondary cycling of the disease and continued pest suppression. The combination of *Btt* and *B. bassiana* have been used for management of Colorado potato beetle as part of a biological IPM program in a long-term study of the potato agroecosystem conducted at the University of Maine Potato Research Farm in Presque Isle, ME. One to four applications of the combination (2.3 l/ha Mycotrol with 3.5–7 l/ha *Btt* products) has successfully reduced populations of large larvae and emerging summer adult beetles below economic thresholds, and to levels as low or lower than those achieved with conventional insecticides since 1993 (Gallandt et al. 1998, Groden, unpublished). Biological management of this severe pest can be regularly achieved with microbials. However, at the cost of ca. $85–125/ha for the *B. bassiana* product and $30–75/ha for *Btt* products, per application, less expensive chemical insecticides have limited its adoption by commercial potato growers in the northeast.

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Footnote

[1] Mention of product names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Microbial control of insect pests of potato in Canada and the Western United States

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At this time, there are only two pathogens available against the Colorado potato beetle, *Leptinotarsa decemlineata*. The entomopathogenic bacterium, *Bacillus thuringiensis* serovar *tenebrionis* has been developed commercially for use against the beetle. Field trials have demonstrated excellent control (Ferro and Gelernter, 1989; Zhender and Gelernter, 1989; Ghidiu and Zhender, 1993), however, timing of application remains critical, as the bacterium is effective only against the younger larvae. Field and laboratory studies have demonstrated the ability of the fungal entomopathogen *Beauveria bassiana* to control both larvae and adults of the beetle (Campbell *et al.* 1985; Poprawski *et al.* 1997; Drummond & Groden , 1996). In most trials, foliar applications have been used and, although effective, are limited in their use, as conidial viability rapidly decreases in the presence of sunlight (Inglis et al., 1993). However, studies have shown that the conidia can remain viable for longer periods in the soil (Inglis et al., 1997).

We compared foliar application of the *B. thuringiensis var tenebrionis, B. bassiana* and aldicarb for control of the beetle in an irrigated desert agroecosystem in Washington State. We also studied the effects of these pathogens on the feeding rates of the beetle and on the effects of solar radiation on spore persistence. Effects of application of *B. bassiana* against soil inhabiting stages of the beetle are being determined in southern Ontario and Alberta.

Comparisons of efficacy of *B. thuringiensis var tenebrionis, B. bassiana* and aldicarb for control of the beetle in an irrigated desert agroecosystem

Field trials of *B. bassiana* (Mycotrol®), a genetically engineered isolate of *Bacillus thuringiensis* (Raven®), and aldicarb (Temik®) (individually, alternated and mixed) were compared for control of Colorado potato beetle in an irrigated desert cropping system during the summers of 1998-2000 at the USDA-ARS Farm near Yakima. Applications of the label rates of *Bt* and *B. bassiana,* separately and in alternation in the same plots and mixtures of half label rates of *Bt* and *B. bassiana* were compared. Four applications were made at weekly intervals from late June through July. The major difference between tests conducted in 2000 and those conducted in 1998 and 1999 were that control plots were not integrated within the blocks used for treatments. The effects of the treatments on tuber yields are presented in figure 1. Controls were nearly defoliated by early August. Aldicarb consistently provided good control of the beetle. Similar levels of control were obtained with *Bt* and with the mixture of half label rates of *Bt* and *Bb* throughout most of the three growing season. The highest numbers of overwintering beetles were found in control plots. The lowest numbers in the microbial treated plots were in the *Bt* treated plots and plots receiving a mixture of *Bt* and *Beauveria*. Contrary to results

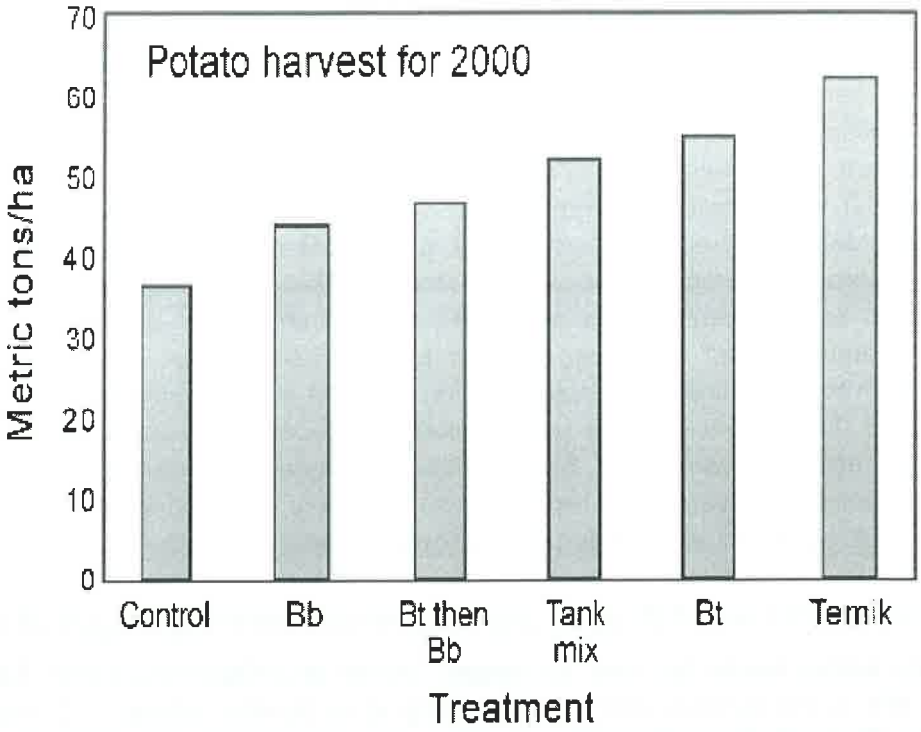

FIG. 1. Tuber yields in plots at Yakima, WA, treated with Temik and various combinations of *Bacillus thuringiensis* and *Beauveria bassiana* for control of the Colorado potato beetleobtained in 1997 (Lacey et al., 1999), plots receiving *B. bassiana* alone and in alternation with *Bt* were comparable to control plots, although the incidence of mycosis was very high in the *B. bassiana*-treated plots. Since mycosis progressively increases throughout the winter, the number of living individuals in *B. basiana*-treated plots could be expected to decline before emergence of the beetles.

Effects of microbial pathogens on feeding rates of CPB

Earlier observations suggested that potatoes that have been treated with the fungal pathogen *B. bassiana* or the bacterial pathogen *B. thuringiensis* tolerate higher densities of Colorado potato beetle than untreated or chemically-treated potatoes, which would mean that the threshold density of beetles that causes yield reduction would be higher for microbial treated potatoes than untreated potatoes. One potential cause of this relationship may be that pathogen-treated beetles feed less than pathogen-free beetles. We ran field and laboratory experiments to test this hypothesis, by collecting beetle larvae from the field and conducting consumption tests. We had 4 treatments: untreated controls; *B. bassiana* applied four times; *B. thuringiensis* applied four times; and, a Temik control. Densities were lowest in the Temik plots (virtually beetle-free all season) and in the *B. thuringiensis*-treated plots, and were highest in the *B. bassiana*-treated plots and the control plots. Yields were highest in the Temik plots (64.2 kg per 10 m of row), intermediate in the pathogen-treated plots (52.7 and 54.0 kg per 10 m of row), and lowest in the control plots (44.2 kg per 10 m of row). Yield-density plots for large larvae (the most destructive life stage) indicate that data for the pathogen-treated plots tended not to group with the control data, consistent with the idea that control and pathogen-treated beetles fed at different rates. Feeding rates for large larvae were in general reduced in insects collected from the pathogen-treated plots compared to those collected from control plots until 10 days following the final application of pathogens made 12 July; by that date, feeding rates were equivalent between larvae collected from treated and untreated plots. Thus, both pathogens caused reduced feeding in beetle larvae, indicating that potatoes should be able to tolerate higher densities of beetles in pathogen-treated plots than in untreated plots.

Solar effects of fungal viability

One of the limiting factors of fungal pathogens used for control of aphids or Colorado potato beetle is their sensitivity to ultra-violet radiation. Initial studies to determine the effect of sunlight on *B. bassiana* were conducted in September over a two day period following application to erect and lush potato foliage using label rates (2.47×10^{13} spores/hectare). Leaf samples were removed at regular intervals from the top and under story of treated and control plants that were touching in and across rows or that were more open. The samples were taken to the lab and vigorously agitated in deionized water containing Tween 80 on an orbital shaker. The suspended spores were then appropriately diluted and plated on SDAY medium. The number of colonies formed (indicating the number of viable spores) was read 72-96 hours later. In addition to determination of solar effects, the data also provided information regarding spore coverage. During the 48 hour period when the tests were conducted, solar radiation was monitored using a pyranometer. Spore viability dropped precipitously after just a half day exposure to sunlight. Lower leaves from plants that were touching were less well covered with the spores, but received slightly better protection from sunlight.

Studies on the effects of *B. bassiana* on the soil-dwelling stages of the beetle

The Colorado potato beetle has two soil stages, pupae and diapausing adults. Mature larvae drop to the soil in mid-summer and pupate, emerging as summer adults 1 - 2 weeks later. In late summer, these adults re-enter the soil where they overwinter until spring. In many areas, the beetle has 2 generations. Field trials inoculating the soil to control pupae have shown considerable promise (Long *et al.* 2000), however, effects of the fungus on overwintering adults have not been studied. One objective of our research study was to determine if prediapausing beetles digging through inoculated soil would pick up and retain enough conidia to incite disease. Another objective was to determine if the fungus would cause mortality in diapausing adults inoculated either by direct application to the beetles, or by pre-application to the soil.

Evaluation of spore retention by digging beetles. We evaluated the amount of conidia that the beetles picked up and retained after burrowing into soil that was surface-treated with *B. bassiana* conidia. Plexi-glass tubes were filled with soil and the surface was sprayed with 10^7 and 10^6 conidia per cm^2. Diapausing beetles were measured, weighed and then allowed to dig into the soil. Beetles were removed 24 hrs later and their depth was recorded. The beetles were then homogenized and plated onto an oatmeal-dodine selective agar medium. After 7 to 10 days incubation at 25° C, the number of colony forming units were enumerated. Beetles picked up significant numbers of conidia, however, numbers of conidia found on the body decreased as depth increased. Sex, weight, width and length did not have an effect on the number of spores lost.

Evaluation of effects of soil moisture on susceptibility. Laboratory dose/mortality assays on beetles pupating in *B. bassiana* inoculated soils at different moisture levels demonstrated that the pupating larvae were highly susceptible to the fungus and that moisture was important for infection to occur. Similar results were obtained when late 4th instar larvae were inoculated topically (i.e. sprayed with spore suspensions). At high concentrations, (1×10^7 to 1×10^8 spores/gm soil), mortality levels of greater than 90% were obtained, regardless of soil moisture levels between 7 and 14 %. At higher moisture levels and lower doses (i.e. 5×10^5 conidia/gm), mortality was much reduced (~20% vs 55 - 70% mortality at 7 - 8% moisture). No mortality was obtained at a moisture level of 4%, regardless of dose. Similar results were obtained in a replicated experiment; high mortality (>80%) was obtained at all spore concentrations at 7% soil moisture, but at greater soil moisture (12 and 14%), lower mortalities

were obtained at the lower spore concentrations, demonstrating the important effect of soil moisture. However, at the highest spore concentration, over 90% mortality was obtained at all soil moisture levels.

Evaluation of effects on overwintering mortality. Field cage trials against overwintering adults were initiated in September, 2000 on (A) fox sandy loam soil in southern Ontario. A wettable powder formulation of *B. bassiana* strain GHA was added to a solution of water plus surfactant and applied to the soil surface using a CO_2 sprayer, prior to incorporating to 0.15m using a rototiller. The experiment consisted of 2 spore concentrations (1×10^6 and 1×10^7 spores/g soil) and a control (sprayed with water and surfactant solution). Each treatment plot contained 3 replicate cages (each with a potato plant and 100 CPB adults) plus an additional cage for monitoring spore persistence. In the spring, adult CPB emerged from the overwintering cages over a period of 5 weeks. Overwintering mortality averaged 59%, 71%, 83% respectively, in control, low and high *Beauveria* treatments. The experiment was repeated in southern Ontario in the fall of 2001 and similar plots established at Lethbridge. Results will be gathered in May, 2002 and reported at the presentation in Iguassu.

Soil persistence. Persistence of spores applied to the soil was monitored in Ontario. Spore persistence in the *Beauveria* treatments was reduced to half (42% low, 50% high) of the original concentration within 60 days, and further reduced to 1/3 (34% low, 20% high) the original concentration by 90 days post application. This concentration then remained fairly constant prior to spring emergence but gradually decreased to 2% of the original concentration by the following August (351d post application). Overwintering spore persistence is being monitored in the Lethbridge plots this year and will be reported in the presentation.

Conclusions

There is great potential in the use of microbial control agents in Integrated Pest Management of the Colorado potato beetle. Pathogens such as *Bt* and *B. bassiana* can be used against the foliar feeding stages, while there is evidence that *B. bassiana* could be used against the soil dwelling stages.

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Integration of insect-resistant transgenic plants, predators and parasitoids, and microbial agents for the control of potato pest insects

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Transgenic potatoes expressing genes for insect resistance are rapidly developing alternatives for controlling major pests such as the Colorado potato beetle (CPB) *Leptinotarsa decemlineata*. To be sustainable, transgenic potatoes with specific insect resistance should be compatible with biological control of target and non-target pests. We study interactions among potatoes, potato pests and their natural enemies and pathogens, to evaluate if and how transgenic potatoes can be used jointly with biological control to develop sustainable potato production systems. We use the stinkbug *Perillus bioculatus* as a model predator of the CPB; the potato aphid *Macrosiphum euphorbiae* as a secondary pest which is not currently targeted by insect resistant transgenic potatoes; and the parasitic wasp *Aphidius nigripes* and the entomopathogen *Verticillium lecanii* as aphid antagonists. Newleaf® potatoes expressing a high dose of *Bacillus thuringiensis* (Bt) Cry3a toxin have low potential for interacting with *Perillus bioculatus*, a specialized predator with low tendency to forage on potatoes in the absence of CPB. By contrast, age-dependent sublethal effects of Bt biopesticides on CPB larvae allowed for positive interaction with predator-inflicted mortality. Cry Bt toxins are not expected to affect aphids, but potato aphids feeding on Newleaf® in lab tests had reduced growth and were poor-quality hosts to the aphid parasitoid. By affecting insect nutrition, protease inhibitors (PIs) are potential factors for developing insect-resistant transgenic potatoes that are more compatible with CPB natural control than Bt toxins. In a study with transgenic potatoes expressing a rice cystatin I (OC1) gene, we found that this approach is complex with versatile pests such as CPB, who can compensate PI effects despite relying on target proteases for dietary protein digestion. When evaluated for non-target effects, OC1-potatoes showed no deleterious prey-mediated effects on *P. bioculatus*, and were highly suitable food to the potato aphid and its parasitoid. The hyphomycete *V. lecanii* is a mycoparasite with biocontrol potential against aphid pests and pathogenic fungi based on versatile antibiotic plus hydrolytic enzyme antagonism, in addition to showing potential for eliciting plant cellular defenses against pathogens. Potential interactions between *V. lecanii* and transgenic insect resistance based on *B. thuringiensis* toxins or PIs remain to be documented. To summarize, our studies indicate that few generalizations are available concerning direct and indirect effects of transgenic plant resistance on non target organisms associating with potato crops in absence of chemical insecticides, suggesting the need for a case-by-case approach to reveal significant interactions.

Workshop
Ethics, Legal and Regulatory Concerns of Transgenic Plants

Development of international scientific biosafety testing guidelines for transgenic plants

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The GMO public perception in Brazil

The interest in alternative strategies for controlling crop pests has been growing considerably in the world in response to the environmental and public health problems caused by the use of agrochemicals in crop protection. As a result of this raised public awareness, in Brazil, the public requests reliable information regarding the commercial release, environmental consequences and food safety of genetically modified organisms (GMO). Consequently, the development of methods for assessing the effects of GMOs has increased in importance.

The general public opinion is divided into those that favor the release of GMOs because they expect benefits from the possible reduction of food costs, increased yields and, hence, higher profits for the farmers, and those opposing the release of GMOs because of potential risks associated with these releases and the lack of well established safety procedures for the environment and public health. Although the Brazilian government recognizes the difficulties involved, they promote the use and labeling of transgenic products.

As indicated in the Cartagena Protocol on Biosafety of Living Modified Organisms (Biosafety Protocol) under the Convention on Biodiversity (CBD) and many other international forums, there is a clear need for comprehensive, transparent, scientific guidelines for meaningful pre-release testing and post-release monitoring of transgenic plants to ensure their environmental safety and sustainable use. The lack of such guidelines globally and the need for such guidelines in developing countries has been repeatedly expressed by both the private and public sector. Chapter 16 of Agenda 21 recognizes that the maximum benefits of genetically modified crops can be achieved only if appropriate guidelines for their biosafety are in place and the relevant capacities to implement the guidelines are acquired.

The same needs are recognized by Brazilian Ministries of Agriculture, of Environment and of Science and Technology: to establish protocols concerned with economic aspects, public health, the conservation of biodiversity, scientific innovation, implicit technological dependencies and the vagaries of the import/export market.

In view of this, the Brazilian Agriculture Research Corporation (Embrapa) understood the importance of participating in a national and an international project to discuss and develop appropriate methods for the evaluation of potential environmental effects for the most relevant GMOs products that Embrapa is developing.

Because of Embrapa´s position, the need to strengthen risk assessments in Brazil, and the need to characterize and detect environmental impacts of Embrapa´s GMOs that are presently under development, a group of Embrapa researchers decided to participate in an international initiative called the "GMO Guidelines Project". It is an international initiative of public sector scientists organized within a global working group on 'Transgenic Organisms in Integrated Pest Management and Biological Control' under the umbrella of the International Organization of Biological Control (IOBC).

The GMO Guidelines Project

The GMO Guidelines Project (GGP) will develop scientific principles and detailed international scientific guidelines for biosafety testing of transgenic plants.

The key elements of this GGP are

An international initiative including expert scientists from leading research institutions from Europe, Australia, China, USA, East Africa, South America, and Southeast Asia.

Coordination of the development and implementation of biosafety testing guidelines as a progressive, dynamic process, which will include scientific and technical capacity building and communication among scientists and between scientists and policy makers Swift, serial publication of sections of the guidelines as they are completed Rapid and timely revision of previously published sections should new scientific biosafety issues arise.

The GGP can be envisaged as a set of interlinked modules consisting of scientific questions related to risk assessment and corresponding scientific methodologies to answer those questions. The guidelines will have no regulatory legitimacy themselves, but regulatory authorities can choose to implement parts or all of the guidelines as they desire or need, with confidence in the scientific soundness behind the evaluations that follow the recommended methodologies. The guidelines will be designed for use on a case-by-case basis, as specified in the Biosafety Protocol and the EU Directive on release of GM plants. They will cover the environmental and agricultural impacts of GMOs, but they will not include the scientific expertise to evaluate human health impacts or ethical implications.

The GGP recognizes that a strong science base may be easier to transfer among countries than a regulatory system, consequently, the project focuses capacity building efforts on a few countries with reasonably developed scientific infrastructures. By strengthening the scientific capacities for risk assessment in these countries, it is expected that the necessary expertise can diffuse more readily to neighboring countries.

Project participants are either members of the core group or the project advisory board. The core group is divided into three regional groups and five scientific sections. The project is coordinated by the steering committee.

The core group is responsible for the development of the Guidelines that will be revised at regional workshops. The core group will be comprised of all IOBC Working Group members who express an interest and actively participate, and external resource people invited to complement group expertise.

The advisory board is invited to review periodically and provide advice on improving the products and process of the project. It is comprised of representatives from international and national organizations who have the scientific expertise and who can influence the international

adoption of the guidelines. All members will be kept informed of the activities of the project via periodic communications and there will be one advisory board meeting. The scientific scope of the Guidelines has been divided into five scientific sections as follows:

Needs analysis / good agricultural practice

This section will provide a framework for evaluating the need for the transgenic plant in specific crop production contexts. This includes providing an approach to evaluating projected changes in crop production practices, such as tillage systems or insecticide use. It will incorporate a precautionary approach to the issues as specified by the Biosafety Protocol and EU legislation.

Characterization of transgene construct and phenotype in the plant

This section will specify how a transgene should be described to enable evaluation of its stability and inheritance, and how the phenotypic effects of the transgene in the plant should be described; what, how, what plant parts, and when product concentrations should be measured in transgenic plants to facilitate assessment and management of environmental effects.

Non-target effects

This section will specify:

scientific procedures to determine the non-target species or function/process that should be tested (=selection procedure) scientific procedures for testing these species/functions/processes (=testing procedure) for the following categories of organisms: natural enemies; pollinators; soil organisms; species of conservation concern; species of cultural significance; non-target pests; other non-target species

Routes of exposure need to be identified. The organisms that are exposed need to be determined through suspected causal chains of impact. Based on this information, protocols and methodologies for appropriate testing can then be developed.

Pest resistance management

This section will specify procedures to determine the resistance risk of transgenic crops, and feasible management responses needed to reduce this risk. It will also consider approaches for developing a practical monitoring and response system to detect resistance and to adapt management appropriately. While this is primarily addressing resistance development in insect pests, resistance development of weeds as a result of commercial planting of transgenic herbicide-tolerant crops will also be considered.

Gene flow and its effects

Gene flow is the route along which transgenes can spread genetically into populations of related species and geographically into other regions including protected areas of sensitive ecological value. Gene flow is considered a risk because of the great uncertainties associated with the possible consequences in the recipient ecosystems. Successful transgene flow will simultaneously affect both recipient plants and their associated organisms.

This section will determine protocols for establishing:

- the likelihood of intra- and interspecific gene flow
- the possibility of geographic and genetic spread of transgenes

- the potential ecological effects resulting from gene flow
- the effectiveness of sterility mechanisms, their breakdown and management

All public sector scientists are welcome to participate in the core group. Other interested people are invited to register themselves on the mailing list of the project. An application form can filled in on the project website {HOPEFULLY WE WILL HAVE AN ADDRESS SOON}, or be printed sent by email or air mail. You can also contact the project secretariat for application details (Evelyn Underwood <underwood@geobot.umnw.ethz.ch>).

Current Situation of Transgenic Organisms in Brazil

In Brazil more than 700applications for field trials have been approved through 2001. The vast majority of these field trials were carried out with corn (80% of these for demonstrational purposes). Among the transgenic traits examined, the dominant ones were for resistance to herbicides followed by insect resistance (*Bt*-genes). The three main plant-trait combinations used in the field release trials were herbicide-resistant corn and soybean and insect-resistant corn. Field trials also included more rare plant-trait combinations such as Bt-soybean (insect-resistant) , herbicide-resistant Eucalyptus, Bt-sugarcane (insect-resistant), herbicide-resistant rice , virus-resistant potato and a number of insect-resistant corn cultivars whose traits were confidential.

The commercial production of Round-Up Ready soybean (belonging to Monsanto) was approved February 2002, by the Federal District Court. The approval, a six-hour oral presentation , was based on technical data presented to National Biosafety Technical Committee (CTNBio). It is important to mention that this matter has been under justice discussion since 1998.

For more information (in portuguese).about CTNBio, visit http://www.ctnbio.gov.br/ctnbio/default.htm For more information on the project go to http://www.gmo-guidelines.info

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A. Hilbeck, (Switzerland) – Project co-coordination and management

D. Andow, USA) – Project co-coordination

N. Birch, Scottish Crop Research Institute, Dundee (UK) – Coordinator of Scientific Section Gene Flow

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J. Songa, Kenyan Agricultural Research Institute, Machakos (Kenya) – Co-coordinator of Regional Group Africa. F-H Wan, Chinese Academy of Agricultural Sciences, Beijing (China)

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Considerations for research in agricultural biotechnology

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Recent controversial articles on agricultural biotechnology published in major journals have caused great consternation within the scientific community, and have led to a decline in public confidence about the use of agricultural biotechnology, and perhaps science in general. Controversies in science can be healthy if they lead to questions which can be investigated, thereby advancing knowledge. However, some of the recent controversies have also involved questions about the integrity of scientific conduct, policies of scientific journal publication, and the proper role of the media in educating the general public about the important issues involving agricultural biotechnology.

As scientists we play a central role in determining the future of agricultural biotechnology. We select the areas to be investigated based on our intellectual curiosity, position responsibility and available resources. Because agricultural biotechnology is a "hot topic", it has attracted considerable attention by regulatory agencies, funding agencies, the general public and scientists. Scientists are interested in this area for a multitude of personal or professional reasons. Science is done by people, each with his and her own prejudices and view of the world, and this can affect how experiments are performed and reported. Furthermore, because many of us depend on competitive funding and recognition to advance our careers, some may also feel an unhealthy pressure to publish their work before it is solid. While perhaps the best science is done by people with passion, that passion must be balanced with ethical behaviors not injurious to science in the long run.

Besides the personal and professional reason(s) for selecting a particular aspect of science (such as biotechnology) to work on, how the science is performed is fundamental to the project's eventual outcome. Two examples of recent controversies in agricultural biotechnology involve corn engineered to produce proteins from the bacterium, *Bacillus thuringiensis*, for insect control. While much has been written about the motivation of the authors for selecting these projects (Knight 2000, Anon. 2002), the central consideration should be about whether the science was conducted soundly and reported accurately in the scientific and lay press. I believe these two examples failed in these areas and have damaged the credibility of science and, particularly, the credibility of agricultural biotechnology. The first example is what can only be considered preliminary findings on the effect of monarch butterfly larvae to be negatively impacted when they consumed milkweed on which pollen from Bt corn had been applied (Losey et al. 1999). This study was strongly criticized in the scientific community because of its poor quality including the unspecified dose of pollen used and the unspecified endotoxin concentration in the pollen, the lack of a choice test, the use of inappropriate controls and the lack of information on the potential for a temporal and spatial overlap of pollen shed, milkweed plants and monarchs under natural field conditions (Hodgson 1999, Shelton and Roush 1999, Shelton and Sears 2001). However, for those opposed to agricultural biotechnology, this study became a rallying cry, not justified by the science, but by their personal feelings about agricultural biotechnology. More detailed laboratory studies and a series of field studies have shown the risk to monarch butterfly populations in the field is "negligible" (Sears et al. 2001).

In 2001, another highly controversial paper was published in the same journal (*Nature*) as the monarch story. In this paper the authors claimed that native Mexican corn had become contaminated by DNA from genetically-modified varieties that were banned in Mexico (Quist

and Chapela 2001). This paper caused a furor in the scientific community because of the techniques used by the researchers. Once it was published in *Nature*, the editorial board of the journal *Transgenic Research* stated that "no credible scientific evidence is presented to support claims that transgenic DNA was introgressed into traditional maize landraces in Oaxaca, Mexico" (Christou 2002). Furthermore, the board argued that an analysis of the procedures used "demonstrate that the data presented in the published article are mere artifacts resulting from poor experimental design and practices." These concerns led to *Nature* publishing a statement concluding that the evidence available was not sufficient to justify the publication of the original paper. A "retraction" of a paper about such a highly controversial topic is very unusual, and begs the question of why more rigor and caution was not exercised prior to publication (and the same question should also be asked about the monarch butterfly paper). While it is clear that no conclusions can be drawn from the *Nature* article on Mexican corn, this does not mean that transgenes will not be detected in Mexican landraces in the future or what those consequences might be. The retraction meant that the experimental protocol used by the researchers lacked the rigor to back up their claims, but it is impossible to "un-ring a bell". Greenpeace used the article to petition FAO for a global ban of the production of all transgenic plants (Piña 2002).

The impact of conducting flawed experiments in agricultural biotechnology can be tremendous and lead to professional discredit within the scientific community. On the other hand, it may lead those who are against this technology to see the authors of the flawed work as being persecuted by the scientific community. High profile journals such as *Nature* have lost some credibility for publishing flawed articles, but they have also gained some level of notoriety. There are about 2,000 academic journals of consequence, and most of them are published by non-profit organizations. However, *Nature* and *Science*, two of the most prestigious journals, are published commercially, supported by advertising (Knight 2000). Whether for good or bad, *Nature* now has more "name recognition" than it had previously. But publication of any flawed article, whether in a journal with relatively high or low "impact' has even further consequences. It presents difficulties to regulatory agencies charged with making decisions for the public good. Furthermore, it entangles the scientific literature with faulty information. Even articles that are retracted continue to be cited frequently in the scientific literature (Campanario 2000), as well as in the public memory.

News reports involving biological sciences are more common than ever and the public quickly hears about scientific controversies. Controversies about the monarch and the potential for spread of Bt pollen have been highlighted in the media and have influenced public opinion and may influence public policy. As noted by Abbott (2001) the reporting of biotechnology issues has changed markedly since 1997 and "moved from being a scientific issue to being a social issue". The media coverage has exploded and the sources of information have changed. In late 1999 the New York Times was running "almost one article per day on this (biotech) topic" (Abbott 2001).

In a world made smaller by our modern communication methods, more care is needed since misinformation or partial information can easily influence public policy (Shelton and Roush 2000). When working in agricultural biotechnology, scientists need to be thorough in conducting their experiments and deliberate when reporting on them. What has become frustrating to many scientists is that the increased publicity about agricultural biotechnology is often due to sensational stories that are flawed scientifically, such as the original stories of the monarch butterfly and the Mexican corn. Furthermore, scientists may feel they are left out of the discussion on these controversies and missing the opportunity to correct scientific misrepresentations. As noted by Abbott (2001) in the media coverage of GMOs in England

and the US from 1997-2000, the *New York Times* and the *Times of London* were using scientists less and less as sources for stories and by Sept. 2000 only 12% of the news stories quoted scientists. In contrast, Abbott (2001) stated that environmental activist groups such as Greenpeace, the Environmental Defense Fund and the Union of Concerned Scientists were used increasingly as sources of news and that the newspapers noted were more than twice as likely to use a quote from one of these sources as compared to scientists.

In agricultural biotechnology there are roles and responsibilities of scientists, scientific journals, the public media, public agencies and those who oppose or advocate a specific technology, and serious consequences for science in general when those roles and responsibilities go awry. Scientists may feel the pressure of competition, especially in an academic setting. Personal views may continue to decide which issues one will work on, but the quality of science must back up those personal opinions. Common sense tells us that scientific inquiry and the publication and reporting of results to the scientific community and general population need to be performed with high standards of ethical behavior, regardless of one's personal perspective on agricultural biotechnology.

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Workshop
Preservation of Entomopathogenic Fungi

Workshop in methods for the preservation of fungal cultures

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Nearly every laboratory that deals with fungal pathogens affecting insects or other vertebrates must also deal with finding some appropriate means to preserve the cultures of the fungi being studied. There is such a diversity of techniques and variants of techniques that might be used that this workshop was proposed to help try to familiarize participants with some of the techniques being used in various laboratories, and to provide visual guidance about how these techniques are applied.

It is hoped—indeed, expected—that participants in this workshop will come prepared to share information about how they preserve fungal cultures in their own laboratories and about any problems or questions that make the job of preserving cultures more difficult for them.

The major techniques to be covered during this workshop were already discussed, and step-by-step instructions given in Humber (1997). Despite the protocols being written down in this manner, however, it must be recognized that the minor modifications in these methods made in many laboratories represent undocumented variants that deserve recognition and documentation because they may be well adapted to the resources, capabilities, and needs of users who do not know about them. Another value in is presenting these variations in established techniques is to dispel any idea that the methods are inflexible and bound to fail unless they are reproduced exactly.

Many fungal entomopathogens are no more difficult to isolate or to grow in axenic culture than any more 'routine' saprobic fungus or many plant pathogenic fungi. These 'easy' entomopathogens are mostly the hyphomycete conidial stages (e.g., species of *Beauveria*, *Metarhizium*, *Paecilomyces*, or *Aschersonia*) of clavicipitaceous ascomycetes (e.g., species of *Cordyceps*, *Torrubiella*, or *Hypocrella*). Not only are these fungi easy to isolate in pure cultures and to grow on an extraordinarily wide range of media that are familiar to any mycology or microbiology laboratory, but they are also extremely easy to preserve by a wide range of techniques with excellent retention of viability and other key phenotypic characters such as the ability to sporulate, virulence, and pathogenicity. For these fungi, the choice of method to use for long-term preservation really can be chosen as a matter of convenience covering the entire range of possibilities from serial transfer to cryogenic preservation under liquid nitrogen.

Many especially fastidious pathogens require increasingly complex and specific methods—particularly on cryogenic approaches, often with a require-ment for relatively controlled initial freezing conditions—for their successful preservation as discussed by Humber (1994, 1997). Nutritional or physiological fastidiousness in the adjustment of a fungus to its invertebrate host, however, may not be the only or most critical factor requiring dependence on cryogenic

preservation techniques. This is one instance where the actual morphology of the fungus to be preserved may require dependence on a much more limited range of preservation options. Fungi whose cells contain high vacuolar volumes are not suitable for lyophilization or any other approach (such as silica gel) that preserves primarily by desiccation; these fungi are best preserved either in distilled water stasis or by one of the cryogenic approaches that leaves the water in place in the cells but depends on the use of a cryoprotectant to prevent the formation or growth of ice crystals. Fungi needing this gentler, fully hydrated approach to preservation include the Entomophthorales and watermolds—either the Chytridiomycetes or those organisms formerly classified as fungi in the Oomycetes but that are now recognized not to be true fungi and are placed in the Kingdom Chromista (= Straminipila); all chromistans produce tinsel-type flagella coated with hairs (mastigonemes).

Ascomycetes and their hyphomycete conidial states generally have cells whose diameter is comparatively narrower than those of the Entomophthorales or watermolds and in which the cytoplasm occupies the great majority of the total cellular volume. Most culture collections routinely preserve these fungi by lyophilization (freeze-drying). Freeze-drying requires equipment whose expense is beyond the capacities of many smaller research collections; these collections might depend on preservation on silica gel crystals or as frozen mats of hyphae on filter paper, but these fungi can also be stored very easily by almost any other convenient technique including cryopreservation (especially in electric freezers operating at -80 deg C).

A CD-ROM incorporating the image files illustrating the range of storage techniques to be discussed along with a range of other documents about the isolation, maintenance, and preservation of fungi will be available for a nominal cost (still to be determined); the SIP Division on Fungi, as the sponsor of this workshop, will receive all proceeds from sales of this disk.

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Workshop
Microbiol Control of the Coffee Berry Borer by Entomopathogens Fungi

Microbial control of the coffee berry borer in Colombia

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Summary

In Colombia, Cenicafé has been conducting research on the entomopathogenic fungus *Beauveria bassiana* to control the coffee berry borer since 1988. Additionally, research on other entomopathogenic fungi recorded as natural enemies have been undertaken. Cenicafé has an extensive fungal entomopathogen collection including seven species of entomopathogenic fungi isolated from the coffee berry borer CBB. Among these fungi, *Beauveria bassiana* has been extensively studied at Cenicafe. *B. bassiana* production has been scaled-up using a low input methodology and an industrial system that has resulted in the production of spores that have been used to conduct pathogenicity bioassays, quality control of formulation, field application and *B. bassiana* DNA characterization. Encouraged by these results and the technical support given by Cenicafé, private companies have made investments in the development of *B. bassiana* mass production system, formulations and spraying equipment. The coffee growers have gained an increased understanding of the mode of action of *B. bassiana* against the coffee berry borer and they are trying to use it according to their incomes after becoming convinced of the advantages in using biological control agents.

Key words: *Hypothenemus hampei*. Biological control. Entomopathogenic fungus. Production. Formulation. Application.

Introduction

In Colombia, Cenicafé has developed a research project on entomopathogenic fungi to control the coffee berry borer (CBB) since this pest reached the country in 1988. Studies started when *Beauveria bassiana* (Balsamo) Vuillemin was first reported attaching CBB in the Colombian border with Ecuador.

At that time, in Colombia there were no installed *B. bassiana* production capabilities and for this reason the National Coffee Growers Federation of Colombia through Cenicafé began its investigations on *B. bassiana* mass production using industrial and low input production systems Cenicafé has encouraged the production of *B. bassiana* by releasing disseminating research results and giving technical assistance to entomopathogenic fungi producers.

It is believed that *B. bassiana* can play an important role as a biological control agent against the CBB because it is found attacking the insect as soon it invades new coffee plantations in different localities of the Colombian coffee region.

Entomopathogenic fungi that attack CBB in Colombia

Since CBB arrived to Colombia, 22 natural enemies attacking adults have been recorded, including seven species of entomopathogenic fungi that were recorded attacking CBB on berries. Cenicafé has a fungal entomopathogen collection consisting of 202 isolates. Among these isolates, *Fusarium* species must be considered with low potential because *Fusarium* group has strains that are highly virulent as plant pathogens and therefore use of this fungus against insects is very risky (Table 1).

Today, both *B. bassiana* and *Metarhizium anisopliae* (Metschnikoff) Sorokin are mass produced to carry out laboratory, field experimentation and application across the coffee growing region to control CBB on berries in trees and on the ground, respectively. The other fungi have been recorded as natural enemies or used to evaluate their pathogenicity in the laboratory.

Laboratory and field trials using entomological sleeves have shown that isolates of *M. anisopliae* caused mortalities of over 80%. However, applications directed to CBB found on the ground resulted in low mortality levels. Even though *M. anisopliae* is considered to be an effective entomopathogenic fungus to control insect pests in the soil, *B. bassiana* exhibited a better performance when used to control CBB on the ground.

TABLE 1. Species of entomopathogenic fungus reported attacking CBB in Colombia.

Fungus	First record	Number of isolates
Beauveria bassiana	1989	137
Beauveria brongniartii	1993	4
Metarhizium anisopliae	1993	40
Hirsutella eleutheratorum	1993	0
Paecilomyces lilacinus	1998	4
Fusarium oxysporum	1996	1
Fusarium solani	2002	1

Isolates of *B. bassiana* from the Colombian coffee region

Cenicafé's *B. bassiana* collection consists of 137 isolates obtained from 47 species of insects and from 48 different localities. There are 45 isolates from CBB collected in different places. Even though they were isolated from the same host, they exhibit different field behavior such as increased sporulation in coffee grown under sun or a high CBB mortality when the insect is inside berries that have fallen on the ground.

These isolates have been an important resource of material to Cenicafé's research program, and especially in the identification of isolates with high virulence that can be used to undertake mass production, formulation, and field application studies. Additionally, they have been used to develop quality control methodology, DNA characterization, coffee growers educational programs, extension programs on integrated coffee berry borer management (ICBBM) and in the near future, genetic transformation to improve field performance against CBB.

Mass production of entomopathogenic fungi in Colombia

The mass production of entomopathogenic fungi in Colombia rose sharply from 0.5 ton in 1992 to 140 ton in 1995 and decreased afterwards. The main production was obtained using the low input methodology of culturing the fungus on rice. The average spore concentration obtained was $4x10^8$ spores / gram. This production allowed the Colombian coffee growers to introduce *B. bassiana* as inoculum in the field and was complemented with harvesting of ripe and infested berries. These control practices keep the CBB population under check and the coffee growers can sell their coffee without penalty.

Developments in low input production of *B. a bassiana* in Colombia

The aim of this methodology was to educate the farmers so they could carry out the *B. bassiana* production in their own farms. This would overcome many of the problems encountered in *B. bassiana* formulations, such as maintaining spore viability and pathogenicity. This production system was made widespread in Colombia through teaching courses aimed at the farmers and field extension workers in which they learned the procedure and got the initial inoculum to start their production with the understanding that they would teach other coffee growers in their neighborhood.

Farmers were taught how to sterilize the rice to be used as substrate in which to culture the fungus. The main contribution to this methodology was made by the growers who changed the original pressure cooker in which only few bottles were sterilized to a large kitchen pot in which the rice media was sterilized using a water bath and in which over 200 bottles were sterilized simultaneously. Coffee growers also learned how to obtain the inoculum from the field; the aim of this objective was to avoid them having to depend on a central laboratory for supplies of the inoculum. In the field, they found the *B. bassiana* inoculum source by picking the infested berries that showed a white spot over them. Additionally they were able to produce the inoculum using CBB adults gathered from dry berries and to infect them with a suspension obtained from their own fungus cultures. Using this methodology, Cenicafé built a pilot plant for the production of the inoculum denominated "Cepa Cenicafé". This material was used to support the *B. bassiana* production either using an industrial or low input procedure in the growers farms.

When this methodology was released, it was used by many growers. Some of them created their own family enterprise and sell the fungus to the farmers that prefer to buy the fungus rather than producing it themselves. Even though the farmers showed high skills in producing a high quality fungus they suddenly started claiming that they were seeking for a more efficient and less time consuming way to control CBB.

It is important to consider that at the beginning of this project, the Colombian coffee growers did not have the appropriate knowledge to manage insect pests. They lacked the skills needed to follow a program in which they were responsible for monitoring pest populations, assessing damage, followed by undertaking pest management practices. They also had weaknesses in using chemical pesticides, including the selection of active ingredients, understanding their toxicity and their efficacy against CBB, the appropriate timing of the application and knowledge of the side effect on non-target organisms, especially biological agents and their own families which live in the treated farm.

Developments in industrial production of *B. bassiana* in Colombia

Several industrial laboratories in Colombia involved in to the production of livestock medicines have taken advantage of their installations, know-how and marketing strategies to carry out the production of *B. bassiana*. In the same way, pesticide companies such as Agrevo have built installations to produce the fungus. The companies have produced several *B. bassiana* products with different formulations and properties (Table 2) that in some cases make it difficult to apply the product with the current spray equipments that are used in the steep slopes of Colombian coffee plantations.

In Colombia, over 11 enterprises have launched more than 16 *B. bassiana* formulations and some of these products have been marketed in Central and South America for CBB and other insect pests.

TABLA 2. *B. bassiana* formulations marketed in Colombia for coffee berry borer control.

Product Name	Formulation	Concentration (spores per gram)	Manufacturer	Address
Cebiopest Matabroca	Rice + spores (dried) Rice + spores	1,2 x10 ^{10} 7,9 x10 ^{8}	F:M:O F:M:O	Fundación Centro de Biotecnología. Bogota, Colombia. Tel. 287 86 11
Brocaril Brocaril	WP (sterile micro-talc) Lactose- peptone (Freeze-dried product)	6,1 x10 ^{9} 4,3 x10 ^{10}	Laverlam Laverlam	Laverlam. Cali, Colombia. Tel. 4474411. AA. 4524
Bassianil	WP (sterile microtalc)	1,0 x10 ^{9}	Biocontrol	Biocontrol - Palmira, Colombia Tel: (922)661340
Conidia Conidia	WP WG (diatomaceous earth)	8,2 x10 ^{9} 5,2 x10 ^{10}	Agrevo Agrevo	Agrevo. Bogotá. Colombia Tel. (91)2576818
Cepa Cenicafé Cenicafé pilot plant Carrie +kerosene	Rice + spores Powder (spores) Bb Oil based formulation	3,0 x10 ^{9} 5,0 x10 ^{10} – 1,0 x10 ^{11} 2,0 x10 ^{11}	Cenicafé Cenicafé Cenicafé	Cenicafé. Manizales, Colombia. Tel. (96)8506550. .AA 2427.
Matabroca	WP (filtrated earth)	2,5 x10 ^{10}	Vecol	Vecol. - Bogota. Colombia. Tel 2633100
Micosplag	WP	1,0x10 ^{8}	Orius	Orius Biotecnologia Villavicencio, Colombia Tel: (098) 6632595.
Agobiocontrol	WP	1,4x10 ^{8}	Onatec	Onatec - Bogotá, Colombia. Tel. 6212301
Biogarden *Bassiana* AC	WP	2x1010	Biogarden Control Biológico	Biogarden Control Biológico. Bogotá, Colombia Tel. 2521474. AA 50824
Sporen 21	WP	2x10^{9}	Bioenlace 21 S. A	Bioenlace 21 S. A. Popayán, Colombia. Telefax: 8392121
Biogarden B. *bassiana*	WP	2x1010	Biogarden	Biogarden LTDA. Bogota, Calle 70 N° 81-36

The companies had problems maintaining the quality control parameters such as viability, pathogenicity and concentration. There was also a low demand for *B. bassiana* formulations by the growers, specially during years 1997 to 1999 when there were heavy rainfalls (The Niña phenomenon) and the *B. bassiana* field inoculum was enough to keep under check the CBB population complemented with the sanitary harvest.

Quality control of commercial *B. bassiana* formulations

Due to Cenicafé´s mission of giving advice to the coffee growers about the more active, less expensive and biologically friendly products, the research center developed methodologies and quality control programs to evaluate the *B. bassiana* industrial formulations. In Colombia all *B. bassiana* formulations marketed for CBB control are tested for quality control at Cenicafé. Formulations are compared against "Cepa Cenicafé" on germination, pathogenicity, concentration, purity, moisture content, particle size, spore suspension, and a spraying test. Higher mortality results are considered an improvement of the formulation and are expected

to exhibit a better performance against CBB. Cenicafé´s results are sent to the companies with the aim of creating a feedback and they have allowed to improve the commercial formulations.

Field evaluation of *B. bassiana* against the coffee berry borer

Formulations

Cenicafé has carried out research using different *B. bassiana* formulations. One of them uses a simple procedure that consists of washing-off the cooked rice media culture after adding oils and water to get the spores in suspension and its application to the coffee plantation. The most complex formulation adds different compounds to the spores at the factory, thus allowing to market the product as a powder or dispersal granule that can be mixed with water in the tank. One example of this product is "Conidia". One experimental oil based formulation was developed for low volume application applied using an ultra low volume sprayer (Motax sprayer). In field evaluations this formulation resulted in 64% CBB mortality. The main constraint in using this product t is the cost of the oils and the Motax equipment. This research project determined that in order to find higher efficacy of *B. bassiana* CBB under field conditions, it is necessary to increase spore concentration in culture media to formulate products with higher spore concentration per gram and to use pieces of equipment and spray technology that allow an adequate droplet coverage / cm^2, in addition to timing the application according to CBB behavior.

Formulations to be applied on the ground to control CBB there are currently not available. Applications have been carried out using a knapsack to spray the spore suspension. Some attempts have been undertaken using ground rice culture containing *B. bassiana*, or just the unground rice cultures plus spores. Application directed to the ground is recommended when the coffee plantations are renewed by pruning the trees or replanting. When this is done, many berries fall on the ground and the CBB may increase in numbers. In the following coffee production cycle, plantations near the renewed plots can have high infestation levels. In this case, the application of a fungal spore formulation or suspension to the ground prior to the pruning can keep the CBB population under control. The research carried out on *B. bassiana* has shown that it has a better performance over CBB found either in the tree or on the ground. *B. bassiana* can be considered as the best natural enemy and a good biological agent to control CBB.

Spray technology

The main areas of coffee plantations in Colombia are located in steep slopes, where the more suitable spray equipment are the knapsack, knapsack mistblowers, and the stationary sprayer. These equipment use high water volumes, requiring high labor, and the droplet coverage is uneven and low. For this reason Cenicafé has carried out research on application technology in cooperation with Micron Sprayers Ltd. (Herefordshire, UK). This program started when coffee leaf rust was detected in Colombia in 1983 and resulted in the development of the low volume sprayer "Motax". Even though it has a good performance to apply *B. bassiana*, its big constraint is the high cost making it unaffordable for the coffee growers.

Field evaluation of *B. bassiana* efficacy

It is well known that the mortality obtained using entomopathogenic fungi under laboratory bioassays is usually superior to that obtained in the field. The data analyses have allowed for the identification of bias in the evaluation of CBB mortality caused by *B. bassiana*. For instance, the fungus efficacy is evaluated only by counting the CBB covered by mycelium and spores, as a white spot over the coffee berry. In this case, the fungus growth depends on the field

environmental conditions. Also the *B. bassiana* performance is underestimated when the coffee berries are dissected because some CBB are found dead but do not exhibit fungal growth. To evaluate the real contribution of *B. bassiana* to control CBB, the field evaluations are focussed on managing coffee commercial plots treated with the following treatments: (1) *B. bassiana* and harvest; (2) insecticides and harvest; and (3) harvest only. The results showed no statistical and economical differences between treatments. In all treatments the coffee was sold without penalty.

More recently, in other experiments carried out as participatory research with smallholders managing commercial plots where treatments were *B. bassiana* and harvest versus harvest and complementary integrated pest control practices without *B. bassiana,* the results showed that the CBB infestation, on average, was less than 5% in the field. Persistence of *B. bassiana* in field conditions has been demonstrated with the appearance of the fungus attacking CBB years before first applications. These results encourage the growers to produce or buy the fungus and apply it in their farms to reinforce the natural inoculum.

Strategies for using *B. bassiana* to control the coffee berry borer

Field performance of *B. bassiana* differs from one farm or field to another and it is affected by formulation characteristics, quality of spray application, and adverse weather conditions. In addition, farmer expectations are based on results obtained with chemical insecticides, and thus, they expect similar results when using *B. bassiana*, which makes its acceptance difficult. Therefore, *B. bassiana* research must consider other strategies different to the biopesticide concept and focus in strategies that sound more friendly and ecological according with the coffee producer incomes, skills and knowledge to manage insect pests.

Cenicafé has conducted research on different ways of using *B. bassiana* such as an inoculum introduction strategy with favorable ecological and sustainable consequences. This inoculum could increase CBB mortality in the field. In the Colombian coffee regions, the natural mortality caused by *B. bassiana* in field has been estimated at around 50%. However, in Colombia the predominant method used for the production and application of *B. bassiana* is focused on using traditional spray technology and the augmentative - inundative biological strategy aimed at obtaining the same results as with chemical insecticides. Cenicafé is also conducting research on the genetic transformation of *B. bassiana* isolates (in co-operation with Cornell University) and in the near future will start a collaborative project with the Insect Biocontrol Laboratory (USDA, ARS) to test *Beauveria* as an endophytic fungus with the objective of developing systemically protected coffee plants.

Non-target B. bassiana effects in the coffee growing region

The non-target effects of B. bassiana on non-target insects have been evaluated using CBB parasitoids (Cephalonomia stephanoderis Betrem and Prorops nasuta Waterston; Hymenoptera: Bethylidae) in laboratory and field conditions. For C. stephanoderis, B. bassiana infection reached 24% when the application was carried out the same day as the wasps were released. For P. nasuta the highest infection caused by B. bassiana was over 80%.

The application of *B. bassiana* on mulberry leaves (*Morus alba* L.) used to feed the silk worm *Bombyx mori* (Lepidoptera: Bombycidae), showed that leaves disinfected prior to feeding the caterpillars or exposed to the sun seemed to be enough to inactivate *B. bassiana* without reduction of silk production.

In the field, one specimen of *Apis mellifera* L. (Apidae) was recorded as infected with *B. bassiana* in a pineapple crop where *B. bassiana* was applied against *Metamasius hemipterus* Oliver (Coleoptera: Curculionidae). In coffee plantations, *Cyanea surinana* var. cyanea F (Vespidae), Xylocopa sp (Apidae), *Ectatomma* sp., and *Crematogaster* sp. (Formicidae) have been recorded as *B. bassiana* hosts. These reports were based on one specimen and never reached epizootic status.

Application of *B. bassiana* mass production methodology to other entomopathogenic fungi in Colombia

The mass production and research developed in the nineties by Cenicafé stimulated the entomopathogen production and in only four years it increased from 0.5 to 140 ton culture in rice. This mass production technology was applied to other entomopathogenic fungus to be used against other organisms that attack coffee or other crops in Colombia (Table 3).

TABLE 3. Fungi produced and formulated to control other organisms in coffee or other crops in Colombia using Cenicafé support.

Fungus	Plant	Target organism
Beauveria bassiana	coffee	*Meloidogyne* spp, *Phyllophaga* sp. *Anomala* sp.
	grass	*Collaria columbiensis* C
	forest	*Cargolia arana* Dognin, *Glena bisulca* Rindge
	apple	*Compsus* spp.
	citrus	*Compus* sp
	avocado	*Heilipus lauri* Boheman, *Stenoma catenifer* W.
	plantain	*Metamasius hemipterus* O., *Cosmopolites sordidus* (Germar)
	pineapple	*Metamasius hemipterus* O.
	cotton	*Anthonomus grandis*
	bean	*Phyllophaga* sp., *Cyclocephala* sp.
	corn	*Phyllophaga* sp., *Cyclocephala* sp.
Metarhizium anisopliae	coffee	*Meloidogyne* spp.
Paecilomyces lilacinus	coffee	*Meloidogyne* spp.
Verticillium chlamydosporium	coffee	*Meloidogyne* spp.
Trichoderma koningii	coffee	*Rosellinia bunodes*

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Microbiological control of the coffee berry borer in Brazil

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The coffee berry borer *Hypothenemus hampei* (CBB) is a worldwide key pest of coffee. It is considered very important because it directly attacks the coffee berries causing severe losses. Control of this pest is mostly achieved by the use of synthetic insecticides (endosulfan). The use of this simplistic control strategy may lead to development of resistant populations, may eliminate natural enemies of several other insect pests, and promote environmental pollution as well as contamination of food and farmers. Among the different biological control agents of CBB, the entomopathogenic fungus *Beauveria bassiana* has shown a high potential. Not only in conventional cropping systems but mainly in organic production systems as well, where together with some species of parasitoids, it is one of the few available alternatives for the control of this pest. The first report on the occurrence of entomopathogenic fungi affecting CBB in Brazil is dated from 1930, in the State of Sao Paulo (Neiva and Averna-Saccá 1930). The fungus was initially described as *Botrytis stephnoderes* (*Beauveria bassiana*). Mesquita (1944) also reported the occurrence of this entomopathogenic fungus on coffee plantations in the State of Rio de Janeiro. After the introduction of organochlorine insecticides, such as BHC, for the control of CBB, no more citations on studies or on the occurrence of *B. bassiana* were found in the literature for a long period. In 1984, Villacorta reported the occurrence of the fungus parasitizing CBB adults on coffee plantations on the coastal and north regions of the State of Paraná. At the same period, Carneiro Filho *et al.* (1984) carried out laboratory and field studies on the control of CBB using *B. bassiana* achieving satisfactory success only in the laboratory studies. After that, the use of *B. bassiana* (Fernandes *et al.* 1985) as well as of *Metarhizium anisoplie* (Lecuona *et al.* 1986) for the control of CBB was reported. The occurrence of *B. bassiana* in Brazil has also been reported in the states of: Bahia (Matiello and Fernandes 1989); Espírito Santo (Benassi 1995); Minas Gerais (Souza and Reis 1997); Roraima (Olzeno Trevisan Jr., personal communication); and Mato Grosso (Orlando Salles Jr., personal communication). However, this pathogen is probably present in all coffee production regions of Brazil. Batista Filho *et al.* (1988) used ethanol traps and *B. bassiana* aiming the control of CBB in the field. The same authors, in 1992, determined that the concentration of 3.7×10^6 conidia/ml was the minimal feasible concentration to be used in field experiments on CBB control. Studies on the use of systemic chemicals associated to application of *B. bassiana* as compared to cupric fungicides and endosulfan sprays for controlling the fungus *Hemileia vastatrix* (coffee rust) and the insect pest *Perileucoptera coffeella* (leaf miner) were conducted (D'Antonio *et al.* 1994; D'Antonio *et al.* 1995; Alves *et al.* 1995). Results have shown a higher persistence of the entomopathogenic fungus where the systemic chemicals were used. The occurrence of *B. bassiana* on CBB populations has also been already reported in Java, Sri-Lanka, Uganda, Cameroon, Jamaica, Mexico, Brazil, Ecuador and Colombia (Bustillo 1990) as well as in Honduras, Guatemala and Nicaragua (Barrios 1992). The use of *B. bassiana* or other entomopathogenic fungi for CBB control as well as any other pest control technology can only be adopted by farmers if economically feasible. Thus, it is important to have in mind that the same principles applied to the use of synthetic insecticides, when generally levels of infestation from which the control methods are established within an IPM philosophy, should not be used. These principles are generally firmly established and are very difficult of being abolished. So, the CBB control is subjected to different steps elapsing from the technology of application

to, mainly, the strategy of the pathogen utilization. Nowadays in Brazil, *B. bassiana* has been used for controlling CBB in some coffee plantations. However, its use has been based on the same principles applicable to insecticides control, leading to non-satisfactory results. The cost of *B. bassiana* formulations is also considerably higher, under-stimulating their use. These formulations are still very simple, made with ground rice grains where the fungus is grown and conidia are produced. Thus, in order to turn the technology of utilization of B. *bassiana* for CBB control fully available to the farmers, some specific actions are needed. The most important one is the development of a program of studies focusing the different strategies of utilization and concomitantly directed to solve problems of application and storage, always considering the final costs of control. Regarding technology of application, studies comparing powder and liquid applications of *B. bassiana* must be carried out. It is necessary to consider that the fungus always evolves and acts as a highly hydrophobic product and may have its efficiency compromised when mixed with water or other products. The use of one or more strains of the fungus in commercial formulations should also be considered; the use of a single strain may limit the optimal performance range of the fungus. This range may be wider if more than one strain is used, once they were previously selected for CBB control. In selecting strains, several aspects such as conidia production on insect cadavers, resistance to adverse environment (temperature, radiation, humidity), range of optimal temperatures for germination, growth and sporulation, among others, should be considered. The provision and constant testing of new strains, more virulent as compared to a previously selected standard strain, should also be considered in the improvement of CBB control products (Bustillo and Posada 1996). The strategy of utilization of the pathogen is linked to these previously described aspects. This strategy should comprise the microbiological control considering the CBB bio-ecology, the plant phenology and other control methods of the borer and other pests and diseases. The later aspect is extremely important since in the Brazilian coffee agro-ecosystem, the coffee rust is an important disease that forces the farmer to spray fungicides that may disable and/or kill *B. bassiana* conidia. Similarly, many pesticides synthetic or not (the so-called organic pesticides) used for the leaf-miner control also inhibit germination of *B. bassiana* conidia (Alves *et al.* 1998; Hirose *et al.* 2001). As far as strategy of biocontrol is concerned, it is important to start the CBB control on the fruits that fell to the soil, mainly after harvest, which harbor the insects that will be responsible for the re-infestation in the following season. *B. bassiana* should be applied at the beginning of the flowering period and should be directed to the lower third of the plant and surrounding soil where most of the remaining CBB are located. With this procedure, a lower amount of fungus will be needed, thus decreasing control costs. Cultural control, using re-passing and sweeping, are also control practices that contribute in reducing the insect population. In some cases, as in small "organic" farms, a selective collection of berries damaged during their development may be performed, leaving on the plant those berries on which the insect pest had been parasitized by the fungus. This method has been successfully used in Colombia (Bustillo *et al.* 1998). Harvest is considered as the main mode of CBB removal from the field. However, part of this population, return to the coffee plants, coming out of the bags where the harvested coffee is being placed as well as from the yard where the coffee is drying. From this point of view it is important to study methodologies for the control of CBB in the yard. This can be achieved by applying the fungus directly on the berries and/or by the use of traps with and without the fungus for attraction and release of parasitized insects that will die in the field disseminating the pathogen. The use of these techniques would probably reduce the number of applications in the field. Besides the direct CBB control by the fungus *B. bassiana*, the integration with other natural enemies should be used. For this, studies on the effect of the pathogen and its selectivity on these parasitoids are needed. It is also necessary to study the direct application of the fungus during fruit development, considering the bio-ecology of the borer and the stage of higher susceptibility of

the fruits to the insect. This way, for each production region, it will be possible to establish the best time of application of the fungus as a function of the higher CBB "transit". Further studies are still needed to turn the use of *B. bassiana* to control *H. hampei* successfully feasible. To have this technology fully available in the near future, it is important to allocate a higher amount of financial support to specific research projects. It is also important that the researchers have in mind that this technology should always be inserted in a series of tactics of insect management, becoming a "control process" and not a philosophy of product utilization, represented by the simplistic use of a synthetic insecticide as it has occurred nowadays. This philosophy of integrated process, most of times, is not used, thus discrediting the technology and almost disabling its further use.

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Use of fungal pathogens for the management of coffee berry borer, *Hypothenemus hampei* – the Indian experience

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Coffee is cultivated in about 340,000 ha. in India, mainly in the three southern states of Karnataka, Kerala and Tamil Nadu, with an annual average production of five million bags. Both the species of coffee viz., *Coffea arabica* and *C.canephora* are cultivated in the country with equal importance. The coffee berry borer, *Hypothenemus hampei* was first detected in India during February, 1990 and at present, it is prevalent in an estimated area of over 130,000 ha covering about 40% of the total coffee area in the country. With regards to the natural control agents of the pest in India, *Beauveria bassiana* is the most important fungal pathogen recorded and the others of rare occurrence include *Fusarium pallidoroseum, Paecilomyces amoeneroseus Paecilomyces fumosoroseus, Hirsutella sp Metarhizium anisopliae. B. bassiana* was found to cause up to 60% natural infection under favourable conditions. A spore concentration of 10^7 spores per ml. was found to be the optimum dosage and addition of oil was found to enhance the infectivity. *Beauveria bassiana* culture developed on rice medium was found better, in causing infectivity to the beetles, than those cultured on other media. The field trials resulted in varying degrees of infectivity depending on the age of the culture and environmental factors. The commercial powder formulations based on *B. bassiana* tried were found ineffective in causing appreciable level of mortality of the beetles. The results of the various studies conducted on the use of *B.bassiana* in CBB management are discussed in the paper. Though *B.bassiana* is found to be a potential fungal pathogen against CBB, it's use is not considered as an important component in the CBB IPM programme, at present, mainly due to the practical difficulties in obtaining quality product at affordable prices. Hence, there is a need to probe further into the development of a viable technology for mass culturing this useful fungus, without loosing its inherent virulence. Another important area of study is the refinement of application technology including development of specific formulations and improvement in the spraying equipment to place the spray deposit exactly on the target to achieve better result.

Symposium (Bacteria 3) *B. thuringiensis* and *B. sphaericus* Mosquitocidal Strains: Use and Necessities

Bacillus thuringiensis and *Bacillus sphaericus* useful tools for mosquito and blackfly control a short history of two insecticides development

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Some 37 years have gone by since *Bacillus sphaericus*, B.s., was described as producing active toxins against certain mosquitoes (Kellen *et al.*, 1965), and it has also been 25 years since the announcement that aerobic Gram-positive cell bacterial spores "were toxic against the larvae of various mosquito genera"(Goldberg and Margalit, 1977), with *Bacillus thuringiensis* serovar *israelensis*, serotype H14, B.t.i., subsequently indicated as a new and highly toxic weapon against mosquitoes (Barjac, 1978).

The practical importance of these two sporulated species was soon felt in various countries, particularly Brazil, a nation of continental dimensions situated in the tropics. In this country, diseases whose vectors represent Diptera Orders (Culicidae and Simuliidae) leading to dengue, yellow fever, filariasis, onchocerciasis, and viral encephalites are of great medical and public health relevance, with serious socioeconomic implications. Insects such as *Aedes aegypti, Culex quinquefasciatus*, and *Simulium* spp., among others, are involved in the transmission of the etiologic agents, respectively the Flaviviruses (*Togaviridae* Family), *Wuchereria bancrofti,* and *Onchocerca volvulus*. At this precise moment a new dengue epidemic is sweeping several States of Brazil, particularly the State of Rio de Janeiro. In this State alone, as of March 2002, the health authorities had recorded 129,920 cases, including 1,271 cases of hemorrhagic dengue and 47 deaths. With a view towards controlling this new epidemic, and together with other prophylactic measures, the Brazilian Ministry of Health introduced a specific granulated formulation based on B.t.i. with a view towards controlling the *Aedes aegypti* population. This measure was extended to other States of Brazil. Some believe that conventional chemical insecticides used to control the above-mentioned vectors are failing to find susceptibility in larval and/or adult insects. Thus the alternative of deploying entomopathogenic *Bacillus*, in general from the species *Bacillus thuringiensis* and *Bacillus sphaericus,* as producers of larvicide entomotoxins, has been carried out to produce so-called biological insecticides, and studies and attempts at industrial-scale production are currently being conducted in the country. As a contribution to this effort, the Laboratory of Bacterial Physiology (LFB) at the Department of Bacteriology, Oswaldo Cruz Institute, in Rio de Janeiro has worked on this line of research and has already filed two patents with the National Institute for Industrial Property, one of which has already been approved.

The LFB has focused on the *Bacillus* genus since 1967, and this interest led at the time to the creation of a Culture Collection of the *Bacillus* Genus and Related Genera (CCGB), whose collection now includes 1047 different strains from 20 species, some of which are no longer *Bacillus*, rather belonging to other genera.

Included in the CCGB are strains isolated at the LFB with high biological activity, apparently competitive with the classical strains (Silva *et al.*, 1998; Rabinovitch *et al.*, 1999; Fonseca *et al.*, 1998; Chaves, *et al.*, 1999; Chaves, *et al.*, 2001).

In 1989, the LFB began developing experiments aimed at the production of emulsive concentrated insecticides based on B.t.i. and B.s. Such insecticides were commissioned by a private company which established a partnership with the Oswaldo Cruz Foundation. The strains employed were the classical IPS-82 (B.t.i.) and 2362 (B.s), for several technical and strategic reasons. The raw materials used in the composition of the culture media were designed as being low-cost and abundant in Brazil. The advantage of producing an emulsive concentrate as the first step for manufacturing such products is related to the low cost, on the order of US$ 9.00 per kilogram for the final product. Such preparations have a good shelf life both with B.t.i. and B.s. and significantly reduce the larval population of dipterans, but the residual action is variable, a fact which has motivated the search for other formulations. The two preparations are currently under experimentation in an industrial plant equipped with fermenters with a capacity of up to 450 hl.

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Bacillus thuringiensis israelensis: a model for improving microbial insecticides for mosquito control

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Bacillus thuringiensis israelensis produces a potent group of crystal toxins, which have been used to develop insecticides to control mosquito larvae. In contrast to other microbial insecticides, such as *Bacillus sphaericus* and lepidopteran-active strains of *Bacillus thuringiensis*, resistance to *B. t. israelensis* has not occurred in any target populations, either in the field or in the laboratory. In view of the increasing importance of microbial insecticides and the growing use of genetically engineered plants that express microbial toxins in their tissues, the risk that insecticide resistance will develop in targeted populations is also rising. Thus the refractoriness of *B. t. israelensis* to inducing resistance is of considerable interest. We have investigated *B. t. israelensis* to identify what factors contribute to this lack of resistance with the goal of exploiting this information for the development of resistance management strategies and for engineering novel microbial insecticides that are refractory to resistance.

Introduction

Bacillus thuringiensis israelensis (Bti) produces a parasporal crystal containing four insecticidal proteins, Cry4A (125 kDa), Cry4B (135 kDa), Cry11A (formerly Cry4D, 65 kDa), and Cyt1Aa (28 kDa) (Höfte and Whiteley 1989). This parasporal body is released during cell lysis and exerts toxicity when ingested by susceptible insects, generally mosquitoes and blackflies. Bti is unusual because it has been used as a mosquito larvacide for many years with no evidence of insecticide resistance in the targeted populations. In contrast, resistance to *Bacillus sphaericus*, another microbial strain active against mosquitoes, has been reported in several areas (Rao et al. 1995; Silva-Filha et al. 1995). Insecticide resistance poses a serious threat to the future of microbial insecticides; therefore the refractoriness of Bti to resistance is of considerable interest. Understanding the factors contributing to such refractoriness may provide useful information for identifying strains that would likely be refractory to resistance and may contribute to the engineering of recombinant microbial strains designed that avoid resistance.

Factors influencing resistance to *Bti.* Mosquitoes selected with native Bti, containing its normal complement of three Cry toxins (Cry4A, Cry4B, Cry11A) and a cytolytic toxin (Cyt1Aa), failed to develop significant resistance after prolonged selection In contrast, resistance could be readily induced if mosquitoes were selected with one, two or three of the Cry toxins in Bti. These results suggested that the presence of the Cyt1Aa toxin was a pivotal factor in the lack of resistance development to Bti since resistance readily developed in its absence (Georghiou and Wirth 1997). We directly tested this hypothesis in two ways. First, we selected mosquitoes with a combination of Cry11A and Cyt1Aa, and compared resistance development to selection with each toxin alone. Second, we selected mosquitoes with *B. t. jegathesan* (Btjeg), another mosquitocidal bacterium which contains a complex mixture of Cry toxins and a Cytolytic toxin. Resistance development was compared to selection with a component toxin from Btjeg, Cry11B.

Selection with toxin mixtures containing a cytolytic component, i.e. selection with Btjeg or with Cry11A + Cyt1Aa showed little or no resistance after prolonged selection pressure. In

contrast selection with a single toxin, Cry11A, or Cry11B, readily induced significant levels of resistance. Therefore the presence of the Cyt1Aa toxin had a significant impact on the development of insecticide resistance, as observed in earlier selection studies with Bti.

Toxin interactions. In addition to selection studies, we evaluated the interaction of Cry and Cyt toxins against resistant mosquitoes. It was well known that the toxins within Bti interact synergistically because the activity of the complete parasporal body exceeded that additive toxicity of its component toxins (Poncet et al. 1994; Crickmore et al. 1995). We assayed combinations of Cry and Cyt toxins against both susceptible and Bti resistant colonies of mosquitoes. These mixtures were synergistic, but the levels of synergism were significantly higher against the resistant mosquitoes compared to the susceptible mosquitoes. Most significantly, these combinations effectively suppressed resistance in the Bti-selected mosquitoes (Wirth et al. 1997). Because Cyt1Aa synergized each of the component toxins in Bti, and our selection experiments demonstrated that the presence of Cyt1Aa prevented resistance development, it appears that the mechanism of resistance refractoriness results from toxin synergism.

Resistance to *Bacillus sphaericus. Bacillus sphaericus* (Bs) is an unrelated microbial strain that is also widely used to control mosquito larvae. Bs is important in mosquito larvae control because it retains activity in polluted water, an important habitat for mosquito larvae. Resistance to Bs has developed in populations of *Culex pipiens* complex in several parts of the world (see earlier references), probably because the binary toxin in Bs acts at a single receptor type in the insect midgut (Nielsen-LeRoux and Charles 1992). Using laboratory selected, Bs-resistant mosquitoes, we tested combinations of Bs and Cyt1Aa. These mixtures were highly active against the resistant mosquitoes, and suppressed their high levels of Bs-resistance. Again, the mechanism of resistance suppression was synergism between the two materials (Wirth et al 2000). These toxin mixtures also were synergistic and highly active against *Aedes aegypti*, an important vector that is not considered to be susceptible to Bs because it lacks the Bs receptor (Wirth et al. 2000).

Engineering a *Bti/Bs* recombinant. Bti has a significant advantage because its complex of interacting toxins helps it avoid causing resistance. Bs has an important advantage because of its prolonged activity in the field, but carries a high risk for resistance. Because Cyt1Aa can synergize Bs as well as Bti toxins, and such synergism can suppress and delay resistance, combining toxins from both strains may be advantageous. Studies on regulatory elements involved in toxin synthesis allow Bti to be manipulated to significantly increase toxin expression (Ge et al. 1998; Park et al. 1998, 1999). Using this approach, a recombinant bacteria strain was constructed that expressed the Bs binary toxin under the control of several regulatory sequences in wild-type Bti. This recombinant shows considerable promise against susceptible and resistant mosquitoes, and may be effective in preventing or delaying the development of insecticide resistance.

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Strains and application strategies for improving the use of *Bacillus sphaericus* and *B. thuringiensis* against mosquitoes

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The worldwide use of chemical insecticides for vector control in the 50's and 60's was succeeded by the recrudescence and re-emergence of vector-born diseases, in particular those transmitted by mosquitoes. In the last few decades malaria has became one of the most important tropical diseases with an estimated 200 million cases each year. Dengue is currently the second most important vector-born disease, with approximately 100 million cases each year. According to Gubler (1998) insecticide and drug resistance, in addition to excessive emphasis on insecticide sprays to kill adult mosquitoes, contributed to the resurgence of diseases such as malaria and dengue. Since 1983, insecticide resistance and public concerns about environmental damage resulted in the increasing use of microbial agents in mosquito and black-fly control. The market of bacterial agents for mosquito control is expected to increase significantly over the next few years, particularly in the control of lymphatic filariasis, malaria and dengue vectors.

Bacteriological products for mosquito control currently available in the market are based on *Bacillus thuringiensis israelensis* (Bti) and a few strains of *B. sphaericus* (Bs). Extensive studies have provided a wealth of knowledge about these bacteria, their toxins and mode of action, technologies for large-scale production and formulation. Worldwide field trials have confirmed its operational feasibility against mosquitoes and black-fly in reducing disease transmission or nuisance. However more research is needed to improve their performance as larvicides. It is particularly important to determine effective means for delaying or avoiding the development of mosquito resistance to the commercially available Bs strains and in the maintenance of Bti toxic activity in mosquito larvae habitats.

Although Brazil was one of the first countries to adopt Bti in blackfly control, its use for mosquito control was introduced on a large scale only recently. The National Programme for Eradication of *Aedes aegypti*, launched in 1997, began to replace temephos with Bti two years ago, in localities where the target mosquito became resistant to the organophosphate. To date, in excess of 500 tonnes of corncob granular formulations have been used. However, due to its floatable characteristics and the organic residues left in the water, corncob formulations have not shown to be appropriate for use in water storage containers, one of the most frequent *Ae. aegypti* breeding sites in Brazilian urban areas. They are being gradually replaced in part by water dispersible granules (WDG) formulations.

In the last few years, studies developed by Brazilian research groups have led to the development of Bti and Bs formulations. Some of them have been evaluated in our laboratory from the fermentation phase until the final product and subsequently tested under simulated field conditions (Table). The field performance of some Latin American Bt isolates, highly toxic to mosquito larvae, is also being evaluated.

Activity of solid formulations of *Bacillus thuringiensis israelensis* tested against *Aedes aegypti* larvae under simulated field conditions at FIOCRUZ, Recife.

Product	Potency* (ITU/mg)	Dose**	Persistence (days)***	
			Sun	Shadow
C4P1[1] (tablet)	1146	1 tablet/ 20 L	25	54[3]
T3[1] (tablet)	1146	1 tablet/ 40 L	ND	ND
Antilarv-Gi[2] (granules)	683	0.5 g/m^2	18	ND
Antilarv-Ci[2] (tablet)	683	1 tablet/ 100 L	ND	ND
VectoBac®-G	–	3.0 g /m^2	18	54

[1]developed by Far-Manguinhos-Fiocruz; [2]developed by UFPE and LAFEP; [3]Melo-Santos et al 2001 *potency of the Technical Grade Power; **causing 100% larval mortality within 24 h *** larval mortality >70%

Environmental management measures to reduce the *Culex quinquefasciatus* population in areas endemic for Bancroftian filariasis in the State of Pernambuco, will soon include the use of *B.spaericus* based larvicides. Previous field trials carried out in this region using either a liquid concentrated formulation or a non-formulated whole culture of Bs 2362 showed encouraging results. In hihgly polluted breeding sites such as cesspits, with high initial larvae densities, there was a recorded control persistence of, on average, two months. After two years of treatment a low level resistance in the mosquito population was detected (Silva-Filha et al 1995). The risk of resistance to Bs in mosquito populations is now well documented (Sinègre et al, 1994, Rao et al, 1995, Yuan et al 2000), therefore this bacterium should be applied rationally. The use of Bti in rotation is a possibility due to the fact that *Culex* populations highly resistant to Bs show no cross resistance to Bti (Regis & Nielsen-LeRoux 2000). Recent work showed that the lab-59 Bs strain is able to kill *Culex* larvae highly resistant to Bs 2362 strain. There is some evidence that lab-59 produces a potent toxic factor apart from the Bin toxin (Pei et al 2002). Preliminary trials indicate that its persistence in mosquito habitats appears to be similar to that of Bs 2362. *Culex* strains showing stable resistance to Bs 2362/ C3-41 are being maintained in the laboratory for screening new Bs strains with the objective of identifying candidates for replacing the currently used strains.

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Molecular characterization of a resistance mechanism to the *Bacillus sphaericus* binary toxin in *Culex pipiens*

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Biopesticides such as *Bacillus thuringiensis* and *Bacillus sphaericus* have been used to control pests and disease vector insects (Schnepf *et al.*, 1998; Becker, 2000). Their insecticidal properties are mainly due to larvicidal toxins, i.e δ-endotoxins produced by *B. thuringiensis*, and the binary toxin (Bin) produced by *B. sphaericus*. Upon ingestion by susceptible insect, the toxins bind to specific receptors present on apical microvillar membranes of midgut cells, leading to the death of larvae. Despite they have been used successfully on a large scale in the field in various countries, the potential benefits of these microbial control agents are threatened by the evolution of insecticide resistance (Van Rie *et al.*, 1990; Nielsen-LeRoux *et al.*, 1997; Gahan *et al.*, 2001; Chevillon *et al.*. 2001). The resistance to *B. sphaericus* has been described in laboratory-selected strains and in several field populations of the mosquito *Culex pipiens* isolated from France, Brazil, India, Tunis, China and USA (Nielsen-LeRoux *et al.*, 1995, 1997; Chevillon *et al.*, 2001; Silva-Filha *et al.*, 1995; Rao *et al.*, 1995; Yuan *et al.*, 2000; Georghiou *et al.*, 1992). Genetic studies have shown that the resistance of a Californian strain, GEO, was about 100,000 times greater than that of IP, a susceptible strain, and is inherited as a single recessive gene (Georghiou *et al.*, 1992; Nielsen-LeRoux *et al.*, 1995). Biochemical studies have demonstrated that the binary toxin (Bin) does not bind to brush border membrane fractions prepared from the midguts of GEO larvae (BBMFGEO), whereas a single class of receptor has been identified in susceptible mosquito larvae (Nielsen-LeRoux *et al.*, 1995). Little is known, however, about the molecular mechanisms by which the mosquito can survive after the ingestion of the toxin.

In this study, we describe the mechanism of resistance to Bin developed by the GEO strain. We reported previously the partial purification of a Bin-binding protein from mosquito midgut brush border membranes (Silva-Filha *et al.*, 1999). This receptor displayed sequence similarity with α-glucosidases and other maltase-like proteins, and was thus named Cpm1, for *Culex pipiens* maltase 1 (Silva-Filha *et al.*, 1999). We have cloned the full-length cDNA encoding this protein from susceptible *C. pipiens* midgut (Darboux *et al.*, 2001). The 580 amino acid-long ORF predicted a protein with a putative signal peptide at the N-terminus and, at the C-terminus, a tripeptide Ser-Ser-Ala followed by an hydrophobic tail which constitute the signature of a putative signal of anchoring of the protein to the cell membrane by a glycosylphosphatidylinositol (GPI) moiety. Cpm1 exhibits α-glucosidase activity when expressed in bacteria (Darboux *et al.*, 2001).

We have checked for the presence and integrity of the receptor Cpm1 in the GEO strain, by using a polyclonal antiserum directed against a C-terminal portion of the protein. Immunoblot analysis showed that the anti-Cpm1 antibody recognized a 67-kDa protein in BBMFIP, the membranes prepared from the midgut of IP larvae. In contrast, no signal was detected for BBMFGEO, suggesting that the receptor is no longer associated with the midgut epithelial cell membranes in the resistant strain (Fig.1).

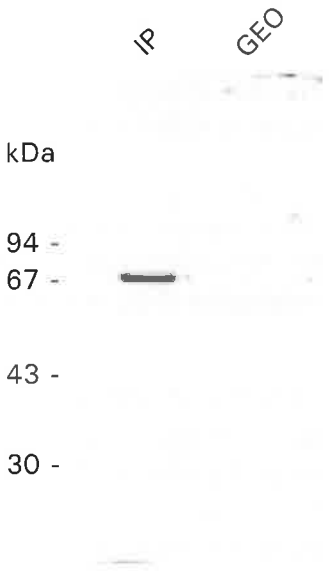

FIG. 1. Western blot analysis of Cpm1 in BBMF prepared from the midgut of mosquito larvae. A polyclonal anti-Cpm1 detected the 67-kDa Cpm1 protein in IP larvae but not in GEO larvae.

Immunohistochemical and *in situ* hybridization experiments showed that Cpm1 was strongly and specifically produced in the brush border membranes of the gastric caeca, and the posterior stomach cells. In contrast, no signal was detected in the midgut of GEO larvae, although the gene was correctly transcribed. Therefore, the lack of detection of Cpm1 protein in BBMFGEO does not result from changes in the pattern of transcription, but, most probably, from changes in the *cpm1* coding sequence.

Sequence analysis revealed that the *cpm1*GEO cDNA differed from the *cpm1IP* one by seven mutations. Six of these mutations were missense mutations that led to amino acid substitutions: Ala95Asp, Lys115Met, Glu178Thr, Asp230His, Asn265Asp and Leu486Met. The seventh was a T to A replacement at nucleotide 1706, which converted the Leu569 codon into a stop codon, leading to the premature termination of translation. To investigate the relationships between these mutations and the resistance of the GEO strain to Bin, we expressed four *cpm*1 constructs in Sf9 insect cells. Two of these constructs Sf9-IP and Sf9-GEO, corresponded to the *cpm1* cDNAs from IP and GEO mosquitoes, respectively.

The third construct, Sf9-IPMut, corresponded to a *cpm1IP* cDNA in which the translation was prematurely stopped at Leu569, mimicking the nonsense mutation in the GEO sequence. In the fourth construct, Sf9-GEOMut, the wild-type Leu569 was reintroduced into the *cpm1GEO* sequence by site-directed mutagenesis. Culture media and membrane fractions from transfected and control cells were analyzed by Western blotting (Fig. 2).

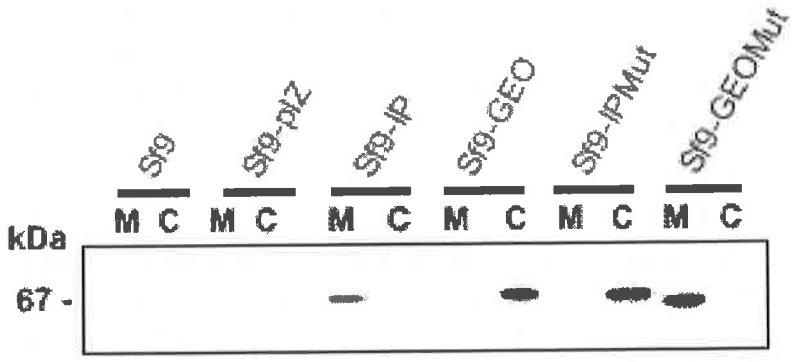

FIG. 2. Membrane-enriched fractions (M) or culture medium (C) collected from untransfected Sf9 cells (Sf9), from cells transfected with the insert-less vector (Sf9-pIZ) or the four constructs were resolved by SDS-PAGE, blotted onto PVDF membrane and probed with the anti-Cpm1 antibody.

Sf9-IP and Sf9-GEOMut proteins were detected in membrane fractions, whereas Sf9-GEO and Sf9-IPMut proteins were detected in the culture medium. Thus, the mutations in the *cpm1GEO* cDNA do not interfere with protein translation or stability. Nevertheless, one of these mutations, the Leu569Stop codon altered the membrane localization of Cpm1, resulting in the production of a secreted form of the receptor.

The nonsense mutation is localized in the COOH-terminus hydrophobic domain involved int the putative GPI-anchorage of Cpm1 to the epithelial cell membranes. To confirm such a GPI anchoring of Cpm1, cell membrane extracts were prepared from transfected cells and then treated with phosphatidylinositol-specific phospholipase C (PI-PLC).

Western blot analysis showed that, after treatment with PI-PLC, Sf9-IP and Sf9-GEOMut were released in the medium, thus demonstrating that Cpm1 protein is GPI-anchored to the membrane. Similar results were obtained when the experiment was performed on BBMFIP (Fig.3). These data suggest that, in the GEO strain, the stop codon shortens the C-terminus hydrophobic peptide required for the GPI anchorage of Cpm1, leading to the secretion of the receptor.

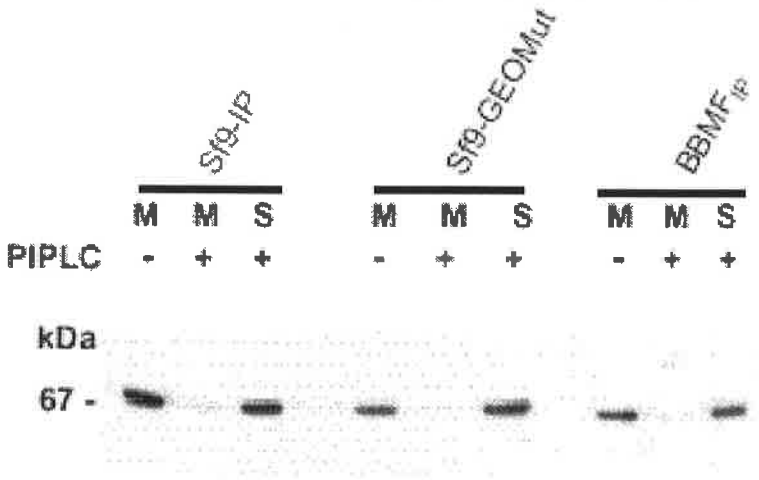

FIG.3. Membrane extracts from transfected Sf9 cells and BBMFIP were incubated in the absence ( ) or presence (+) of PI-PLC. Both soluble (S) and membrane (M) fractions collected after treatment were analyzed by Western blotting with the anti-Cpm1 antibody.

Binding experiments performed with [^{125}I]-Bin showed that membranes prepared from Sf9 cells expressing Sf9-IP or Sf9-GEOMut bound the toxin with a K_d value of 5.8 nM, that is similar to the value reported for the native receptor expressed in BBMFIP. These data indicate that the six missense mutations in the *cpm1GEO* coding sequence did not affect the affinity of the receptor for the toxin. Moreover, the soluble form of the receptor fully retained its physiological α-glucosidase activity.

We have demonstrated that the replacement of the Leu569 residue by a stop codon alters the GPI anchoring of the Bin receptor to the epithelial cell membranes by releasing the molecule in the extracellular compartment. This mutation had no side effects on the binary toxin binding nor on the enzymatic activity of the receptor. Thus Bin is not toxic to GEO larvae because it cannot interact with the midgut cell membrane, even though its receptor is present and functional (Darboux et al., 2002). This unique mechanism contrasts with other known resistance mechanisms in which point mutations decrease the affinity of binding between the receptor and the toxin.

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