PRACTICAL GUIDE
TO IDENTIFY THE MOST FREQUENT FUNGI IN SOYBEAN SEEDS

Ademir Assis Henning

Embrapa

Brazilian Agricultural Research Corporation
Embrapa Soja
Ministry of Agriculture, Livestock and Food Supply

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PRESENTATION

This manual is directed to the technicians who work in the soybean seed pathology laboratories. The photographs and drawings of the structures of the main fungi that occur in soybean seeds are very illustrative and allow the analyst to correctly identify the pathogens. To facilitate understanding, microorganisms (fungi mainly) were classified into three groups: i) important pathogens (plant pathogens); (ii) storage fungi and (iii) contaminants and / or saprophytes.

The sanitary analysis of the seed, especially in the case of soybeans, together with the tetrazolium test, in addition to other physiological tests such as the germination and vigor test (Accelerated aging) can provide important information about the reasons of poor seed quality, allowing the identification of causal problems and help to make decisions to correct their causing factors, such as: mechanical damage, stink bug damage, field weathering or storage deterioration

Ricardo Vilela Abdelnoor
Head of Research and Development
Embrapa Soja

SUMÁRIO

1. FILTER PAPER METHOD (BLOTTER TEST)

Photos: Agnes Izumi Nagashima and Samantha Rigo Segalin

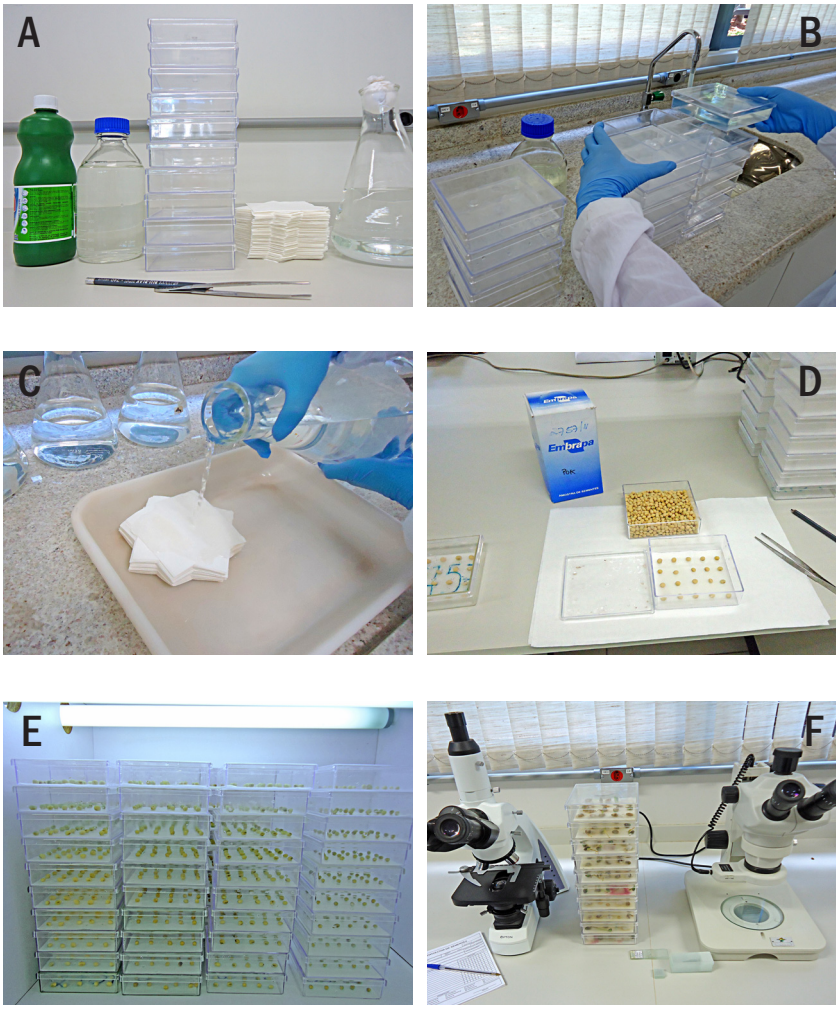


Figure 1. Assembling the blotter test. A) materials to be used; B) gerbox disinfestation with NaOCl at 1.05%; C) autoclaved distilled water to moisten sterile filter paper; D) twenty seeds per gerbox; E) samples in the incubation chamber at 20 ± 2°C for 7 days and F) evaluation of pathogens and saprophytes.

1.1. NECESSARY MATERIALS

· Plastic boxes (gerbox) with dimensions 11.0 x 11.0 x 3.5 cm (Figure 1A)

· Qualitative filter paper 80 g m2, four sheets 10.5 x 10.5 cm previously sterilized in oven at 160°C for 20 min (Figures 1A and 1C)

· Distilled and autoclaved water (preferably) or sterilized in microwaves (Figures 1A and 1C)

· Straight tweezers, forceps, stylus, dropper flasks with water and with lactophenol with dye (cotton blue), glass slides, coverslips, tissue paper (Figures 1D and 1F)

· Biological (compound) microscope with at least 400 x magnification (Figure 1F)

· Stereoscopic microscope with at least 50 x magnification (Figure 1F)

· Clorox solution containing 20% sodium hypochlorite (NaOCl 1.05%) for gerbox disinfestation (Figures 1A and 1B)

· Rubber gloves, non-slip gloves or surgical gloves (Fig. 1B and 1C)

· Water markers (Figure 1D)

· Apron (Figure 1B)

· Incubation chamber at 20°C ± 2°C with white fluorescent light (or NUV) (Figure 1E).

1.2. METHODOLOGY

The filter paper method (blotter test) is the most commonly used method in the soybean seed health testing. The experience has proven that this method is perfectly feasible, being the most effective one for analyzing soybean seeds (HENNING, 2005). In specific cases, the method can be changed by varying the temperature and incubation period to detect important pathogens such as *Sclerotinia sclerotiorum* (white mold).

For the execution of the test, the plastic boxes (gerbox) can be reused for a long time, if they are properly washed with detergent, after each use, rinsed and dried. Before use, they should be disinfested with a 1.05% sodium hypochlorite solution (bleach at 20%). Before setting up the test it is necessary to prepare filter paper (80g m2) sheets (10.5 cm x 10.5 cm), pile them up, package them in brown paper bag(s) and sterilize the package(s) at 160°C for 20 minutes in an oven. After sterilization the oven cannot be open until its contents have cooled down next to the ambient temperature. The test is set up placing four 10.5 cm x 10.5 cm filter paper sheets in each gerbox previously disinfested. Water (preferably autoclaved) is added to the filter papers just to sufficiently moisten them. One must avoid excess of water since it will favor the occurrence of bacteria and *Alternaria* spp. For each sample of at least 400 soybean seeds, 20 gerboxes are prepared and identified. Then, 20 seeds are randomly taken from the sample and disposed in a 5 x 4 arrangement on the moist filter paper.

After completion of the preparation, the identified gerboxes are incubated for seven days at 20 ± 2°C, under continuous fluorescent light or NUV (near ultra violet) in alternating periods of 12h darkness/12h light (REGRAS...2009). Evaluation is done in each seed, being annotated in an appropriate form the occurrence of the various pathogens. *Aspergillus flavus* and *Penicillium* spp., despite of being considered saprophytic by some authors, these fungi must be counted as being storage fungi, responsible for deterioration of the seed, when the conditions of storage are inadequate (high humidity and temperature).

2. IMPORTANT PATHOGENS

2.1. *Cercospora kikuchii*

It is the most frequent soybean-seedborne fungus, in Brazil. However it does not affect seed quality!

Photos: Agnes Izumi Nagashima

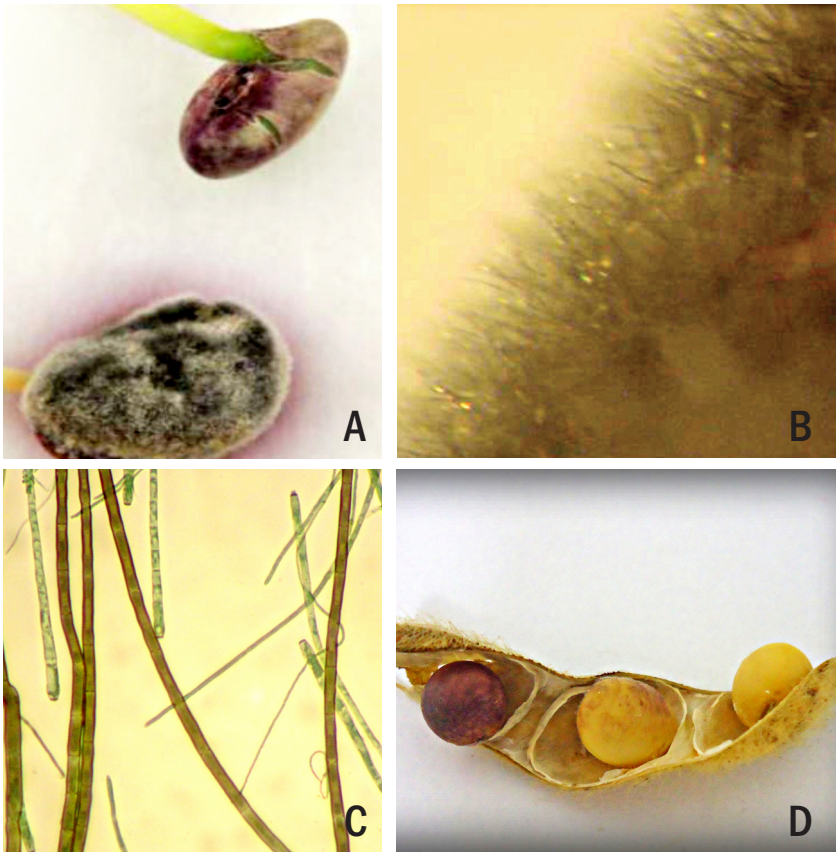


Figure 2. *Cercospora kikuchii.* A) seed with typical purple stain symptom; B) fungus sporulating on the seed; C) conidia (blue stained) and conidiophores (brown); D) infected seed within a pod.

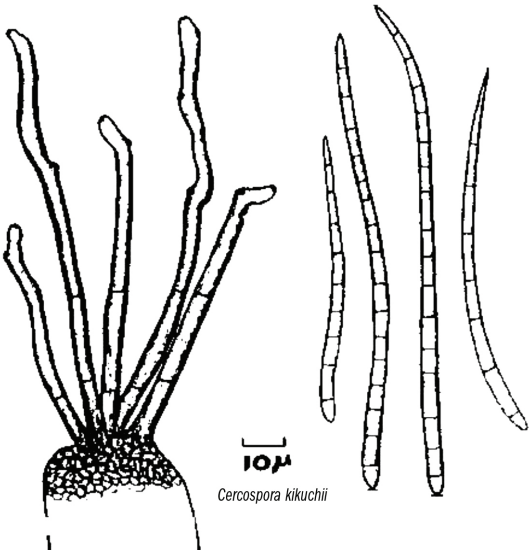


Figure 3. Conidiophores (left) and conidia or multi-septated spores (right)
Source: Sinclair e Shurtleff (1975).

2.2. *Colletotrichum truncatum*

Causal agente of soybean anthracnose

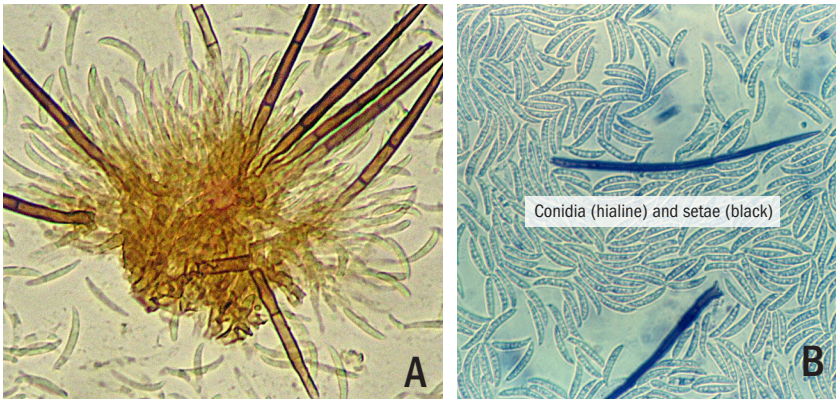
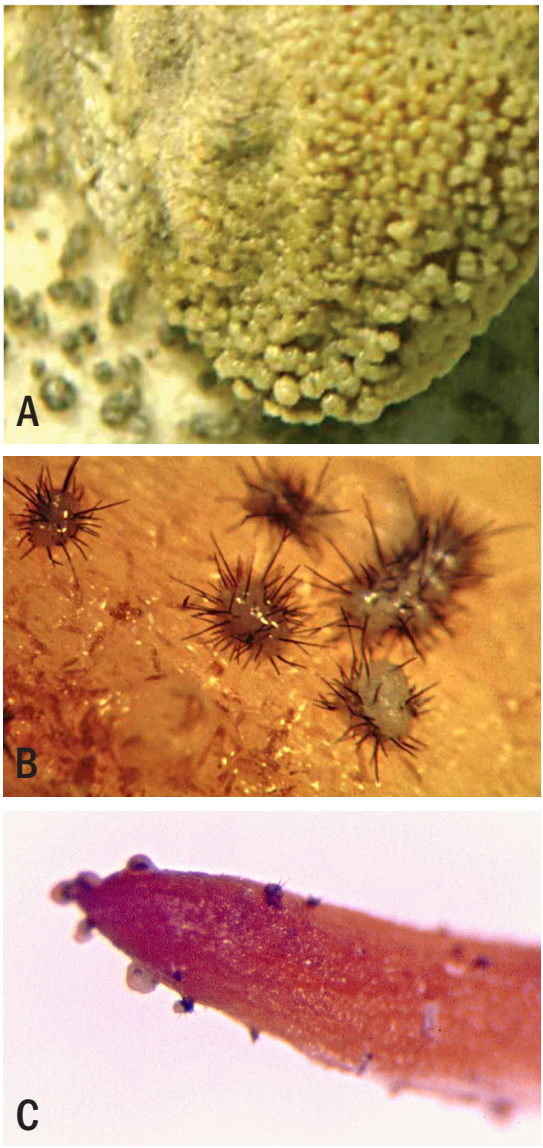


Figure 4. *Colletotrichum truncatum*. A and B) conidia (spores) and arrows of *Colletotrichum truncatum*.



Photos: Agnes Izumi Nagashima (A) and Ademir Assis Henning (B and C)

Figure 5. *Colletotrichum truncatum*. A and B) acervuli with setae and mass of spores (cream coloration) on seed coat; C) radicle showing various acervuli.

2.3. *Fusarium* spp.

The two most frequente *Fusarium* species in soybean seeds. They may reduce germination in laboratory tests but do not cause disease in soybean plants, in the field.

Photos: Agnes Izumi Nagashima

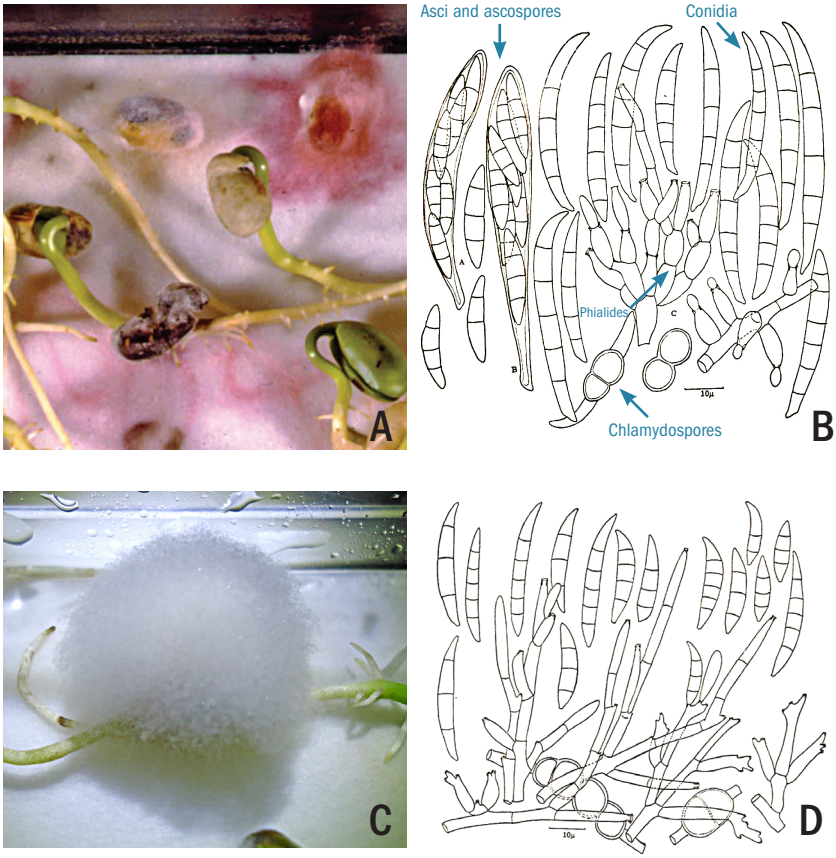


Figure 6. *Fusarium* spp. A) *Fusarium graminearum*; B) conidia, asci, chlamydospores of *Fusarium graminearum*; C) *Fusarium pallidoroseum*; D) conidiophores, conidia and chlamydospores of *Fusarium pallidoroseum* (*semitectum*).

Source: Booth (1971).

2.4. *Macrophomina phaseolina*

Typical soilborne fungus, may eventually contaminate externally the seed (concomitante seed transmission)

Photo: Agnes Izumi Nagashima

Figure 7. Conidia (spores) of *Macrophomina phaseolina*.

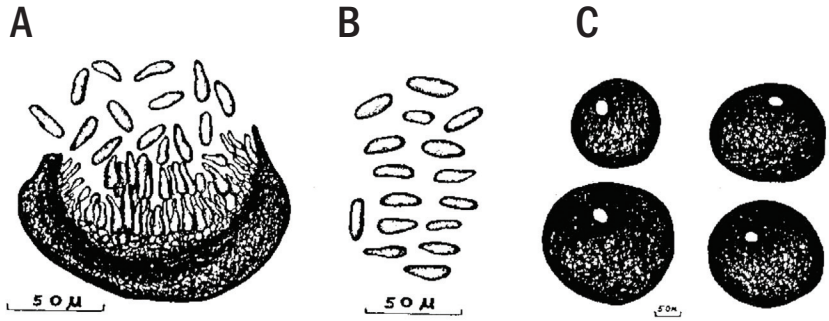


Figure 8. *Macrophomina phaseolina*. A) cut of pycnidium with spores; B) spores; C) pycnidia.

Source: Sinclair e Shurtleff (1975).

Photos: Agnes Izumi Nagashima

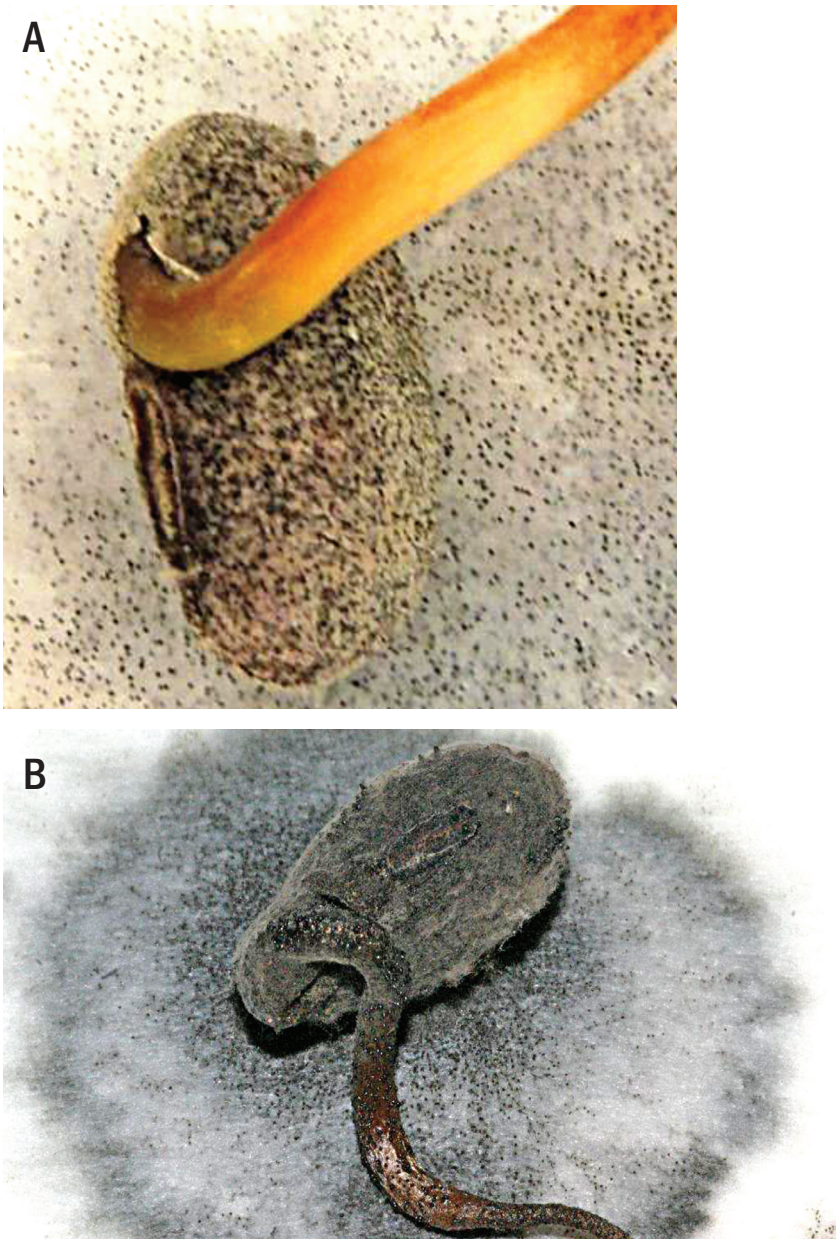


Figure 9. *Macrophomina phaseolina*. A) microesclerotia spread over the filter paper (blotter); B) seed infected with *Macrophomina phaseolina.*

2.5. *Phomopsis* sp.

It is the main soybean seedborne pathogen. Along with *Fusarium pallidoroseum* (*semitectum*) interfere with the results of the standard germination test (rolled paper towelling). Seed infection levels may be very high when harvest coincides with moist, rainy weather during seed maturation and/or harvest.

Photos: Agnes Izumi Nagashima (A, B, C and E) and Ademir Assis Henning (D)

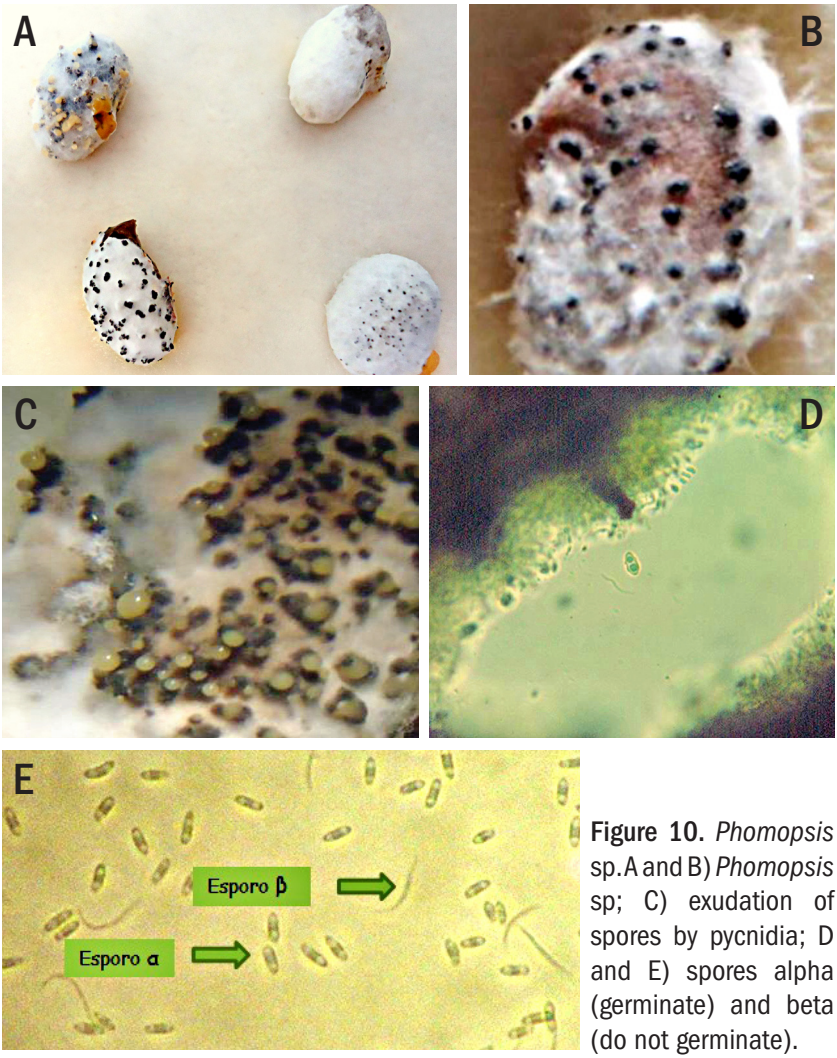


Figure 10. *Phomopsis* sp. A and B) *Phomopsis* sp; C) exudation of spores by pycnidia; D and E) spores alpha (germinate) and beta (do not germinate).

2.6. *Rhizoctonia solani*

Very common soilborne pathogen. May contaminate the seed during harvest

Photos: Ademir Assis Henning

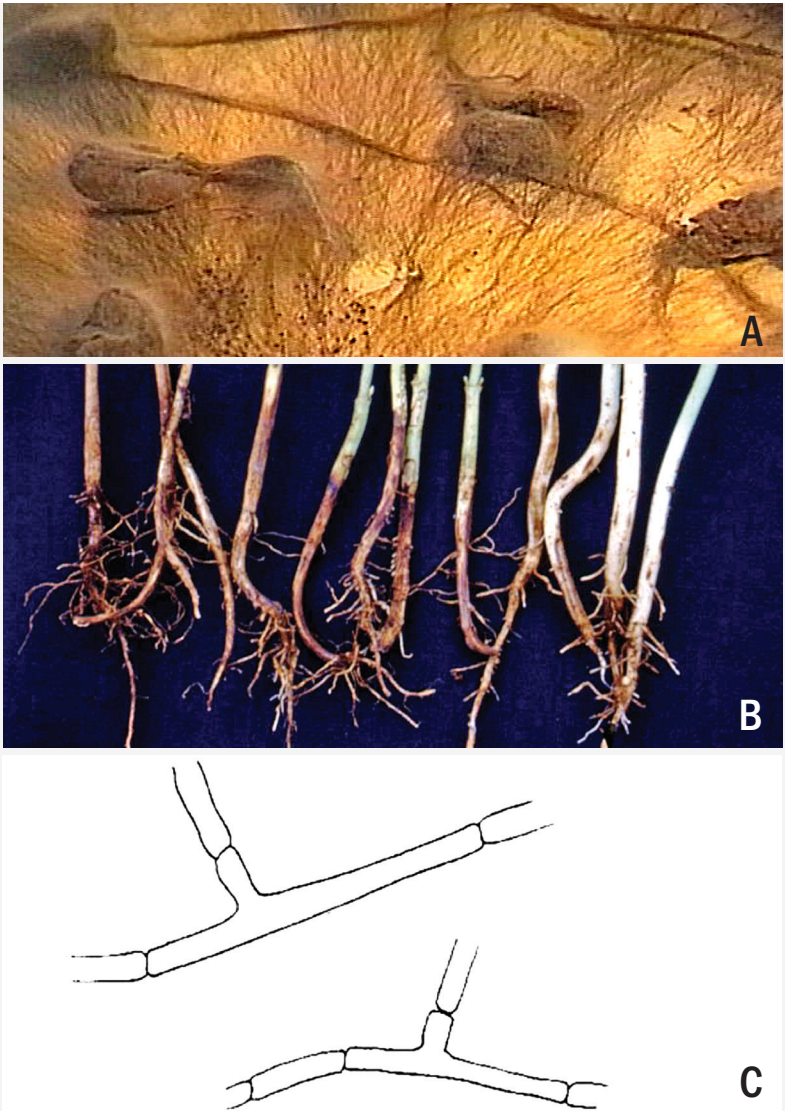


Figure 11. *Rhizoctonia solani.* A) rhizomorphs in filter paper method; B) plant symptoms; C) septated hyphae with typical right-angle branching.

Source: Henning et. al (2002).

2.7. *Sclerotinia sclerotiorum* - White mold

Very importante soybean plant pathogen. However seed transmission rate, as internal dormant mycelium, is very low! (< 0,1%). The biggest problem is the sclerotia mixed with seed.

Photos: Ademir Assis Henning

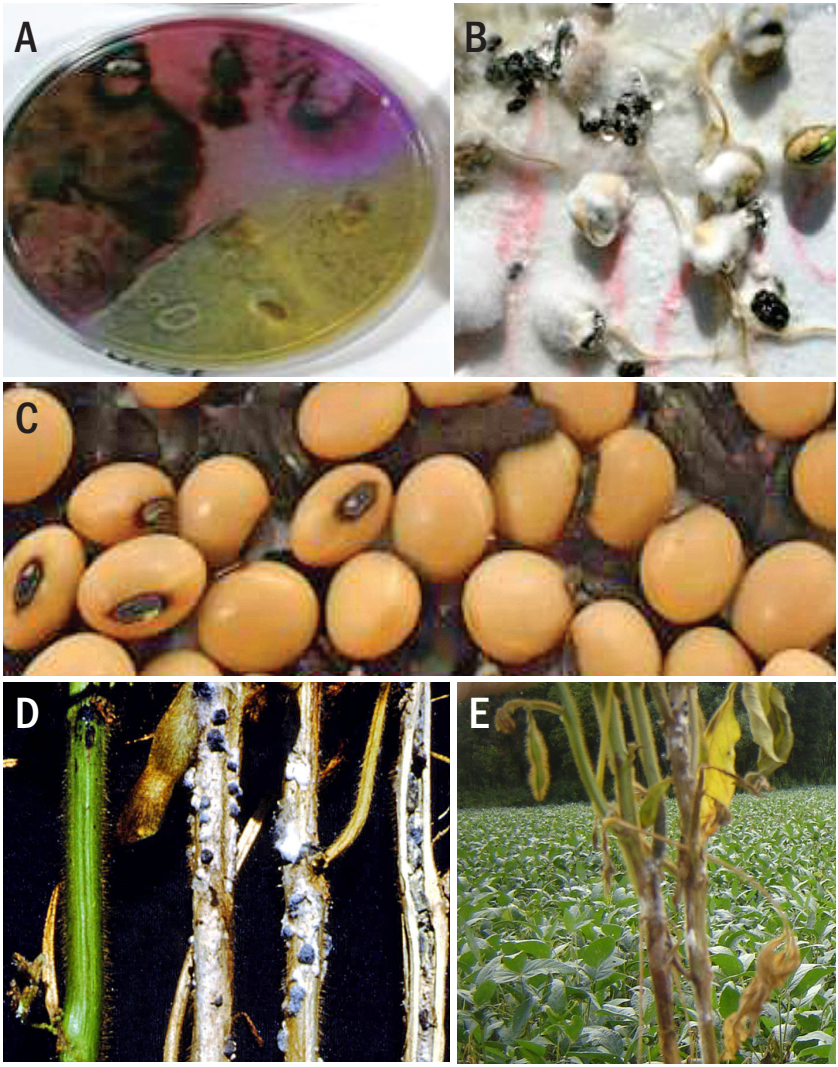
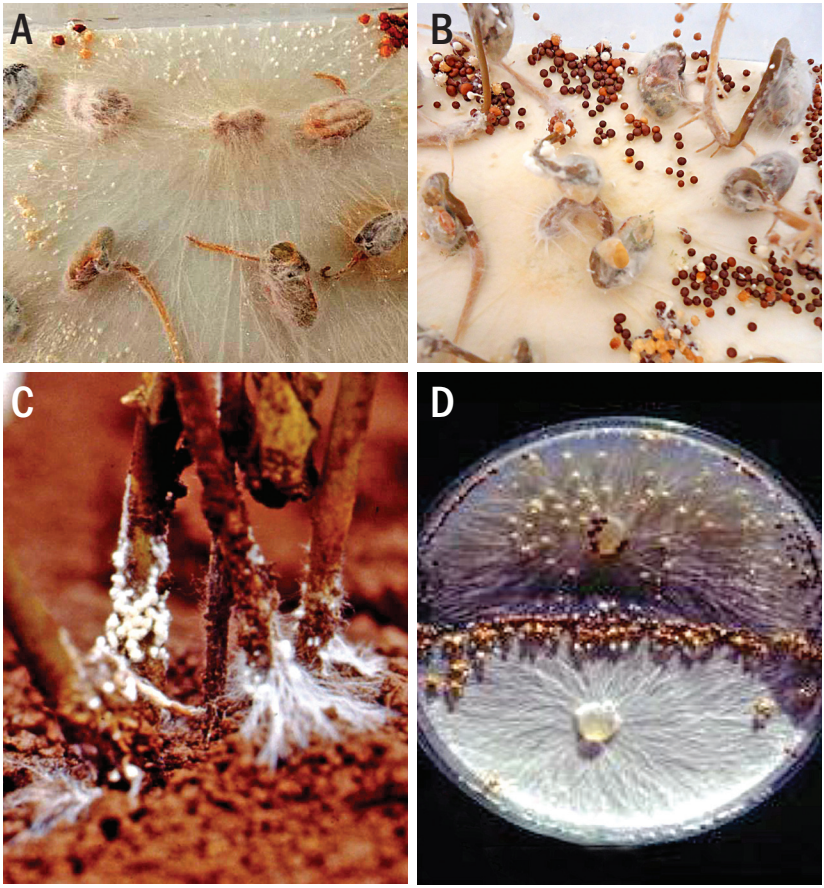


Figure 12. *Sclerotinia sclerotiorum.* A) neon Method; B) sclerotia in gerbox in the filter paper method; C) sclerotia mixed with seed; D) stems with sclerotia and E) symptoms on the plants in the field

2.8. *Sclerotium rolfsii*

Typical soilborne fungus. Sclerotia mixed with seed may sometimes occur. Sclerotia is initially white and later turn dark brown (but the center remains white)



Photos: Agnes Izumi Nagashima (A and B) and Álvaro Manoel Rodrigues Almeida (C and D)

Figure 13. *Sclerotium rolfsii.* A and B) production of sclerotia in filter paper method; C) symptoms in seedlings and D) production of sclerotia in culture media (PDA).

3. STORAGE FUNGI

3.1. *Aspergillus* spp.

Most important species is *Aspergillus flavus.*

Photos: Agnes Izumi Nagashima

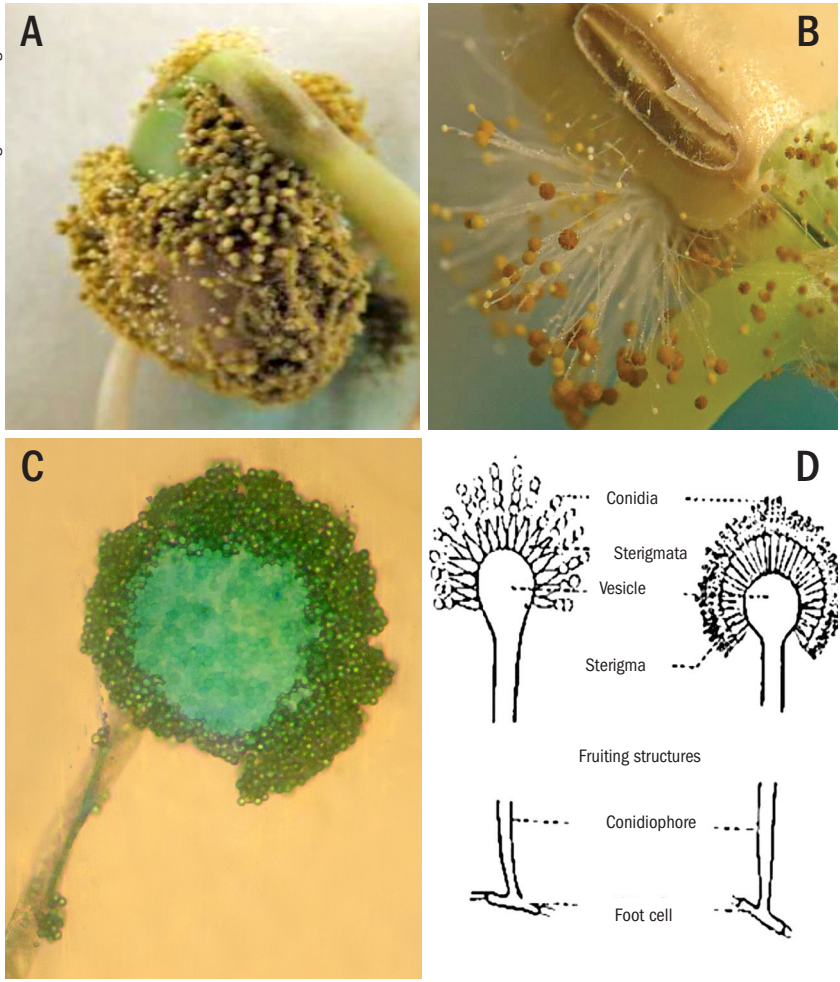


Figure 14. A) *Aspergillus* spp. B) *Aspergillus flavus*; C) structures of *Aspergillus flavus* under compound microscope (400 x); D) fruiting estructures (conidiophore, vesicle and spores) of *Aspergillus flavus.*

Source: Silveira (1968).

3.2. *Penicillium* sp.

Storage fungus, but less frequent than *Aspergillus* spp. in soybean seeds.

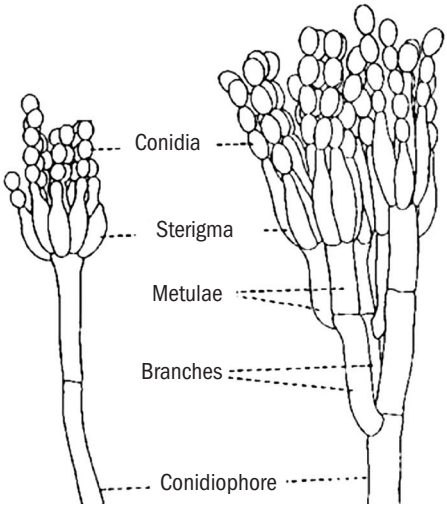


Figure 15. Structures of *Penicillium* sp.

Source: Silveira (1968).

Photos: Ademir Assis Henning

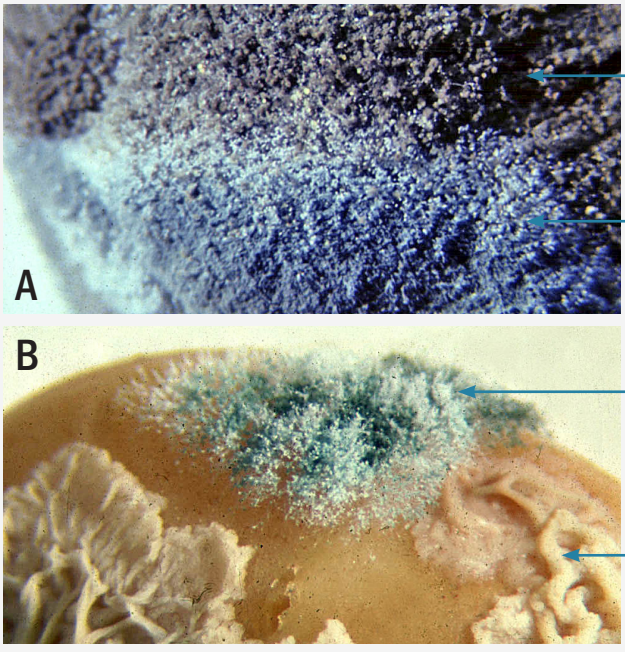


Figure 16. *Penicillium* sp. A) seeds with *Penicillium* sp.; B) *Penicillium* sp. in seeds with *Bacillus* sp.

4. CONTAMINANTS OR SAPROPHYTES

4.1. *Alternaria* spp.

The most common species in soybean seeds is *Alternaria tenuis* or *A. alternata.*

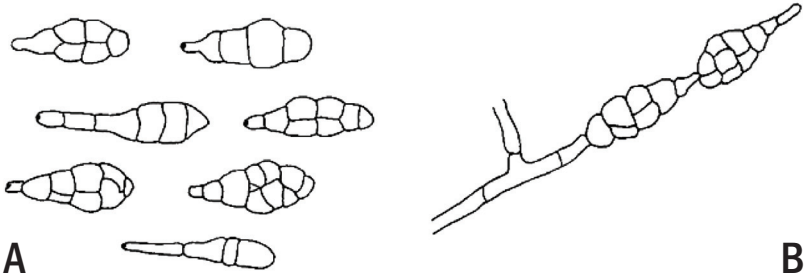


Figure 17. *Alternaria* sp. A) different forms of conidia (spores) with transverse and longitudinal septa; B) spores (conidia) in chains (catenulated).
Source: Henning et al. (2002)

Photos: Agnes Izumi Nagashima

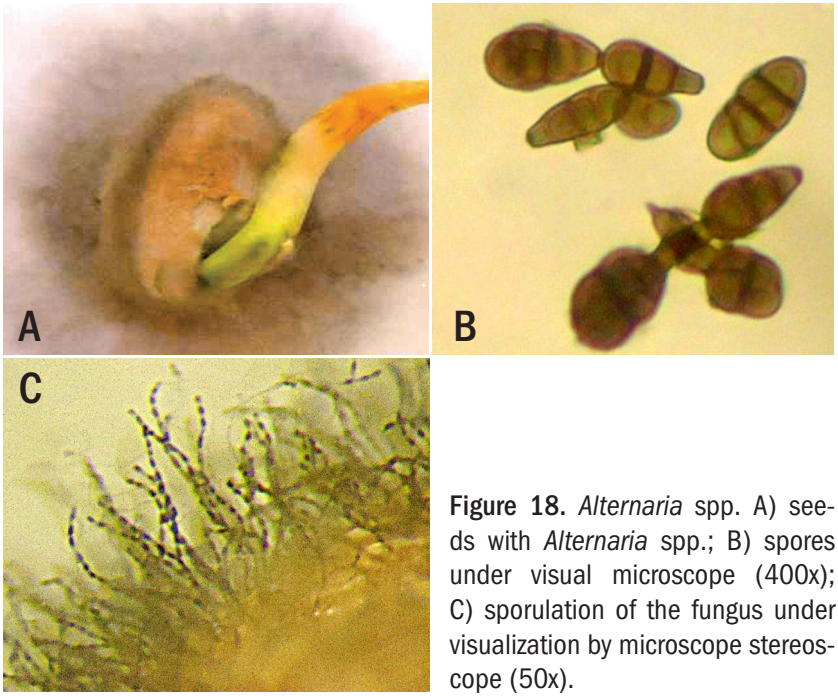


Figure 18. *Alternaria* spp. A) seeds with *Alternaria* spp.; B) spores under visual microscope (400x); C) sporulation of the fungus under visualization by microscope stereoscope (50x).

4.2. Bacteria

Saprophytes, some species are used in the biological control of important pathogens.

Photos: Agnes Izumi Nagashima and Ademir Assis Henning

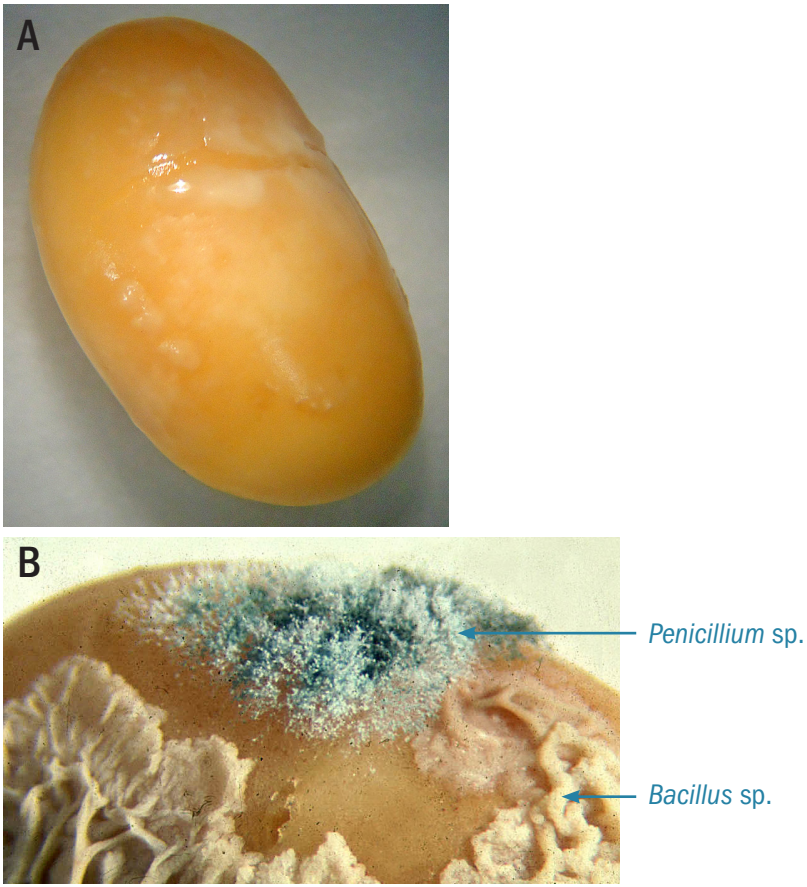


Figure 19. Bactéria. A) bacteria – normaly associated with dead seeds, deteriorated by physiological problems: such as mechanical damage, stink bug damage, or even weathering damages; B) *Bacillus* sp. saprophyte.

4.3. *Botryodiplodia* sp.

Contaminant, not pathogenic

Photo: Agnes Izumi Nagashima

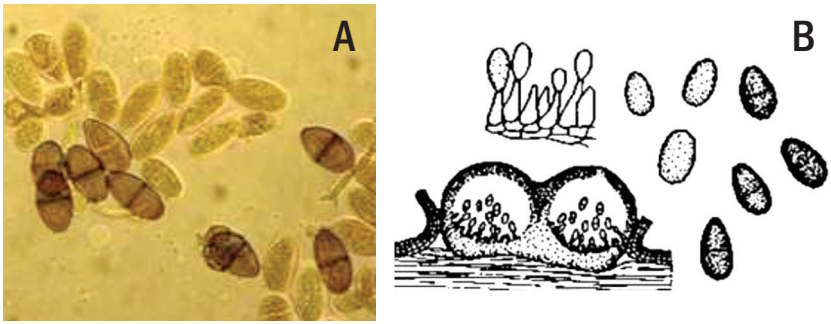


Figure 20. A) Young conidia (light brown) and mature, old conidia (dark brown) with transversal septum. B) diagram ou picnidia and conidia.

Source: Barnett e Hunter (1972).

Photos: Ademir Assis Henning

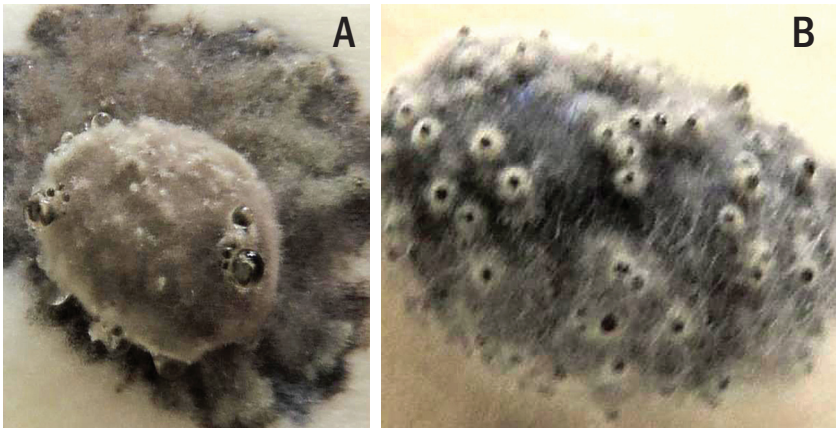


Figure 21. A) Fungus growing ond seed and spread on blotter and B) seed with *Botryodiplodia* sp., showing various picnidia.

4.4. *Chaetomium* sp.

Saprophyte, contaminant! – Be carefull not to confuse with *Colletotrichum!* The fungus produces perithecia, with extensive hairy appearance that may be confused with the setae in acervuli of *C. truncatum!*

Photos: Agnes Izumi Nagashima (A, B and D) and Ademir Assis Henning (E)

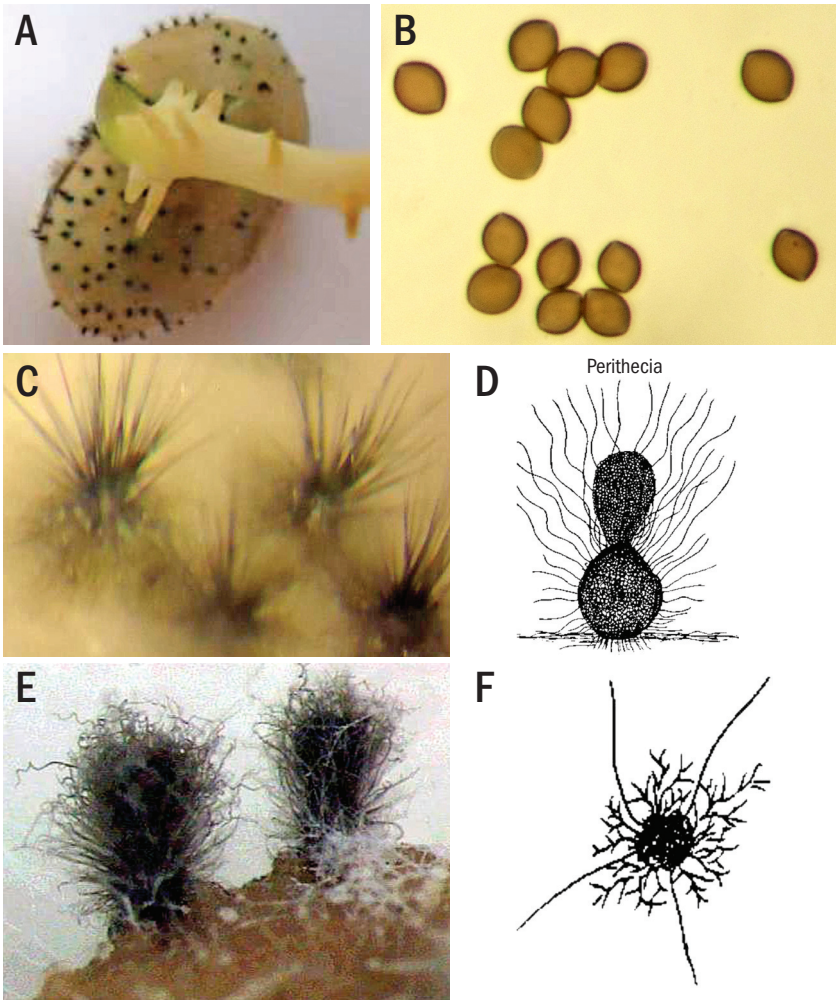


Figure 22. *Chaetomium* sp. A) seed with *Chaetomium* sp.; B) ascospores (spores) under microscope (400x); C, D, E and F) perithecia & different kinds of "hairy structures".

Source: Henning et al. (2002).

4.5. *Cladosporium* spp.

Saprophyte, contaminant.

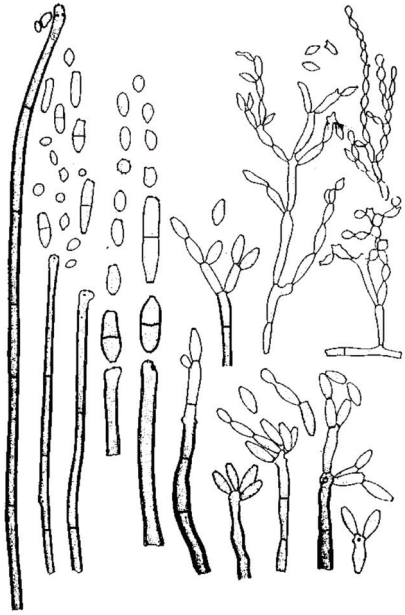


Figure 23. Conidiophores (dark) and conidia of varying shapes and sizes.

Source: Ellis (1976).

Photos: Agnes Izumi Nagashima

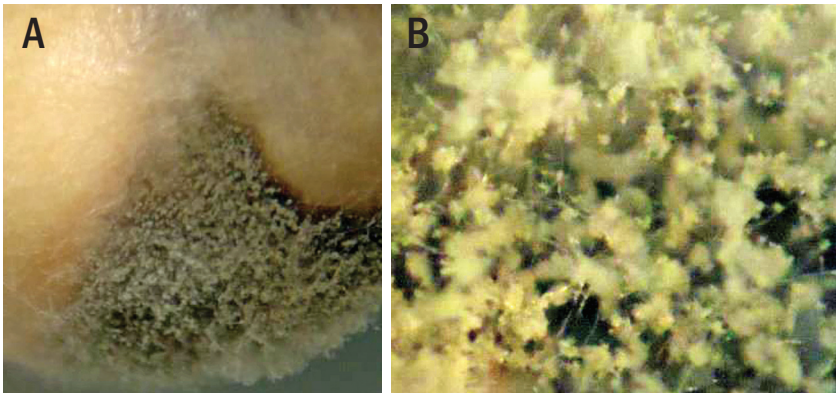


Figure 24. A and B) *Cladosporium* spp.

4.6. *Rhizopus* spp.

Saprophyte, contaminant - produces “rhizoids” that distinguishes it from *Mucor* sp.

Photos: Ademir Assis Henning

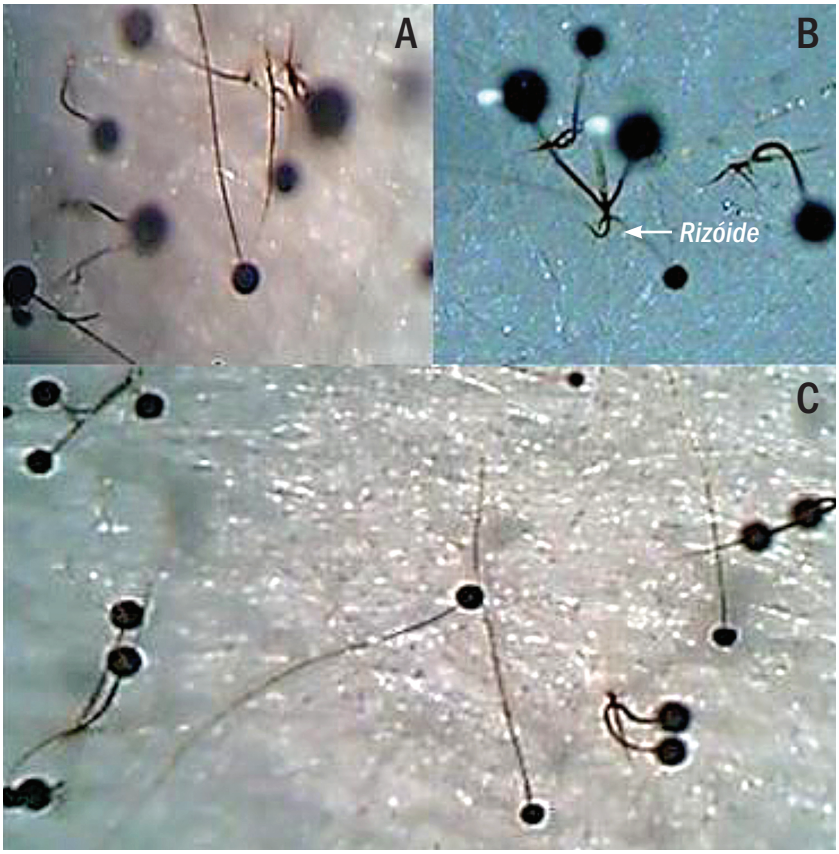


Figure 25. A, B and C) structure showing the rhizoids on the substrate (blotter) at 50X magnification, under stereoscopic microscope.

Photos: Agnes Izumi Nagashima

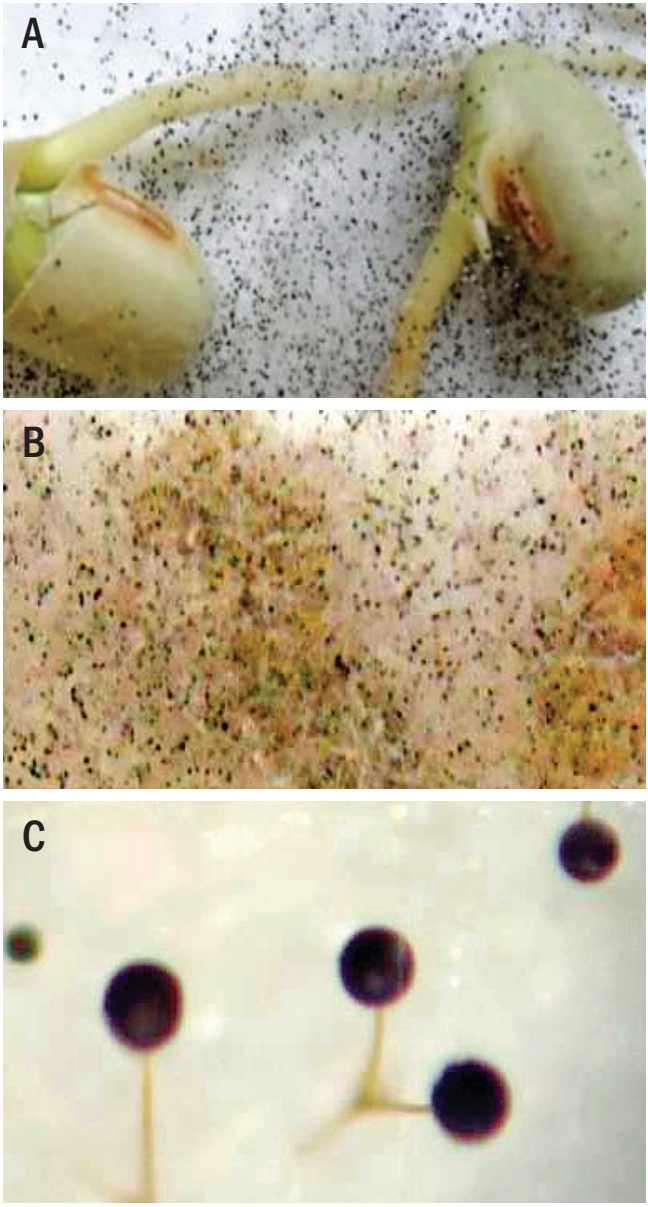


Figure 26. A and B) *Rhizopus* spreading on seeds and on paper filter; C) structure of *Rhizopus* spp. in viewing by optical microscope (400 x).

4.7. *Trichoderma* spp.

Contaminant, but is also used as a biological control agent against white mold, and other fungi.

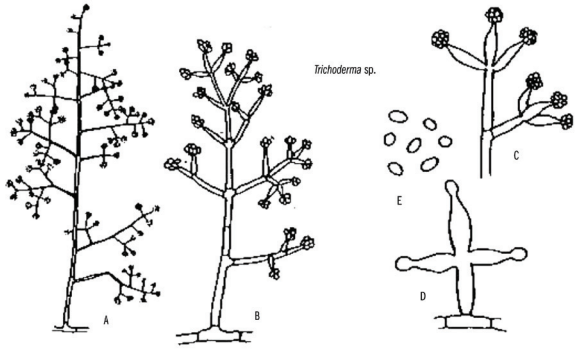


Figure 27. *Trichoderma* spp. A, B, C and D) conidiophores are hialine, profusely branched; E) conidia are small and eliptical

Source: Barnett e Hunter (1972).

Photos: Agnes Izumi Nagashima

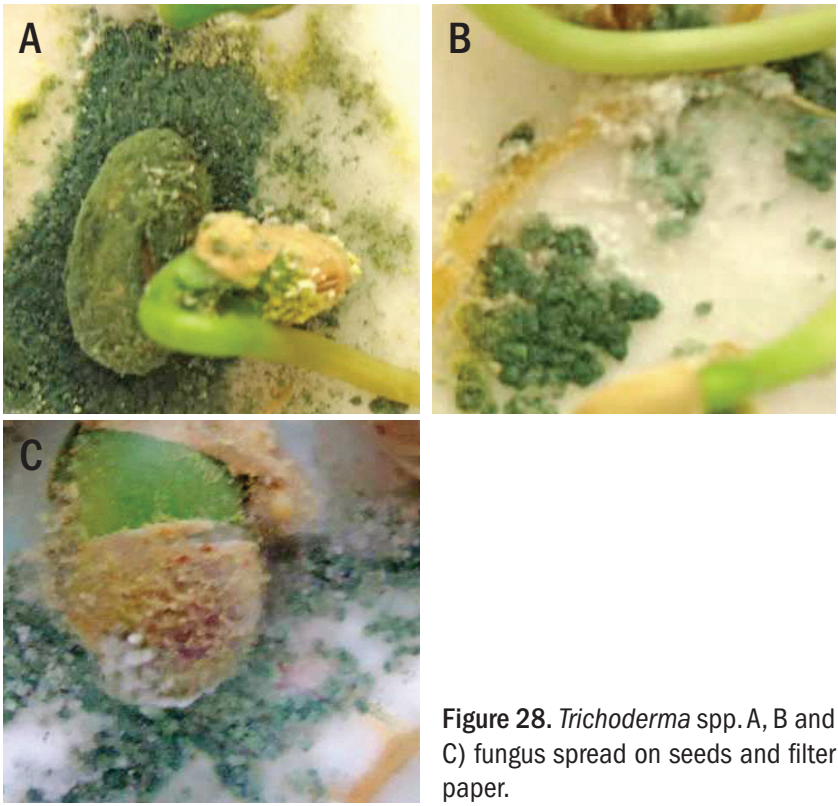


Figure 28. *Trichoderma* spp. A, B and C) fungus spread on seeds and filter paper.

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ANNEX

EVALUATION FORM

Embrapa

LABORATORY OF SEED PATHOLOGY

SAMPLE NUMBER_____

METHOD
☐ Blotter Test
☐ OTHER _____

PERIOD OF TEST
START_____ END _____

SPECIE SOYBEAN
VARIETY _____

HARVESTING DATE

LOCATION OF THE PRODUCTION FIELD:

RECEIVING DATE

SENDER:
NAME
ADDRESS:

PATOGENS (FUNGI)	REPLICATIONS										%
	1	2	3	4	5	6	7	8	9	10	
Aspergillus flavus											
Aspergillus spp											
Cercospora kikuchii											
Colletotrichum trunchatum											
Fusarium pallidoroseum (semitectum)											
Phomopsis sp.											
Macrophomina phaseolina											
BACTERIA											
HARD SEED											
MECHANICAL DAMAGE											
GERMINATED SEED											

SAPROPHYTE: ☐ *Alternaria* sp. ☐ *Curvularia* sp. ☐ *Nigrospora* sp. ☐ *Trichoderma* sp.
☐ *Chaetomium* sp. ☐ *Helminthosporium* sp. ☐ *Penicillium* spp. ☐
☐ *Cladosporium* sp. ☐ *Mucor* sp. ☐ *Rhizopus* sp. ☐

OBSERVATIONS:

DATE__/__/____

_____
ANALYST

Embrapa

Soybean

MINISTRY OF
AGRICULTURE, LIVESTOCK
AND FOOD SUPPLY

BRASIL
BRAZILIAN GOVERNMENT

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