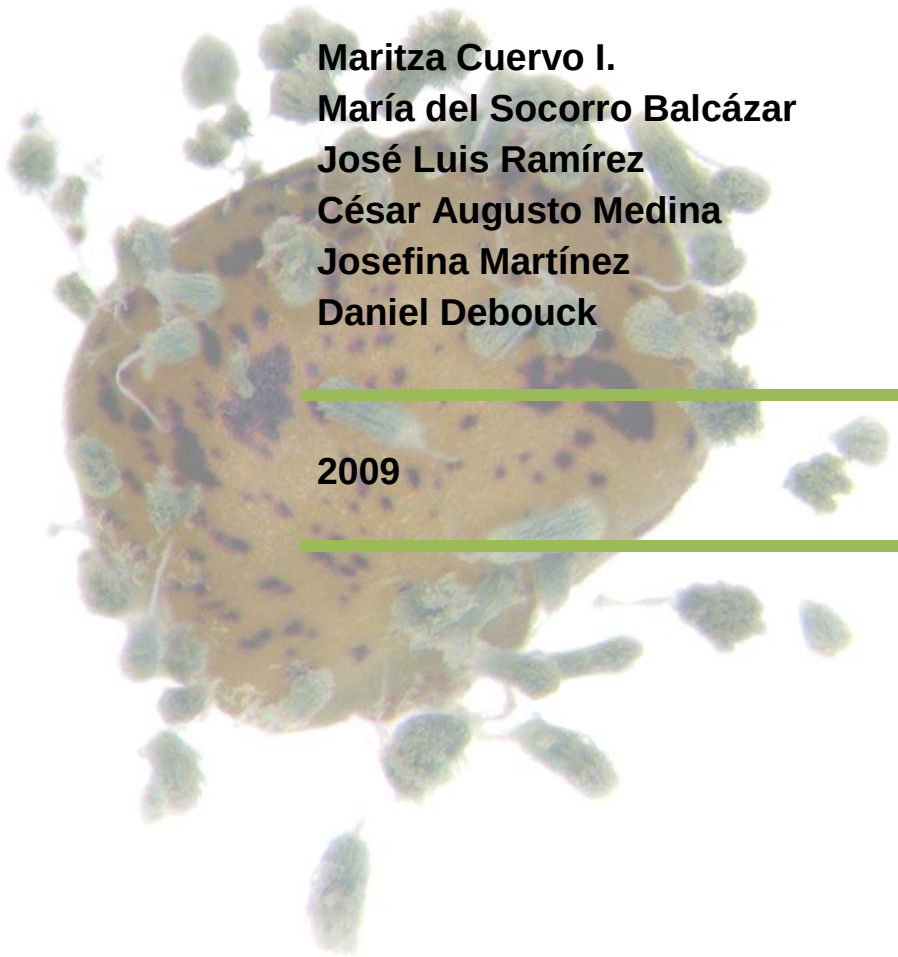
HANDBOOK OF PROCEDURES OF THE GERMPLASM HEALTH LABORATORY GENETIC RESOURCES PROGRAM

Maritza Cuervo I.
María del Socorro Balcázar
José Luis Ramírez
César Augusto Medina
Josefina Martínez
Daniel Debouck



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CIAT
Centro Internacional de Agricultura Tropical
International Center for Tropical Agriculture
Consultative Group on International Agricultural Research

Genetic Resources Program
Let us keep a seed of solutions

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1. Introduction

The collections of the Genetic Resources Program (GRP) of the Centro Internacional de Agricultura Tropical (CIAT) contain 66.000 accessions as samples of seeds and other plant reproductive materials. This germplasm includes 720 species of beans, cassava and tropical forages and corresponds, for the most part, to non-improved landraces.

Each year, the GRP of CIAT distributes on average 5,000 to 6,000 samples of genetic material in response to requests that are received internal- and externally from different parts of the world. These materials of the germplasm bank can be used in research, crop breeding, field trials, seed multiplication, training, and conservation itself.

The conservation of germplasm involves a set of activities ranging from acquisition to distribution, including the processes of growing-out multiplication, rejuvenation and storage, where health plays a crucial role in all stages of the process. It should be noted that the baseline of GRP is that all conserved material must be a certified material, and immediately available for distribution.

The transfer of this germplasm free of pathogens and pests initially involves a under conditions production of seed or vegetatively propagated material received from exchange. It is followed by a postharvest management plan that keeps the health of the materials, and finally a laboratory analysis process that certifies their phytosanitary quality. The first part of the handbook of procedures of the Germplasm Health Laboratory (GHL), focuses on the collections of seeds (beans and tropical grasses), and the second part relates to the health certification of cassava.

To minimize the risks associated with the movement of germplasm, especially the transport of pathogens and pests of quarantine concern, there is, by prior agreement, on the premises of CIAT headquarters, an office of the Division of Plant Health (Department of Inspection and Quarantine) of the Instituto Colombiano Agropecuario (ICA), an agency under the Ministry of Agriculture of Colombia. The objectives of this agreement are:

- To prevent the spread of seed borne diseases and to minimize the risk of accidental introduction of pests and pathogens into Colombia.
- To monitor quarantine greenhouses where the imported germplasm is increased.
- To inspect the multiplication fields and greenhouses where the germplasm is being increased.
- To test the seed health status of germplasm for international export based on the results obtained by the Germplasm Health Laboratory (GHL).

Upon fulfillment of the steps of production and certification, materials are regularly dispatched to the concerned country, after obtaining a phytosanitary certificate of the Colombian Government. In the country of the recipient, the shipments are inspected by the quarantine authorities concerned.

2. Facilities

The Germplasm Health Laboratory (GHL) is in the GRP with responsibilities to report about the phytosanitary status of germplasm distributed for the GRP and other programs of CIAT, certifying that it is free of quarantine diseases. In this laboratory all the health tests for the diagnosis of pathogens such as fungi, bacteria, viruses, and occasionally nematodes and beetles affecting seeds are performed. Additionally, investigations are conducted on the management and characterization of quarantine pathogens and on the standardization of new methods of diagnosis that are more

efficient and sensitive. The facilities of the GHL occupy 144.5 m^2 distributed in different areas that are described in Table 1 and Figures 1 and 2.

Table 1. Description of the areas of the Germplasm Health Laboratory (GHL).

Section	Purpose	Area (m^2)	Equipment
Central room	Reception, labeling, registration, subsampling, sample preparation and microscopic analysis.	53.7	Incubator (2) Precision Incubator (1) Fridge 4 ° C (1) Freezer - 20 ° C (1) Extraction chamber (1) Magnifier (1) Stereoscope (3) Optical microscope (2) Fluorescence microscope (1)
Room for molecular diagnostic	Performing molecular tests (RT-PCR).	6.9	Thermocycler (1) Vortex (1) Refrigerated microcentrifugal (1) Microcentrifugal (1) Blue light trans-illuminator (1) Power supply for electrophoresis (1) Electrophoresis chamber (2)
Incubation Room	Incubation of samples under controlled conditions of optimal light and temperature for growing fungi.	6.9	Digital Thermometer (1) Air Conditioning (1) Shelf White light lamp Near UV light lamp
Isolation room for the analysis of fungi	Seed planting in petri dishes and culture media for diagnosis of fungi.	7.3	Horizontal laminar flow chamber (1) Shelf
Isolation room for the analysis of bacteria	Seed planting in petri dishes and culture media for diagnosis of bacteria.	7.0	Horizontal laminar flow chamber (1) Vertical laminar flow chamber (1) Refrigerator (1) Incubator (1)
Detection of virus	Processes for the diagnosis of virus.	3.6	ELISA Dishwasher machine (1) ELISA reader (1)
Preparation of media	Preparation, storage of culture media and storage of reagents.	9.3	Heating plates with magnetic stirrer (4) pH meter (1) Analytical Balance (2) Shelf
Washing area	Washing process of laboratory material.	14.4	Petri dishes washer machine (1) Dishwasher (1) Shelves with laboratory material
Sterilization room	Sterilization of media and discarded material.	6.5	Small autoclave (1) Big autoclave (1)

Section	Purpose	Area (m^2)	Equipment
Cold room	Storage of solutions and samples.	4.1	Thermo of 25 l for liquid N2 (1) Shelf
Room for the maceration of samples	Maceration and storage of laboratory implements.	10.1	Large seed macerator (1) Small seed macerator (2)
Main office	Data entry to the database and meetings.	7.48	Documentation shelves (2) Book shelf (1) Computer (1)

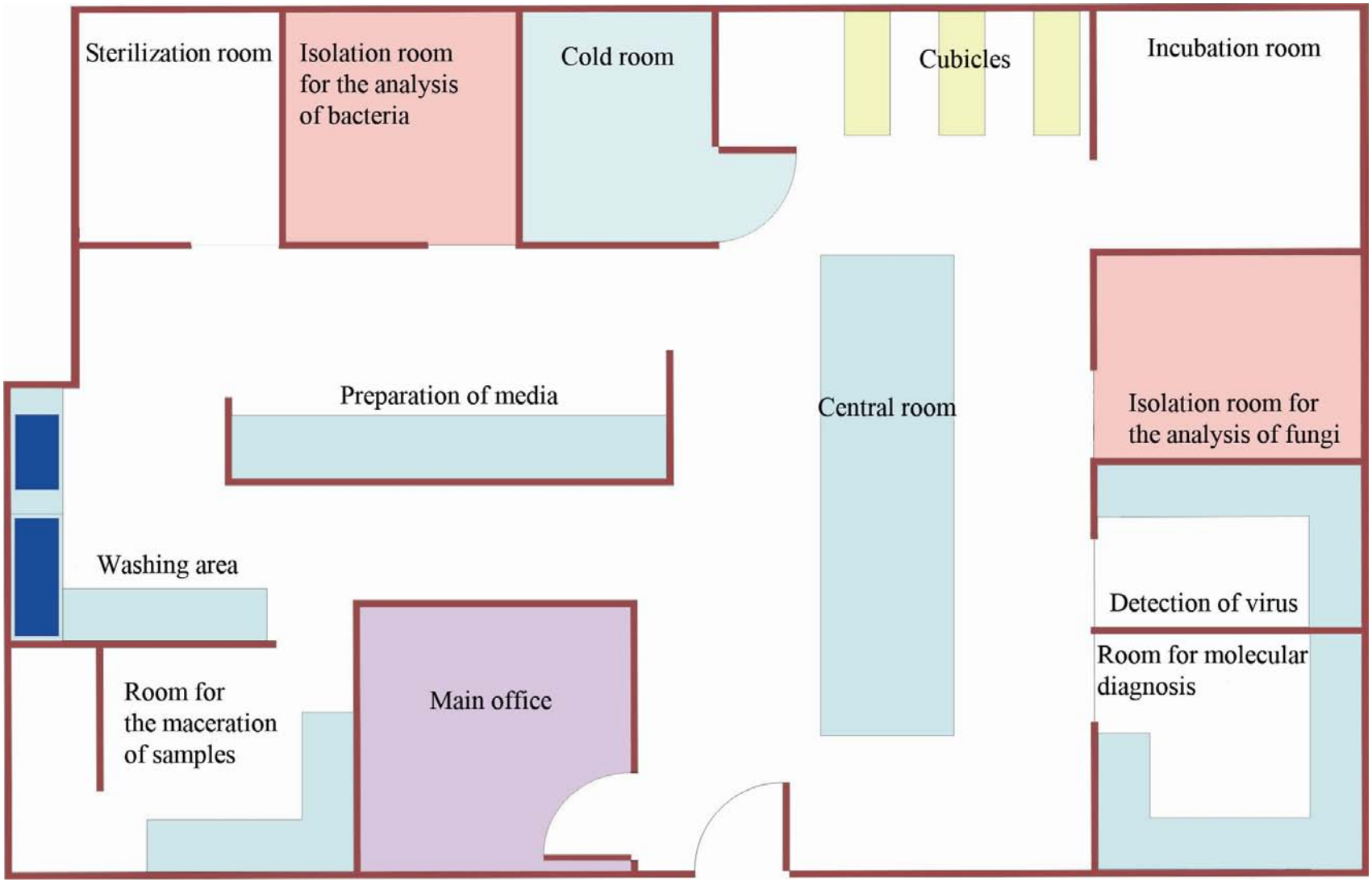


Figure 1. Map of the Germplasm Health Laboratory (GHL).

Figure 2. Premises of Germplasm Health Laboratory (GHL). a) area for preparation of media, b) isolation room for the analysis of bacteria, c) isolation room for analysis of fungi, d) sterilization room, e) central room, f) incubation room, g) room for the maceration of samples, h) room for molecular diagnostic, i) detection of virus.

⚠ **Health hazard: The laboratory design (distribution of areas, facilities, work processes, etc.) should be suitable for keeping enough safety. The laboratory, including the transit areas, exits, doorways, equipment and facilities must be in perfect order and cleanliness, establishing a regular maintenance for that purpose.**

⚠ **Health hazard: The GHL should have facilities for emergency action and fire elements that can be easily accessed requiring that all personnel know their operation.**

3. Production and postharvest handling

For the GRP of CIAT the production and conservation of seeds of beans, tropical forages, and cassava must satisfy standards of quality required for international distribution.

Bean and forages germplasm is produced on controlled plots in the stations of Palmira, Popayan and Santander de Quilichao, and in isolated fields, glass-houses or mesh-houses (Palmira, Popayán) where, in addition to other measures of plant health control, any plant showing symptoms of a known disease considering as transmitted by seed, is removed immediately.

During the production process, in addition to the supervision by scientists of the program or respective unit, the production places are visually inspected by an officer of ICA, who verifies the proper handling of plant health in the lots for seed production, and collects information to issue then a phytosanitary certificate.

The conservation of germplasm and its distribution includes a set of activities such as procurement processes, increase, multiplication, regeneration, characterization, physical and physiological quality control and storage, in which health plays a decisive role (Flowchart 1).

Once the materials have reached their physiological maturity, they are harvested, classified, carefully selected, inspected to discard any that at visual signs shows symptoms of plant health problems, and checked to not be mixed with sclerotia, cysts, insects, part plants, seeds of other crops or any other strange material. After this cleaning, the material is sent to the GHL where health is assessed for pathogens of quarantine significance.

4. Methodology to certify the genetic material for future transfer

Not all organisms, viruses and other agents that are housed in the seeds internal or externally are pathogenic, only those capable of producing an infectious process (disease) in the seed or in the plant, are considered as seed-borne pathogens; the rest can be saprophytic microflora or involved in the decay, or merely a contaminant that does not cause any harm or benefit and has no quarantine significance.

The vast majority of seed-borne pathogens are the same that have hit the mother plant during its development and, in one way or another, are set out in this important reproductive structure. Generally, infections occur under field conditions because it is the place where crops are grown normally and where plants interact with other components of the ecosystem.

Health germplasm mainly refers to the status of disease and the presence or absence of pests and microorganisms such as fungi, bacteria, viruses and nematodes in a sample of seeds, which represents a lot of seeds or one accession. This procedure is done to facilitate the exchange of seeds, avoiding the dispersion of pests and diseases into new regions [Kameswara et al. 2006].

The methodology used to certify the health of the genetic material to be transferred is based on the results of the inspections done during the production and processing of the seeds making preventive health control during the production cycles, minimizing the risk of pathogen transmission by seed, in addition to the results obtained in the analysis of health in the laboratory (Flowchart 2).

The laboratory inspects samples of seeds for pathogens (fungi, bacteria, virus) of quarantine concern that occur in places of production or that are considered a significant health risk in the host country or the country of destination, and that are appearing in the technical guides for the movement of germplasm (Frison et al. 1990). Additionally, it inspects the presence of nematodes (*Meloidogyne spp*. and *Pratylenchus spp*.) and some beetles (*Acanthoscelides sp*. and *Zabrotes spp*) that can cause damage to the seeds (Table 2).

The methods to detect pathogens can vary for each specimen and host, and definite procedures for the precise identification of most pathogens are required [Kameswara et al. 2006].

When a country requires additional tests with regard to the health of seed for other pathogens, the GHL is able to perform them. In addition, the laboratory has the physical and human resources for the carrying out of molecular diagnostic tests for various phytopathogens.

We should note that the seed samples to be analyzed in the case of beans, tropical pastures and forage legumes come from the Area of Conservation and packaging, after cleaning and quality control. In the case of cassava, they come from the *in vitro* conservation laboratory.

To perform laboratory analysis, samples are taken at random from the packages to be sent or stored of not less than 200 seeds; the main sample is divided into two main sub-samples of 100 grains and is packed separately in new paper bags. The samples are received in the GHL in this way. Upon entering the germplasm into the GHL an internal sequence number is assigned to facilitate their management and evaluation, which we will record as the GHL sample number.

Upon receiving the samples, at the same time we receive an electronic report of the accessions with their respective backgrounds, which is checked for corresponding to the samples received. Immediately, an Excel table is generated where are all the analyses to be performed are registered, with their respective number of GHL sample (Annex 1).

This GHL sample number is written on the two bags received with the seeds for the carrying out of the respective analyses. Each sub-sample is processed according to the group of pathogens to be tested through established procedures for each one, which are described below.

4.1 Detection of fungi

The fungi are defined as eukaryotic organisms, producers of spores, without chlorophyll or other photosynthetic pigments, which reproduce sexual and asexually, and may be amoeboid or unicellular, but usually show divided somatic structures, filamentous ones called hyphae and surrounded in typical way by cell walls containing chitin, cellulose, or both substances, along with many other complex organic molecules [Alexopoulos & Mims, 1979; Argawal & Sinclair, 1987].

These pathogens can be carried and transmitted by seeds and this way is the most important means of perpetuation of plant pathogenic fungi. Some fungi enter the plants and seeds through natural openings such as hydathodes, hilum, micropyle, stomata, and through injuries by hail, heavy rain, sand, animals, insects, humans and other micro-organisms, although other fungi use means of pressure, enzymatic action or both to penetrate directly into the plants and seeds [Argawal & Sinclair, 1987].

Infectious fungal plant diseases produce a wide variety of symptoms including seed rots, pre-and post emergence damping-off, chlorotic and necrotic spots, canker, seedlings blights, sooty molds,

bunt, galls, smuts, scabs, pod and seed disease, wilt and pustules. Disease symptoms result from the action of toxic or growth-stimulating metabolites, depletion of hosts nutrient, and/or mechanical displacement of host tissues [Argawal & Sinclair, 1987].

In the Health Germplasm Laboratory one determines the genera of fungi that appear in the seeds, taking special interest in quarantine fungi (Flowchart 3). This determination is made according to the methods of Blotter and Agar plate approved as standard methods by the International Association for the Evaluation of Seeds (ISTA) [Kameswara et al. 2006] and based on taxonomic keys for identification of imperfect fungi by Gilman, 1957; Ellis, 1976; Nelson et al. 1983; Zillinsky, 1983; Hanlin, 1990; Hanlin, 1998; and Barnett et al. 1998.

4.1.1 Major fungi affecting the bean crop

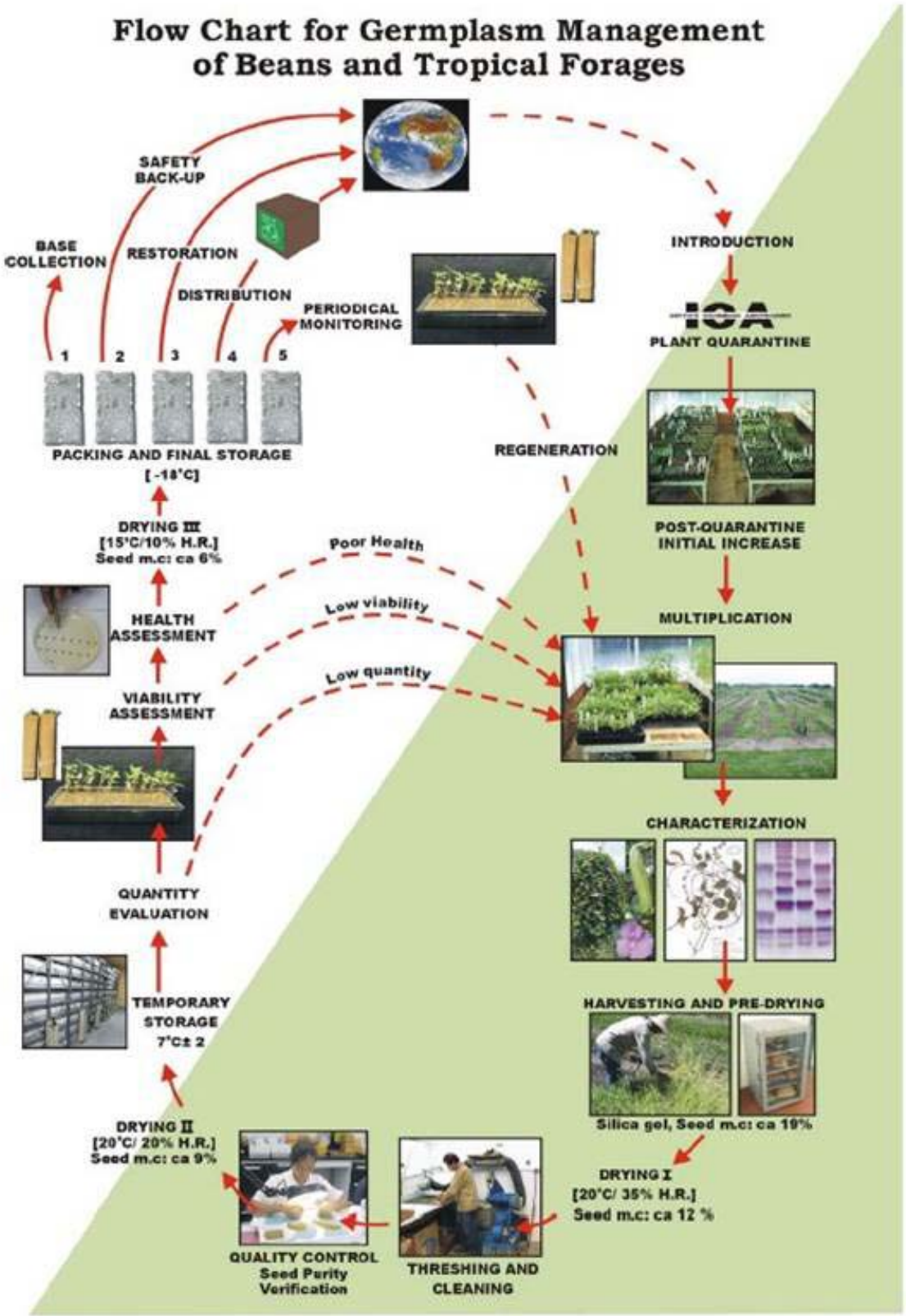
The bean production is limited because of severe attacks of diseases and pests. Many fungi can be borne internally or as surface contaminants in seeds of *Phaseolus* sp. Most internally borne fungi are located inside the seed coat and some infection may occur in the cotyledons or embryo. [Schwartz & Morales, 1994].

The effect that the bodies transmitted by seed have in the germination of beans is not well documented; nevertheless, it is known that many fungi transmitted in seeds decrease the germination in the field [Schwartz & Morales, 1994].

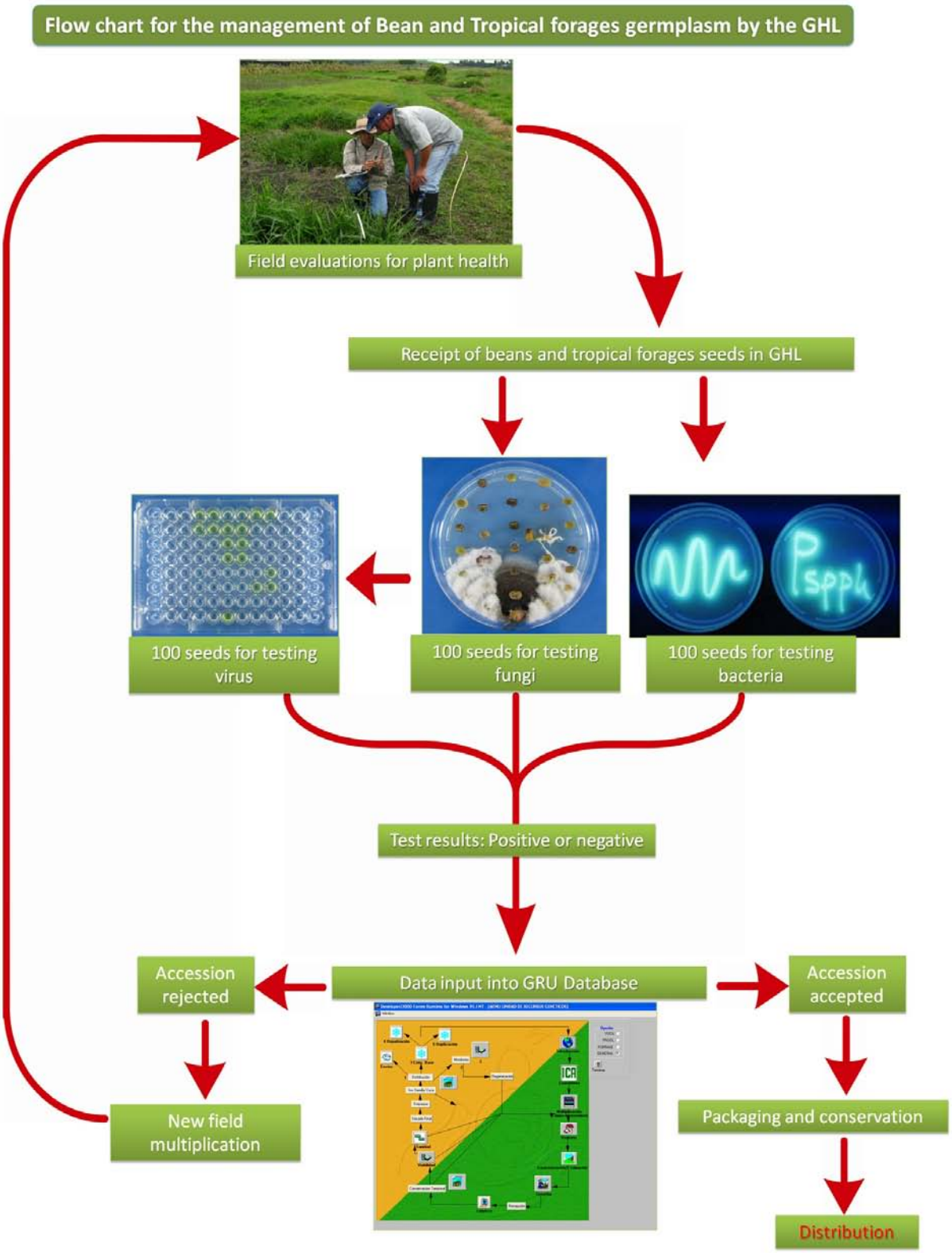
4.1.1.1 Spot caused by *Alternaria* spp.

Alternaria spp. are wound parasites. They usually form lesions only on older or senescent plant tissues during cool periods of high humidity. However, *A. tenuis* can also penetrate the leaf directly or through stomata [Schwartz, 1994]. In general, losses in yield by *Alternaria* are not significant [Cardona et al. 1995]. *Alternaria* conidia have transverse and longitudinal septa, and are known as dictiospores, besides they are brown and pointed (Fig. 3).

Figure 3. Conidia of *Alternaria alternata* as viewed through optical microscope.



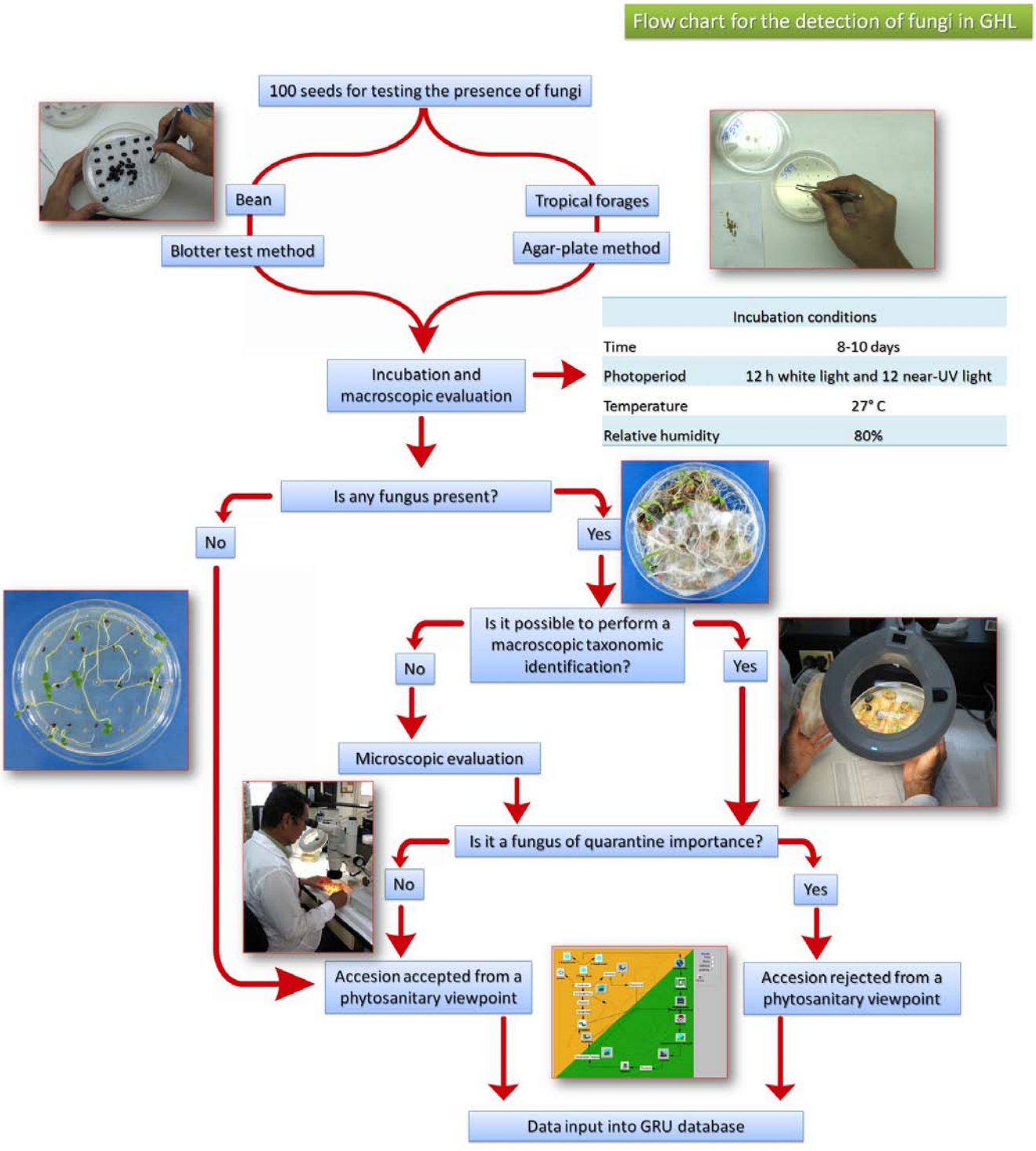
Flow chart 1. Diagram for the management of bean and forages germplasm.



Flow chart 2. Activities for carrying out diagnostic tests of plant quarantine in the GHL.

Table 2. Quarantine pathogens evaluated in the GHL.

Bean	Tropical Pastures
Fungi	
Alternaria spp.	*Alternaria alternata* (Fr.) Keissler, Synonym*: Alternaria tenuis* (Nees).
Ascochyta phaseolorum Sacc. Current name: *Phoma exigua* var. *exigua* Sacc. 1879	*Ascochyta* sp., *Ascochyta graminicola* Sacc., *Ascochyta paspali* (H. Sydow) Punith
Botrytis cinerea Pers. (Teleomorph. *Sclerotinia fuckeliana* (of Bary) Fuckel)	*Botrytis cinerea* Pers. (Teleomorph. *Sclerotinia fuckeliana* (de Bary) Fuckel)
Cercospora canescens Ellis & G. Martin	*Cercospora canescens* Ellis & G. Martin Synonym: *Mycosphaerella cruenta* Latham,
Colletotrichum gloeosporioides (Penz.) Penz. And Sacc.	*Colletotrichum gloeosporioides* (Penz.) Penz. And Sacc.
Colletotrichum lindemuthianum (anamorph.), Gloromella cingulata (teleomorph.)	*Colletotrichum truncatum* (Schwein.) Andrus
Colletotrichum truncatum (Schwein.) Andrus	*Colletotrichum* spp.
Fusarium oxysporum Schltdl. Y *F. solani* f. sp. *solani*	*Curvularia* spp.
Macrophomina phaseolina (Tassi) Goid	*Drechslera setaria* (S. Ito and Kuribay.) Dastur.; *D. sacchari* (Butler) Subram. and Jain
Phoma exigua Desmaz. var. *diversispora* (Bubak) Boerema	*Fusarium oxysporum* Schltdl.
Phomopsis subcircinata (anamorph.), *Diaporthe phaseolorum* (teleomorph)	*Helminthosporium* spp.
Phaeoisariopsis griseola (Sacc.) Ferraris	*Macrophomina phaseolina* (Tassi) Goid
Rhizoctonia solani Kühn (Teleomorph *Thanatephorus cucumeris* (frank) Donk.)	*Phoma exigua Desmaz.* var. *diversispora* (Bubak) Boerema
Sclerotinia sclerotiorum	*Phoma sorgina* (Sacc.) Boerema, Dorenb, and van Kest.
Sclerotium rolfsii Sacc. (Teleomorph. *Corticium rolfsii* Curzi)	*Phomopsis sp.* (Teleomorph. *Diaporthe phaseolorum* (Cooke and Ell.) Sacc.)
	Pestalotiopsis sp.
	Phaeoisariopsis griseola (Sacc.) Ferraris
	Pyricularia oryzae Cavara o *Pyricularia grisea* (Cooke) Sacc.
	Rhizoctonia solani Kühn (Teleomorph *Thanatephorus cucumeris* (Frank) Donk.)
	Sclerotium rolfsii Sacc. (Teleomrph. *Corticium rolfsii* Curzi)
	Sphaceloma arachidis Bitanc. & Jenk.
	Sphacelia sp. (Teleomorph. *Claviceps* spp.)
	Tiletia aryesii Berk.
Bacteria	
Xanthomonas axonopodis pv. *phaseoli*	*Xanthomonas axonopodis* pv. *phaseoli*
Pseudomonas syringae pv. *phaseolicola*	*Pseudomonas syringae* pv. *phaseolicola*
Curtobacterium flaccumfasciens	*Curtobacterium flaccumfasciens*
Pseudomonas fluorescens Biotype II	*Pseudomonas fluorescens* Biotipo II
Viruses	
	Centrosema Mosaic Virus (CMV)
Peanut Mottle Virus (PeMOV)	Peanut Mottle Virus (PeMOV)
Bean common mosaic virus (BCMV)	Bean common mosaic virus (BCMV)
Bean southern mosaic virus (BSMV)	Bean southern mosaic virus (BSMV)
Insects	
Acanthoscelides sp.	*Acanthoscelides* sp.
Zabrotes spp.	*Zabrotes* spp.
Nematodes	
Meloidogyne spp.	*Meloidogyne* spp.
Pratylenchus spp.	*Pratylenchus* spp.



Flow chart 3. Diagnosis of quarantine fungi in the GHL.

Leaf symptoms include small spots of irregular shapes, and reddish brown color, surrounded by a darker edge that when enlarged can make concentric rings in the leaf blade. Seeds can be carriers of Alternaria spp. by internal way (cotyledons, embryo) [Cardona et al. 1995, Schwartz, 1994], although it is commonly considered as a saprophytic or a weak parasite contaminating also the seed cover and the pericarp [Neergaard, 1977], and is not considered as an important cause for phytosanitary rejection of the seeds.

4.1.1.2 Spot of Ascochyta

This disease caused by *Ascochyta phaseolorum* and *Phoma exigua* var. *diversispora* is very severe in regions with altitude higher than 1,500 masl, with cold to moderate temperatures and high humidity. The injuries are spots of brown to gray color almost circular and concentric; the result of these injuries is the severe burn of the leaves [Cardona et al. 1995]. The injuries may form rings around the stem and cause plant death [Schwartz, 1994].

The fungus can spread systemically throughout the plant including the seeds and cause premature leaf drop and death of the plant. The fungus can be transmitted by contaminated residues of past harvest and by seed, establishing firmly in the testa. It is difficult to control by seed treatment [Cardona et al. 1995; Neergaard 1977, Schwartz, 1994].

4.1.1.3 Gray mold caused by *Botrytis cinerea* (anamorph) and *Botrytinia fuckeliana* (teleomorph)

Gray mold is a common rot in beans, favored by injuries in plant tissues and can be more severe during periods of high humidity. Seedlings may be attacked, but usually the damage is more common in older plants that have pods in contact with the soil [Cardona et al. 1995].

The tissues infected by this pathogen can form various structures such as black stromata and sclerotia on which conidia and apothecia may develop similar to those produced by *Sclerotinia sclerotiorum* [Cardona et al. 1995]. The sclerotia of this fungus are usually attached to the casing of the seeds and to the pericarp [Neergaard, 1977].

4.1.1.4 Leaf spot caused by *Cercospora canescens*

Symptoms include brown or rust-colored lesions, which may coalesce and vary in shape and size. An often found lesion by *C. canescens* occurs in older leaves, but also on pods and branches, and it is irregularly shaped. The lesions may have a grayish center with a slightly reddish border. Lesions may dry and portions fall out, leaving a ragged appearance (Cardona et al. 1995).

This fungus can become seed-borne (Schwartz, 1994]. It has been reported that for *Cercospora kikuchii* in soybean the infection is in seed coat [Neergaard, 1977].

4.1.1.5 Anthracnose caused by *Colletotrichum lindemuthianum*

Anthracnose is probably the most important bean disease throughout the world. The disease can be devastating. It causes complete yield losses on susceptible bean cultivars, or when badly contaminated seed is planted and favorable conditions prevail during the growing season [Pastor-Corrales & Tu, 1994].

Disease development is favored by moderate temperatures between 13 and 26 °C and relative humidity greater than 92%. Symptoms appear on the lower surface of leaves, located along the veins and petioles, as small brick-red to purple spots [Cardona et al. 1995].

The fungus can survive in both seed and infected crop residues. In the seed it can survive for at least two years, but this can vary depending on environmental conditions. Symptoms on pods appear as nearly circular spots that become dark cankers delimited by a black ring; these, in conditions of low temperature and high humidity contain pink masses of spores [Cardona et al. 1995]. This fungus can become seed-borne after penetrating pod walls [Schwartz & Morales, 1994].

Colletotrichum lindemuthianum survives as dormant mycelium within the seed coat, sometimes even in the cells of cotyledons, as spores between cotyledons, or elsewhere in the seed. Humidity of more than 92% is required during conidium's germination, incubation and sporulation [Pastor-Corrales & Tu, 1994].

4.1.1.6 Fusarium yellows or wilting caused by *Fusarium oxisporum* f. sp. *Phaseoli*

Infection usually occurs through wounds in the roots or the hypocotyl and causes a reddish discoloration in the vascular system of the root, the hypocotyl, the stem and the petioles. The symptoms are the yellowing and premature aging of the leaves, and progress to the young leaves located at the top of the plant, and result in a decrease in the production of pods and seeds [Cardona et al. 1995]. The pathogen is capable of penetrating intact root tissue, usually near the root tip. After penetration, hyphae of the pathogen move inter- and intracellularly and invade the developing xylem vessels. The fungus can also grow on the surface of infected plant tissue by producing abundant pink mycelium and conidia. In *F. solani* f. sp. *solani* conidia can remain dormant in the soil or in infected tissue, with little mobility for a long time [Abawi, 1994].

The pathogen can be carried by seed in the form of spores adhering to the surface of the testa, by infected soil, crop residues, or drainage and irrigation water [Abawi, 1994]. The attacks on seedlings produce smaller stunted plants [Cardona et al. 1995]. In Blotter tests the infected seed forms a cottony white mycelium that easily colonizes nearby seeds (Fig. 4).

Figure 4. Bean seeds attached by *Fusarium oxisporum*.

4.1.1.7 Ashy Stem Blight caused by *Macrophomina phaseolina*

The plant pathogen has a wide range of hosts. The disease is more prevalent and damaging to beans that are exposed to drought and warm temperatures [Cardona et al., 1995].
Symptoms may appear after soil-borne mycelia or sclerotia germinate and infect seedling stems near the soil line at the base of developing cotyledons. The fungus produces black, sunken cankers, which have a sharp margin and often contain concentric rings. The plant's growing tip may be killed or the stem broken where it is weakened by the canker. Infection may continue into the hypocotyl and root region or the primary leaf petioles. Older seedling and plant infections may cause stunting, leaf chlorosis, premature defoliation, and plant death [Schwartz, 1994].

In bean seed the disease develops as a dark mycelium affecting the cotyledon and root of the seedling. The fungus produces black globose pycnidia that contain large, colorless, one-celled, fusiform conidia, which are pointed at one end and rounded at the end. The straight or slightly curved conidia are 15-30 µm long [Schwartz, 1994] (Fig. 5).

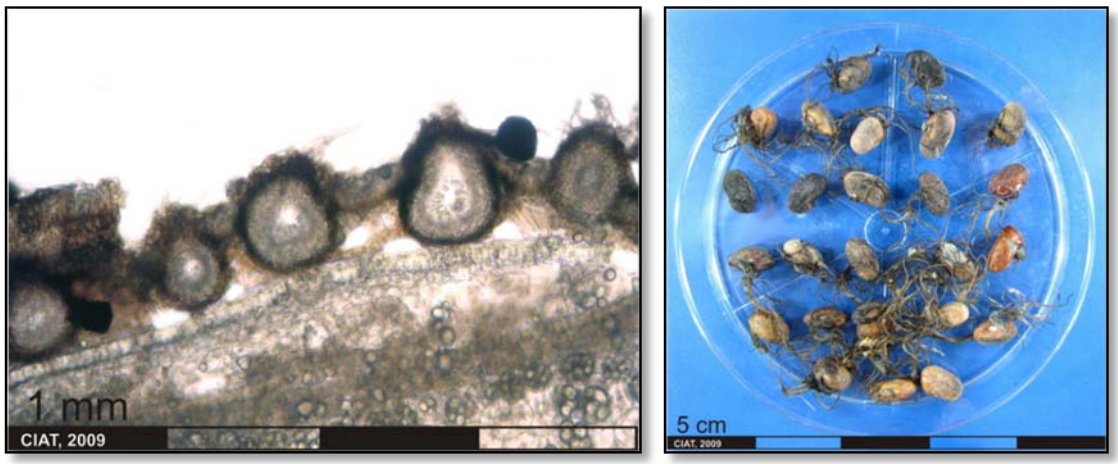


Figure 5. Bean seeds infected with *Macrophomina phaseolina*. On the left side you can see a longitudinal cut of pycnidia on the testa of the bean seed (note the conidia within pycnidia). On the right side you can see the symptoms on bean seeds with *M. phaseolina* (black coloration on bean seed testa and radicle rot).

4.1.1.8 Angular leaf spot caused by *Phaeoisariopsis griseola*

Angular leaf spot of beans, caused by the fungus *Phaeoisariopsis griseola* is a serious disease of beans, which has occurred in several tropical and subtropical countries.

Symptoms of angular leaf spot caused by *Phaeoisariopsis griseola* occur in all aerial parts of the plant. The lesions are most common in the primary leaves. Initially they are gray or brown and may be surrounded by a chlorotic halo, with defined margins. Approximately nine days after infection the spots become necrotic, and well defined with a typical angular shape [Correa-Victoria et al. 1994].

Lesions on pods can be seen as oval to circular spots with reddish-brown centers, sometimes surrounded by darker edges. Infected pods have poorly developed or wrinkled seeds [Correa-Victoria et al. 1994].

Moisture is probably the single most important factor in the development of epidemics of angular leaf spot, and is a prerequisite for infection and sporulation. Infection and disease development may occur at a wide temperature range, 16 to 28 °C. Contaminated seed is a source of primary inoculum. The fungus is usually associated with the hilum area of the seed coat, although the fungus may be in the hilum or in other parts of the seed coat [Correa-Victoria et al. 1994].

4.1.1.9 Diaporthe pod blight caused by *Phomopsis subcircinata* (anamorph) y *Diaporthe phaseolorum* (teleomorph)

Initial symptoms on leaves appear as irregularly shaped patches of brown color, with well-defined border. Dark pycnidia (sexual structures) and occasionally perithecia (asexual structures) are distributed over the entire surface of the lesion. Infections may occur on the pods, as discolored lesions with presence of pycnidia. The fungus can be seed-borne transmitted in soybeans and beans [Cardona et al., 1995].

Diaporthe phaseolorum produce hyaline and oblong ascospores measuring 10-12 by 2-4 µm and having one septation. The ascospores are produced inside black perithecia, which measure 300 µm in diameter.

4.1.1.10 Web blight caused by *Rhizoctonia solani* (asexual stage), *Thanatephorus cucumeris* (sexual stage)

This disease is more prevalent in the humid tropics with moderate to high temperatures, and under these conditions the disease can cause very high losses and completely destroy a crop of beans [Cardona et al. 1995].

The first symptoms are small circular water-soaked lesions on the leaves. In wet conditions the lesions grow rapidly and coalesce to form brown areas surrounded by dark borders. Eventually the affected area may cover the whole plant, joining the leaves, petioles, flowers and pods with a web-shaped mycelium, if environmental conditions permit [Cardona et al. 1995].

Bean pods may become infected during the grain-filling stage. Young pod infections appear as light-brown, irregular-shaped lesions which frequently coalesce and destroy the pod. Seeds can become infected in the endosperm and radicular end of the embryo and on the seed-coat surface [Galvez et al., 1994]. The main source of inocula that can initiate infection are sclerotia and mycelium fragments, either free in the soil or present in colonized debris [Cardona et al., 1995].

4.1.1.11 Root rot caused by *Rhizoctonia solani* (asexual stage), *Thanatephorus cucumeris* (sexual stage)

The disease develops under moderate to low temperatures and soil moisture from moderate to high. The pathogen carried internally in the seed can attack severely the seedling and cause rot of lower stem (damping off), root rot and stem canker [Cardona et al. 1995].

The characteristic symptoms on infected plants are reddish brown, sunken lesions on the stem and taproot. As infection progresses, sunken cankers enlarge and those that are close together may coalesce and griddle the stem, retard growth, and eventually kill the plant.

Many times you can find sclerotia on the canker or inside the stem. Sclerotia serve as a source of inoculum and can survive in the soil. This pathogen can infect pods and branches in contact with the soil surface, causing water-soaking spots and sunken reddish brown lesions with distinct margins around them. The infected seeds are discolored and can carry the fungus [Abawi, 1994].

4.1.1.12 White mold caused by *Sclerotinia sclerotiorum*

This pathogen is spread worldwide, but is more important in temperate zones of the northern hemisphere. The pathogen colonizes senescent plant tissues, and then enters the host through the wound tissue. Initially it causes wet or watery lesions followed by the growth of white, cottony mold [Cardona et al. 1995].

Infection of the stem is accompanied by the wilting of foliage. After infection the fungus produces sclerotia, which when they fall to the soil spread by forming reproductive structures (apothecia) that release spores and infect plant tissues. The fungus can also be transported internally by the seed in the cotyledons. The infected seeds are completely colonized by the fungus before germination and/or seedling emergence [Cardona et al. 1995; Schwartz & Steadman, 1994]. The growth of this fungus is similar to that of *Sclerotium rolfsii*, but differs by the production of thick, dark sclerotia.

4.1.1.13 Southern blight caused by *Sclerotium rolfsii*

The disease is found in many regions, especially in those with high temperature and humidity. Initially symptoms appear as a yellowing of lower leaves accompanied by a dark and watery lesion, located on the stem or hypocotyl below the soil surface. At the base of the stem the presence of a white mycelium with round and white to light brown sclerotia is often seen. Bean pods in contact with the soil may also become infected and rot [Cardona et al. 1995].

Mycelial strands originating from infected debris or germinating sclerotia, penetrate bean tissue through natural openings, wounds, or by direct penetration of intact tissue. After penetration, the fungus ramifies very rapidly in stem and root tissues resulting in hydrolysis and death of tissues in advanced stages of invasion [Abawi, 1994]. The form of transmission in seed is not yet entirely understood [Neergaard, 1977].

When the diagnosis is done by for the blotter test the fungus produces in the seeds a white densely woolly mycelium with production of numerous globose sclerotia of olive brown to light brown colors (Fig. 6). Notably, its mycelium is sterile because it does not produce conidia [Gilman, 1957].

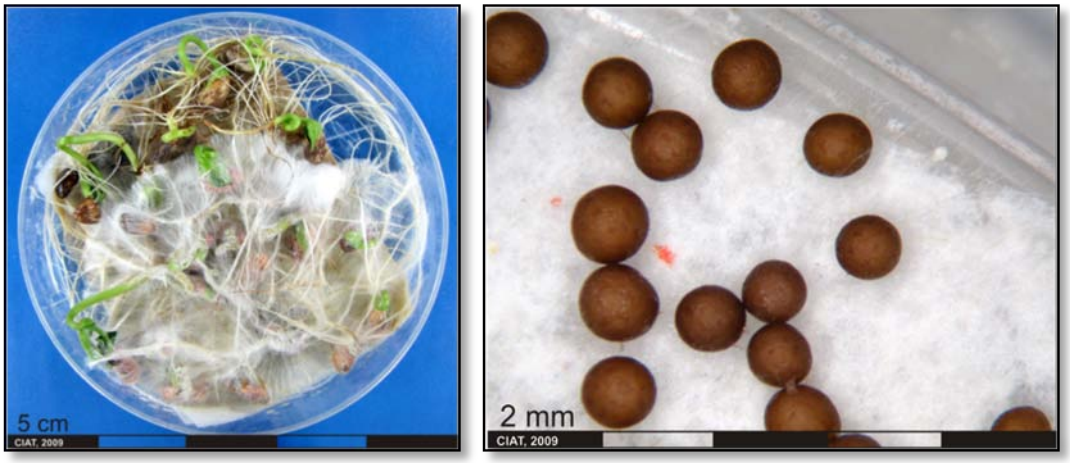


Figure 6. Bean seeds infected with *Sclerotium rolfsii* (left) and sclerotia produced after 8 days of mycelial growth (right).

4.1.2 General procedures for the diagnosis of fungi in bean seeds (*Phaseolus* spp.)

For the diagnosis of fungi in bean seeds one uses the moist chamber technique or Blotter Test [Kameswara et al. 2006], which is described next:

The 100 seeds used in this analysis are divided into 4 plastic boxes (25 seeds/dish). Each box is marked with the sample number initially assigned by GHL, taking care that the number on the bag of seeds be equal to the number marked on each box. The four boxes of the same accession are attached with tape (Fig. 7).

Figure 7. Bean seeds being deposited in petri dishes with paper moistened with distilled water (Blotter technical test).

Place the seeds at uniform intervals on blotting paper moistened with 20 ml of sterile water inside a plastic box (Fig. 8).

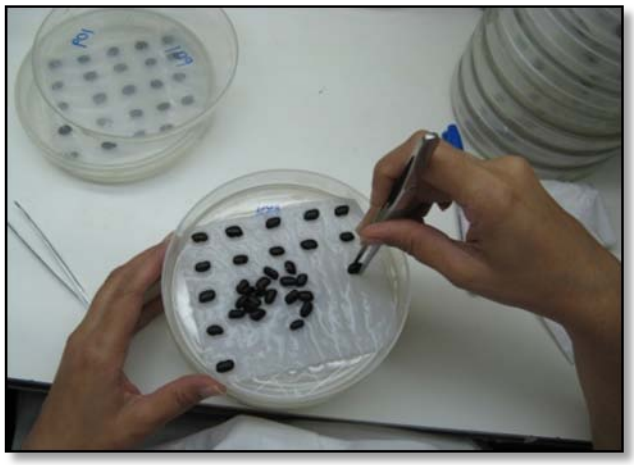


Figure 8. Distribution of seeds in 5 rows and 5 columns per box.

At the end of the seed setting, the dishes properly identified and sealed, have to be moved to the incubation room set at a temperature between 22 and 28 °C, with cycle light/darkness of 12 h/12h, whose light source corresponds to fluorescent cool white light with good emission of near to ultraviolet wavelength, where they remain for 8 to 10 days (Fig. 9).

After incubation, we proceed with the corresponding readings. Each reading is recorded in the Excel table designed for the recording of results, taking into account the GHL sample number (Annex 1). In this table the quarantine and saprophytic fungi that sometimes occur are registered. If any of the seeds appear to be infected with any quarantine fungus, the accession is rejected.

Figure 9. Incubation of seeds under controlled conditions.

To determine the presence of the characteristic structures of each of the fungi listed in Table 2, we first observe them with a magnifying glass and then under the stereoscope. If there is any difficulty in the characterization of a fungus, a montage on slides is done, making a sweep of it, allowing the observation of reproductive structures and thus a better identification (Fig. 10).

Figure 10. Macro and microscopic analysis of the seeds in order to identify the presence and growth of fungi genera.

The material already germinated and evaluated that shows no fungus is used to carry out virus testing (see 4.3.3).

⚠ Health hazard: The manipulation of fungi and bacteria should be performed in laminar flow chamber to avoid contamination and human health risks.

⚠ Health hazard: When using lighters one must be careful to avoid fires.

4.1.3 Principal fungi in tropical pasture crops and forage legumes

Many seed-borne pathogens can cause death to promising legume seedlings before and after the emergence of these, or may reduce its vigor. In grasses, most of the pathogens found in seeds are smut, false rusts, ergot, and inflorescence disease. Most of them reduce seed viability and cause death of the seedings at the stage of pre- and post-emergence [Lenné & Ordoñez, 1991].

Determining the health of tropical forage seeds is a bit more complex because many species of legumes and grasses considered as important forages are involved. The used methodology is similar to the one used for beans, taking into account the pathogens and recommended culture media. For the analysis of these seeds the PDA (Potato Dextrose Agar) test is used. A summary of the main fungi that affect this group are shown in Table 2.

4.1.3.1 Leaf spot caused by *Alternaria*

Alternaria leaf spot is caused primarily by the genera *Alternaria alternata* (Fr.) Keissler, synonym: *Alternaria tenuis* (Nees). The spots are formed as concentric rings of dark tissue. Symptoms appear first on the tip of young leaves as small light spots on the edges [Lenné & Trutmann, 1994].

This species of *Alternaria* usually is characterized by long conidial chains and the conidia are markedly polymorphic. The characteristic of this fungus is its straight conidial growth, with

cylindrical bottle shaped form, honey-colored or blackish green. Its mycelial growth on PDA is dark green or blackish (Fig. 11).

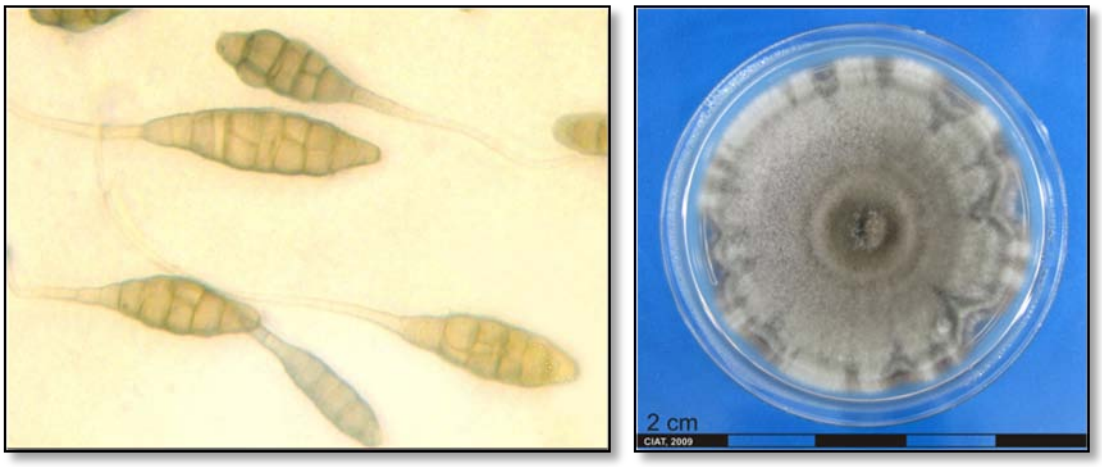


Figure 11. Conidia and mycelial growth of *Alternaria alternata.*

The fungus has been reported in a wide range of hosts within which are species of the genera *Desmodium* and *Stylosanthes.* In the Germplasm Health Laboratory it has been mainly found in seeds of beans, *Brachiaria* spp. and *Centrosema* spp. [Lenné & Trutmann, 1994].

Its geographical distribution is cosmopolitan. Besides, its infectious process occurs in seed externally on the seed coat and pericarp [Neergaard, 1977], although it has also been found in embryos.

4.1.3.2 Leaf spot caused by *Ascochyta*

This disease is caused by fungi of the genera *Ascochyta* such as *Ascochyta graminicola, Ascochyta paspali,* in addition to the fungus *Phoma exigua* var. *diversispora*. Lesions develop as yellowish brown to grey-coloured elongate spots, spreading down the leaf to the tip and subsequently affecting the whole leaves [Lenné & Trutmann, 1994]. This fungus is also present in seeds (Fig. 12) and differs from *Phoma* spp. for its papillate pycnidium (with nozzle shorter than in *Phoma* spp). This fungus is found mainly in the testa of seeds, but as it relates to *Phoma* it can be carried in the internal tissues.

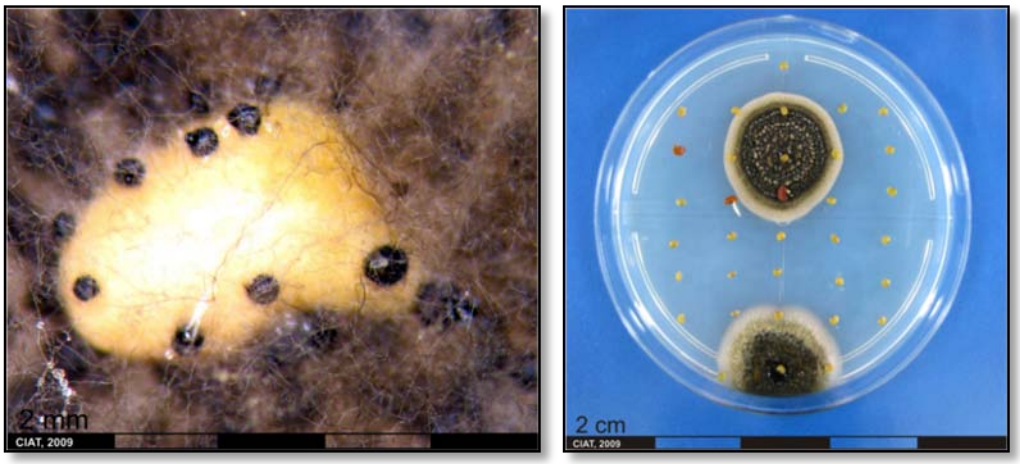


Figure 12. Seed with presence of pycnidia of *Ascochyta graminicola* and its mycelium growth on PDA.

In tropical forages its host genera are: *Andropogon* spp., *Macroptilium atropurpureum* and *Paspalum* spp., and its geographical distribution extends over Africa, Asia, Australia, New Zealand, the Caribbean, Pacific Islands and South America. Conidia are spread by raindrop splash and the fungus has been isolated from seeds.

4.1.3.3 *Botrytis* head blight or inflorescence blight and stem rot caused by *Botrytis cinerea*

Botrytis cinerea Pers (teleomorph. *Sclerotinia fuckeliana* (of Bary) Fuckel) is an important pathogen of many crops inside and outside the greenhouse. The fungus produces brown, water-soaked lesions that can become dry and grayish, which can ruin the inflorescence and the apical part. Brown lesions on the stem develop where the disease occurs [Lenné & Trutmann, 1994].

Its main host is the genus *Stylosanthes*, being reported in the species *S. guianensis, S. hamata, S. humilis* and *S. viscosa*, and with a distribution in Colombia and Zimbabwe. Conidia are mainly airborne and may also be carried by raindrop splash [Lenné & Trutmann, 1994]. This fungus is transported mainly by seed in the seed coat (testa), and pericarp and occasionally in the hilum [Neergaard, 1977].

Colonies are grey or grayish brown and powdery with stout, smooth, brown, branched conidiophores, frequently 2 mm or more long.

4.1.3.4 Leaf spots caused by *Cercospora* y and related genera

Leaf spot caused by *Cercospora* species have been widely reported in tropical forages. The most common species are *Cercospora canescens* and *Pseudocercospora bradburyae*. The disease symptoms are leaf spots from angular to circular, black or brown with chlorotic haloes, which expand, especially under humid conditions, causing chlorosis, necrosis and premature defoliation [Lenné & Trutmann, 1994]. The fungus affects mainly the stems and occasionally the seeds of legumes, being carried in the seed coat as in the case of *Cercospora kikuchii* in soybean [Neergaard, 1977].

Its geographical distribution is reported for Central and South America and in Australia, Barbados, Malaysia, Philippines, Puerto Rico and Sudan. The hosts are: *Arachis* spp., *Aeschynomene* spp., *Centrosema* spp., *Desmodium* spp., *Macroptilium atropurpureum* and *Stylosanthes* spp. [Lenné & Trutmann, 1994].

4.1.3.5 Anthracnose

The anthracnose is the most widely distributed and damaging disease affecting several forages through the tropics. The disease is mainly caused by the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., (teleomorph *Gloromella cingulata* (Stonem.) Spauld. and Schrenk, and *Colletotrichum truncatum* (Schweinitz) Andrus and Moore, causing a reduction in dry matter production with associated reduction in nutritional value and seed losses [Lenné & Trutmann, 1994].

The symptoms include leaf and petiole lesions of 1-3 mm in diameter with cream to light grey centers and dark margins, and dark lesions of 2-6 mm in length and similar in color to leaf lesions. Leaf and petiole lesions cause defoliation and develop into cankers [Lenné & Trutmann, 1994]. The seed fungus infection is within the testa and sometimes even in the cells of the cotyledons, or elsewhere in the seed [Pastor-Corrales & Tu, 1994].

Both species exhibit wide variation in morphological and cultural characteristics and it is difficult to provide a standardized description. In general, both species grow well on artificial culture media as PDA. *Colletotrichum gloesporoides* produces white to light or dark grey colonies with variable amounts of mycelium and presence or absence of setae. Spore masses vary from pale pink to bright orange; the conidia are simple oblong or ovoid (Fig. 13). *Colletotrichum truncatum* produces white to grey colonies, usually abundant setae and grey to light salmon spore masses. *C. truncatum* differs from the former by its filiform to crescent to crescent-like conidia and its mycelia growth occurs with pycnidia with structures similar to trichomes (Fig. 14).

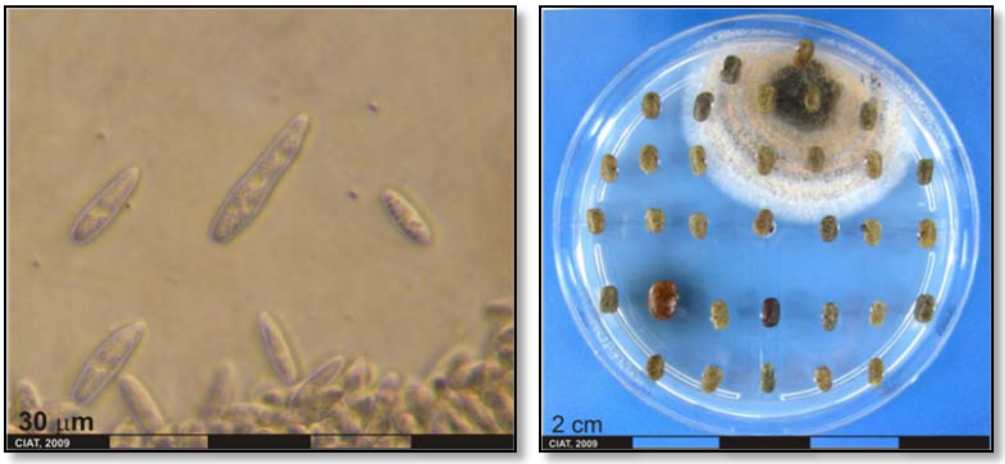


Figure 13. Conidia and mycelial growth of *Colletotrichum gloeosporioides*.

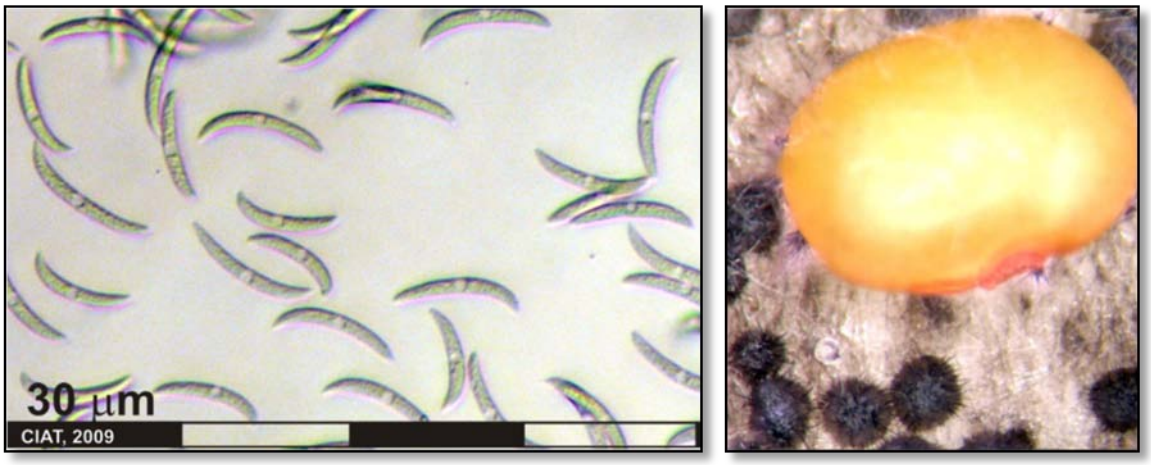


Figure 14. Conidia and mycelial growth of *Colletotrichum truncatum*.

Its hosts include *Arachis* spp., *Aeschynomene* spp., *Calopogonium* spp., *Cassia* spp., *Centrosema* spp., *Desmodium* spp., *Leucaena* spp., *Macroptilium* spp., *Pueraria* spp., and *Stylosanthes* spp., and its distribution is worldwide [Lenné & Trutmann, 1994].

4.1.3.6 Leaf spot and blight caused by *Curvularia*

This disease caused by *Curvularia lunata* and *Curvularia* sp. has been reported for Australia, Florida, North Carolina, Malaysia, Colombia and Trinidad. The leaf spot by *Curvularia* is commonly associated with rounded and small brown color lesions which rarely cause serious damage.

This fungus appears more frequently in grass seed, within the seed tissues namely the embryo, invading it from 25 to 38% in rice [Neergaard, 1977]. As its name implies, the characteristic of this genus are its curved conidia, divided into 3 or 5 segments (Fig. 15).

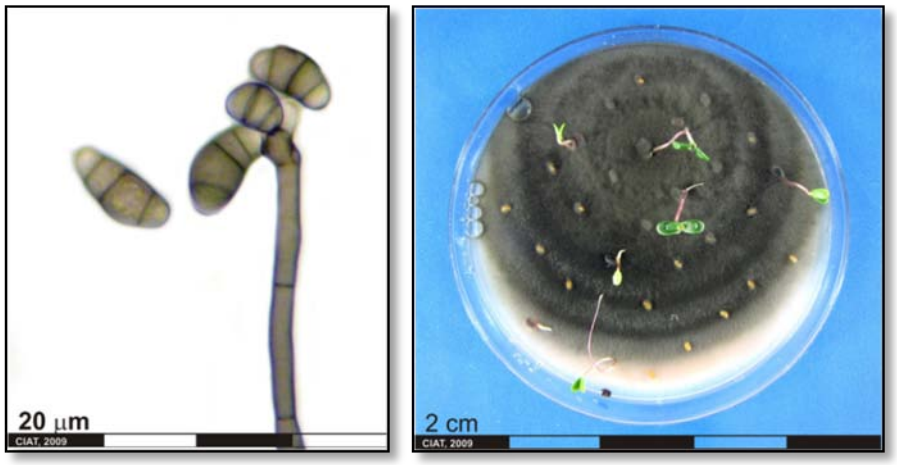


Figure 15. Conidia and mycelial growth of *Curvularia*.

Hosts are species of the genera *Centrosema, Desmodium, Stylosanthes, Zoysia matrella, Z. japonica, Sorghum halepense, Brachiaria platyphylla, Setaria glauca, Cynodon dactylon,* and rice.

4.1.3.7 Leaf and Eye spot caused by *Drechslera*

Several species of *Drechslera* such as *Drechslera setariae* (S. Ito and Kuribay.) Dastur.; *Drechslera sacchari* (Butler) Subram. and Jain, are important pathogens and are associated with symptoms such as black spots on the leaves and root rot in seedlings. Their main hosts are *Cynodon dactylon*, *Brachiaria* spp., *Pennisetum purpureum* and sugarcane [Lenné & Trutmann, 1994].

Mycelial growth on PDA is grayish with whitish spots (Fig. 16). In seeds this fungus is transported in tissues such as glumes or pericarp. Besides, the symptoms develop during the growth of the parasite in the host. The starting point of infection is outside the embryo. The remaining mycelium persists mainly in the epidermis of the pericarp. As the seed germinates, hyphae penetrate through coleoptyle, coleorhiza or root and grow upwards into the seedling [Neergaard, 1977].

Conidiophores of *D. sacchari* arise singly or in fascicles. They are straight or flexuous, mid-dark brown or olivaceous brown, up to 200um long and 5-8 µm wide. Conidia are slightly curved or occasionally straight, cylindrical or narrowly ellipsoid, pale to golden-brown, smooth, five-to nine-pseudoseptate.

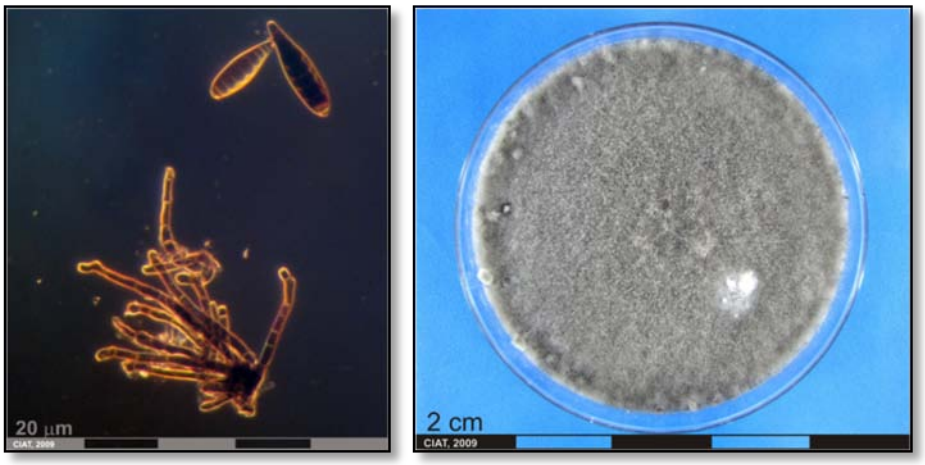


Figure 16. Conidia and mycelial growth of *Drechslera setariae*, where the present septa in their reproductive cells are noted.

Symptoms in plant appear as purple to brown lesions, of 1-4 mm long on the leaves. Lesions tend to form concentric eye-shaped spots. The severely affected leaves turn reddish brown, wilt and die. The geographical distribution is across the Americas [Lenné & Trutmann, 1994].

4.1.3.8 Leaf spot caused by *Helminthosporium* sp.

Helminthosporium species are causal agents of several diseases that occur in a huge range of plants, including wild and cultivated species. The disease is characterized by the formation of eye-shaped lesions, followed by their increased size and reddish-brown discoloration that extends to the tip of the leaf [Lenné & Trutmann, 1994]. This fungus is closely related to the genus *Drechslera.*

Hosts of this disease include *Axonopus* spp., *Cynodon dactylon, Stylosanthes guianensis*, oat, rye, rice, sugarcane, sorghum, *Brachiaria decumbens* and *Panicum maximum* [Lenné & Trutmann, 1994].

4.1.3.9 Decay by *Fusarium* and root rot caused by *Fusarium oxysporum*

In legumes the causal agent is *Fusarium oxysporum* Schlecht, synonyms: *Fusarium lateritium f. ciceris, Fusarium merismoides f. ciceris, Fusarium orthoceras* var. *ciceris* and symptoms include wilting of leaves and shoots, chlorosis of lower leaves, defoliation, internal root browning and death. Older plants are more tolerant to the disease [Lenné & Trutmann, 1994]. In this fungus one can observe fusiform conidia with numerous septa, and its growth on PDA is white and woolly profuse (Fig. 17).

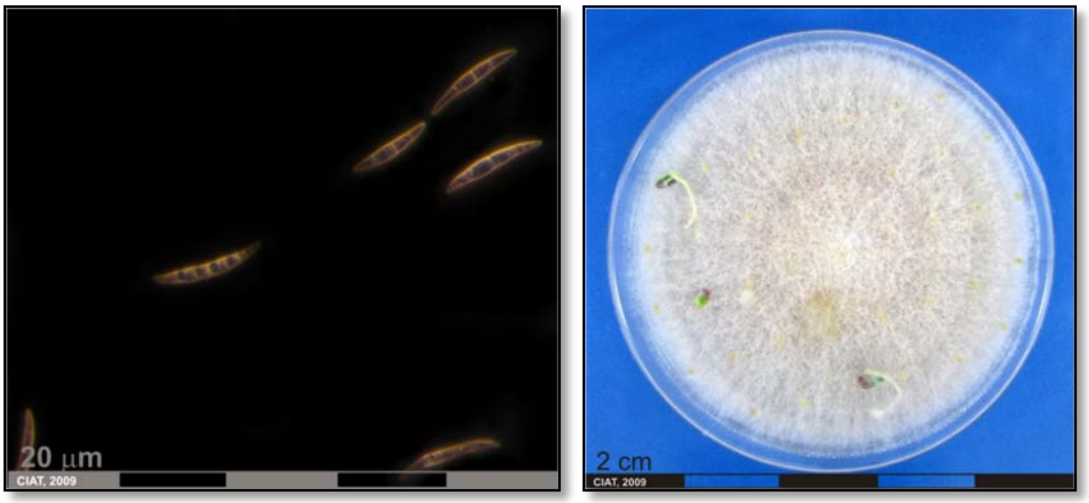


Figure 17. Conidia and mycelial growth of *Fusarium oxysprorum*.

Different species of *Fusarium* contaminate seed covers and pericarp. In addition, there have been extensive studies of blight by *Fusarium* in rye, demonstrating that the strongest infection occurs during flowering. At this point, hyphae penetrate the hilum and the primordia of coleoptile. The hilum is blocked and the transport of nutrients is hindered, leading to an incomplete grain filling. If the infection takes place later, during the maturation of the grain, the latter is affected in lower grade. This time the germinating tubes of conidia are able to penetrate only up to the pericarp. At any point between those two stages of grain development, the infection leads to gradual damage and a reduced grain weight [Neergaard, 1977].

The disease has been reported in: *Cicer arietinum, Cajanus cajan, Lens culinaris, Leucaena leucocephala* and its geographical distribution is: Algeria, Bangladesh, Chile, Ethiopia, India, Iran, Italy, Lebanon, Malawi, Mexico, Morocco, Myanmar (Burma), Pakistan, Peru, Spain, Sudan, Syria, Tunisia and USA [Lenné & Trutmann, 1994].

4.1.3.10 Root smut caused by *Macrophomina phaseolina* (Tassi) Goid

This disease causes chlorosis, severe wilting and death of most seedlings. It produces a rot in developing stalks. The root that is covered by it turns black and then rots. The stem is also attacked; the pathogen infects the fruit quickly. The infected seeds are discolored, small, wrinkled and have dirty appearance (Fig. 18) [Lenné & Trutmann, 1994]. Although some seeds show no external symptoms, the infection may be inside the seed in the hypocotyl, the radicle or in the petioles of primary leaves [Schwartz, 1994].

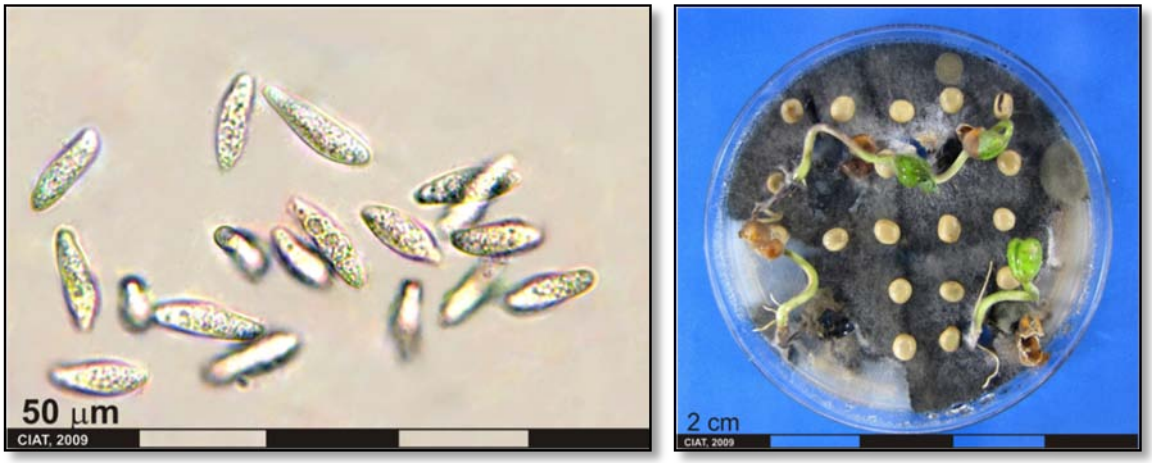


Figure 18. Conidia and mycelial growth of *Macrophomina phaseolina* on PDA artificially inoculated on *Centrosema* seeds.

The disease is found in many cultivated species: *Arachis hypogaea, Cicer arietinum, Glycine max, Stylosanthes guianensis* var. *pauciflora, Stylosanthes capitata* and *Stylosanthes humilis*, of worldwide distribution in warm and temperate regions [Lenné & Trutmann, 1994].

4.1.3.11 Leaf spot caused by *Pestalotiopsis* sp.

Initial symptoms on stems appear as scattered bright yellow spots of 1 to 2 mm of diameter that later come to stand in relief. As the disease progresses, lesions combine to form big cankers which show up on nonfunctional stems [Lenné & Trutmann, 1994]. This fungus is present in legume seeds and its mycelial growth is characterized by the presence of dark droplets with conidia that have more than 4 divisions (usually 5) and appendixes on one of its ends (Fig. 19).

Its main hosts are: *Desmodium* spp., *Cassia* spp., *Psidium guajava, Vicia faba* and various fruits and ornamental plants [Lenné & Trutmann, 1994].

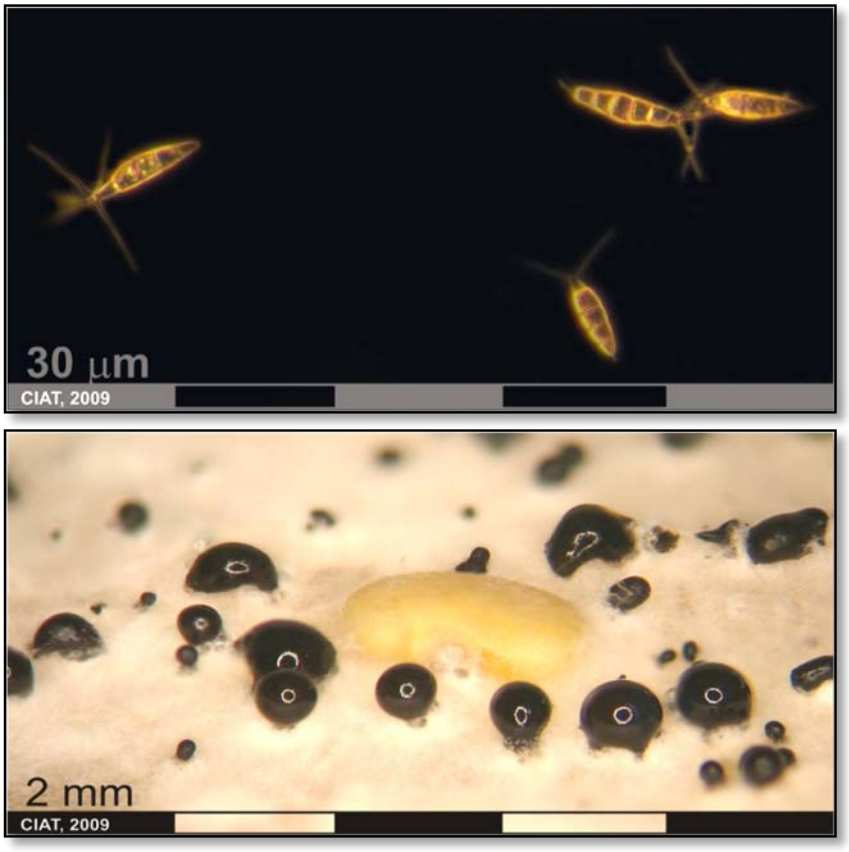


Figure 19. Conidia and mycelial growth of *Pestalotiopsis* sp.

4.1.3.12 Angular leaf spot caused by *Phaeoisariopsis griseola* (Sacc.) Ferraris

This disease causes moderate to severe defoliation in cold conditions; for this the angular leaf spot has a considerable effect on animal production. The first sign of the disease is the development of dark, grey-green fungal structures on the lower surfaces. Coalescence of these grey-green patches on the under surfaces of leaves is commonly associated with the development of chlorotic, often angular lesions on leaf upper surfaces [Lenné & Trutmann, 1994]. The lesions in bean pods have not been reported in the forage legumes; the contamination of the seed with the fungus is usually associated with the area of the hilum of the seed coat, although the fungus may be in the hilum or in other parts of the testa [Correa -Victoria et al. 1994].

The hosts of this disease are: *Desmodium cephalotus, Desmodium gangeticum, Desmodium pulchellum, Dolichos lablab, Macroptilium atropurpureum, Phaseolus lunatus, Phaseolus coccineus, Phaseolus vulgaris, Pisum sativum, Vigna unguiculata*. It is distributed in Brazil, Colombia, Costa Rica, Ecuador, Mexico, Panama, Peru, USA (Florida) y Venezuela [Lenné & Trutmann, 1994].

4.1.3.13 Black stem caused by *Phoma sorghina*

The causal agent of this disease is the fungus *Phoma sorghina* (Sacc.) Boerema, Dorenb & van Kest., which causes collapse of seedlings in pre- and post emergence of several legumes. This pathogen is also commonly associated with necrotic lesions on leaves of *Stylosanthes* spp. in the tropical zone of America [Lenné & Trutmann, 1994]. In seeds it is characterized by the presence of black pycnidia with tips, and its growth on PDA is usually pink (Fig. 20).

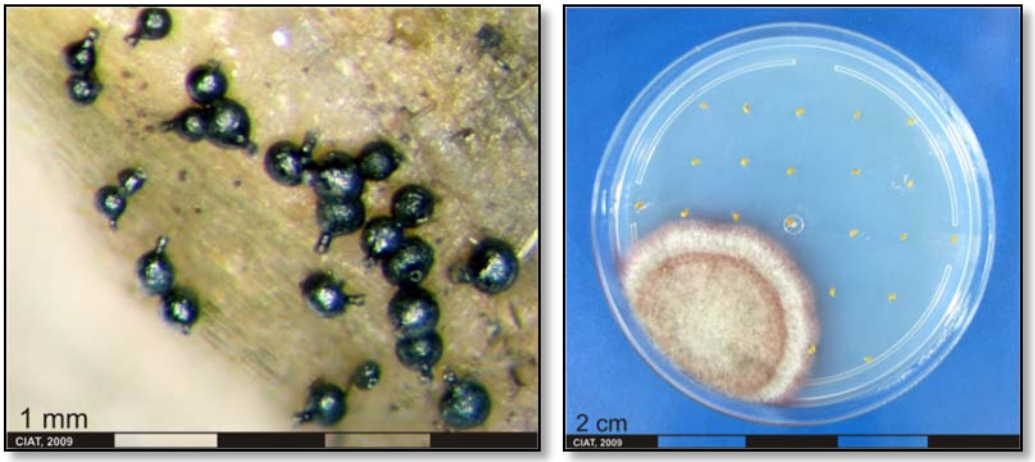


Figure 20. Pycnidia in *Brachiaria* seed husks and mycelial growth on PDA.

Its hosts are: *Desmodium* spp., *Brachiaria* spp., *Centrosema* spp., *Macroptilium atropurpureum, Stylosanthes* spp. and its geographical distribution is Nigeria and South America [Lenné & Trutmann, 1994]. This fungus can be transmitted internally by seeds [Neergaard, 1977].

4.1.3.14 Leaf spot caused by *Phomopsis sp.* (Teleomorph. *Diaporthe phaseolorum* (Cooke and Ell.) Sacc.)

The most noticeable symptom of the disease is the presence of pycnidia on infected material. The pycnidia are black fruiting structures, which are first seen on the petioles. Another key feature is the appearance of lines of pycnidia, which may cover big sections of the stem or may appear in clusters around the nodes. Through the stereoscope dark pycnidia are observed, immersed, almost globose, but the defining characteristic is the presence of two forms of conidia, some ovoid and some others filiform [Lenné & Trutmann, 1994].

Its mycelium on PDA is white and after 8 days turns yellow (Fig. 21). Because of its relationship with *Phoma* spp., this fungus has the same form of seed transmission (internally in embryo and/or cotyledons). It has been reported that *Phomopsis leptostromiformis* remains viable more than three years in seeds of *Lupinus luteus* [Neergaard, 1977].

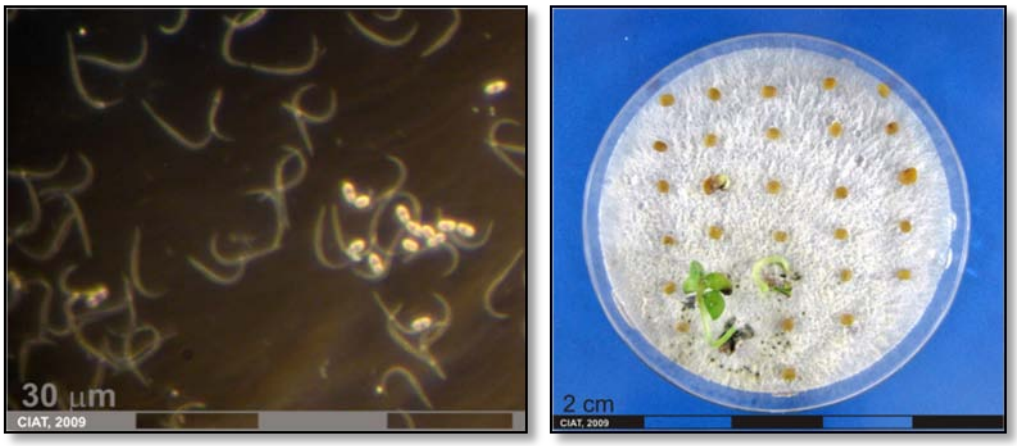


Figure 21. Conidia, pycnidia and mycelium growth of *Phomopsis* sp.

Its distribution includes: Brazil, Colombia, Africa and the Pacific, and their hosts in legumes are: *Centrosema* spp., *Desmodium* spp. and *Macroptilium atropurpureum*. Crop rotation with non-legume species reduces the amount of inoculum available in the seed bed [Lenné & Trutmann, 1994].

4.1.3.15 Leaf spot caused by *Pyricularia* and blight of rice

Pyricularia orizae causes blight in rice and is one of the most important pathogens of this crop due to its distribution and destructive nature. In addition, this fungus can be found in the seeds of grasses, especially in *Lolium perenne*, *Pennisetum clandestinum* [Lenné & Trutmann, 1994]. The fungus presents most of the time long, slender, simple conidiophores with conidias of form obpyriforme to ellipsoide, presenting hialinas with 2 or 3 segments (Fig. 22).

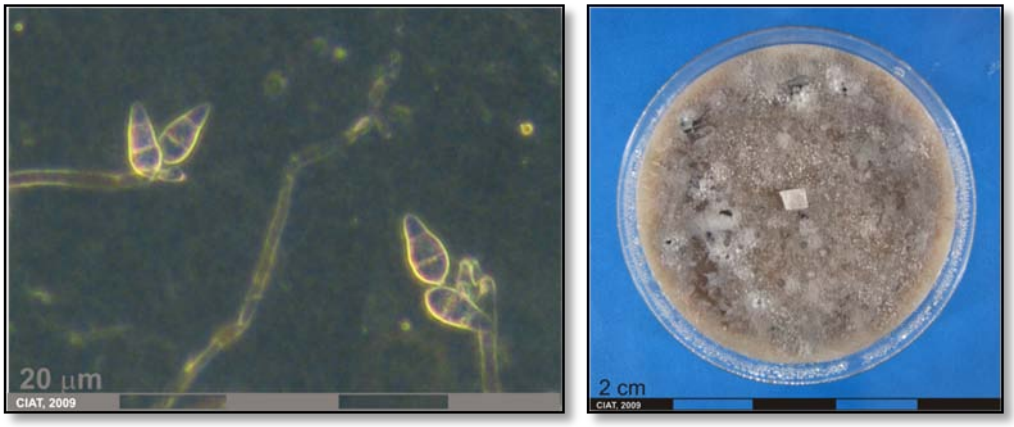


Figure 22. Conidia seen through the phase-contrast microscope and growth of *Pyricularia orizae* in oatmeal agar.

The fungus produces in the leaves spots or injuries of elongated form, of brown uniform color and later with grayish centers and edges of brown color. Also it produces damages in the nodes and in the different parts of the panicle and the grains.

Seed-borne transmission of *P. orizae* was first reported in Japan and then spread throughout the world [Lenné & Trutmann, 1994]. Research suggests systemic transmission of the fungus from seeds to the seedlings. It was established that the conidia of *Pyricularia oryzae* in stored rice seeds under dry conditions at room temperature live longer than 1 year, while the mycelium can survive for at least four years [Neergaard, 1977]. The fungus is carried by seed in external and internal tissues.

4.1.3.16 Leaf and foliar blight caused by *Rhizoctonia solani*

This fungal attack caused by *Rhizoctonia solani* Kühn (Teleomorph *Thanatephorus cucumeris* (Frank) Donk.) initially appears as water-soaked patches in the foliage canopy. Under prolonged humidity conditions, the patches may spread, causing considerable decay and death of foliage. Mycelial growth is similar to a spider web and affects the whole seed and the germinating seedling [Lenné & Trutmann, 1994]. Its sclerotia do not have a definite shape, but its key feature is seen through the microscope since the septa always form a "T" in its mycelium and there is no presence of conidia as it is sterile (Fig. 23).

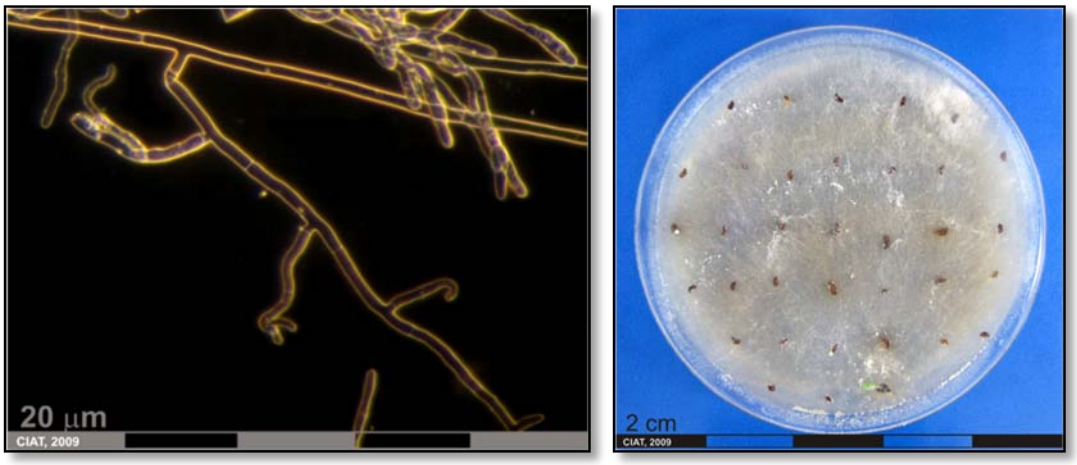


Figure 23. Mycelium seen in phase-contrast microscope, showing septa forming a "T" and mycelial growth on PDA.

This disease is well documented and its hosts are: *Aeschynomene americana, A. histrix, A. brasiliana, Arachis pintoi, A. glabrata, Brachiaria brizantha, B. decumbens, B. dictyoneura, B. humidicola Calopogonium sp., C. mucunoides, Cassia rotundifolia, Centrosema acutifolium, C. arenarium, C. brasilianum, C. macrocarpum, C. pascuorum, C. plumieri, C. pubescens, C. schiedeanum, C. tetragonolobum, C. virginianum, Desmodium ovalifolium, D. uncinatum, Desmodium sp., Macroptilium atropurpureum, Neonotonia wightii, Panicum maximum, Pueraria phaseoloides, Stylosanthes capitata, S. guianensis, S. hamata, S. humilis and S. sundiaca.* Its geographical distribution is: Central and South America, Florida, Malaysia, Papua New Guinea, Solomon Islands, Zambia and globally through the tropics [Lenné & Trutmann, 1994]. The fungus can infect seeds in the endosperm, the radicle end of the embryo and the surface of testa [Gálvez et al. 1994].

4.1.3.17 Inflorescence blight, collar rot and sclerotium wilt caused by *Sclerotium rolfsii* Sacc. (Teleomrph. *Corticium rolfsii* Curzi)

The first sign of attack by *S. rolfsii* is usually a partial or complete decay and collapse of isolated plants. The bottom of the plants is affected, especially the collar region and the region around the soil, frequently colonized by profuse white mycelium and small coffee sclerotia. The mode of transmission of the fungus is poorly known, suggesting an external transmission through contact with inocula present in the soil [Neergaard, 1977].

This fungus is a non-specialist pathogen with extensive host range of legumes such as: *Stylosanthes* spp., *Centrosema* spp., *Desmodium* spp., *Macroptilium atropurpureum*, *Cassia* spp, *Cajanus cajan*. Its geographical distribution is: Australia, Brazil, Colombia, Florida, Malaysia, Panama, Papua New Guinea, Peru, Thailand and Venezuela [Lenné & Trutmann, 1994].

4.1.3.18 Ergot or inflorescence blight caused by the conidial state of *Claviceps* spp. (*Sphacelia* sp.)

Sphacelia sp., the anamorphic state of various *Claviceps* species, is a serious problem in the production of seed of different grasses such as: *Andropogon gayanus*, *A. tectorum, Brachiaria spp., Cynodon, Hyparrhenia, Panicum maximum, Paspalum dilatatum, P. notatum* y *P. plicatulum* identified by its white coating (Fig. 24). Its geographical distribution is: Africa, Australia, Asia, the Caribbean and the Americas [Lenné & Trutmann, 1994].

Figure 24. Conidia, in the infected spikelets of *Brachiaria* and mycelial growth on PDA. Note the whitish coating of the spikelets of grasses.

The ergot first appears as a sugared exudation or sphacelial state in young inflorescences a few weeks after spikelet formation. This exudation is a sticky liquid, sugared, which attracts insects and facilitates the growth of other fungi [Lenné & Trutmann, 1994]. Although the fungus directly infects the ovary, it hasn't been found to be carried internally in the seed since after seed disinfection it does not grow on the PDA. The infection by the ergot fungus occurs at the onset of anthesis, even when the flower is not fertilized. The germ tube invades the ovary near the base of the egg, and the mycelium spreads intracellularly in the ovary wall, and subsequently in the ovary [Neergaard, 1977].

4.1.3.19 Scab caused by *Sphaceloma arachidis*

The scab is caused by *Sphaceloma arachidis* Bitanc. & Jenk. and is common in leaves, petioles and stems. It affects the plants by apparently burning them. Small chlorotic spots disperse uniformly or in clusters close to the veins on both sides of the leaves. The maximum spot size is less than 2 mm. The stems and petioles show a burn look [Lenné & Trutmann, 1994].

The fungus produces fruitings under high humidity [Lenné & Trutmann, 1994]. This fungus is carried externally on the seed testa, but its incidence is very low. Its geographical distribution includes Brazil, Colombia, Argentina and Japan, and is reported in *Arachis glabrata, Arachis pintoi* and *Zornia* spp. There is some evidence of possible seed-borne transmission, but these observations require additional research [Lenné & Trutmann, 1994].

4.1.3.20 Smut caused by *Tiletia aryesii* Berk

The smut causes substantial losses in seed production in tropical America. Although seed production is reduced, the vigor of the plant appears to be unaffected. The spikelets of inflorescences are filled with grayish spore masses, which are released in a gray cloud when the inflorescence is moving. Its host is the grass *Panicum* spp. and its geographic distribution is across Africa, Asia, the Caribbean and tropical America [Lenné & Trutmann, 1994].

As the spores are airborne, infection occurs quickly in exposed flower. Wild plants around the pastures are the major source of infection. The hyphae of the fungus cause a systemic infection of seedlings, and may be carried by the testa and husks of the seeds [Neergaard, 1977].

4.1.4 General procedures for the diagnosis of fungi in seeds of forage legumes and tropical grasses

For the analysis of seeds of legumes the methodology of Agar Test is followed [Kameswara et al. 2006], which is described below:

Make a washing of the seeds soaking them for 10 minutes in a solution of sodium hypochlorite 1% and let them to dry on absorbent paper, taking care to avoid any confusion in the respective numbering (Fig. 25).

Figure 25. Washing of seeds in 1% sodium hypochlorite solution and dried on absorbent paper.

Similarly, as in the bean diagnosis, the 100 bean seeds used are distributed in 4 plastic boxes (25 seeds/dish) duly marked and filled with PDA (Preparation of media, Annex 2). The GHL sample number initially assigned is written with a marker on each box; the four boxes of the same accession are attached with a tape (Fig. 26).

Figure 26. Distribution of seeds in the boxes filled with PDA media.

At the end of the seed planting, the duly marked plates must be sealed to be stored in an incubation room set at a temperature between 22 and 28 °C with light/dark cycle of 12/12 h. The light source corresponds to fluorescent cool white light with good emission wavelength near ultraviolet. The plates remain under these conditions for 8 to 10 days (Fig. 27).

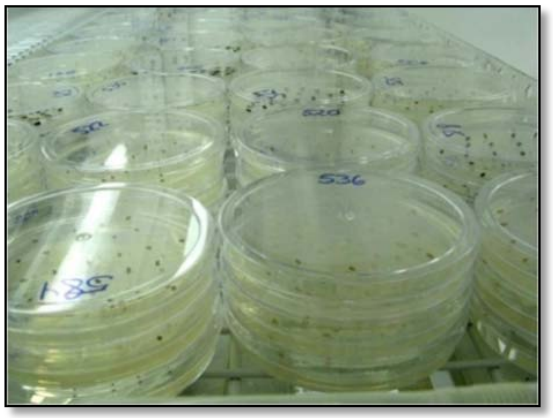


Figure 27. Incubation of seeds under controlled conditions.

Finally the corresponding readings are performed in the same manner as in beans. Each reading is registered in the Excel table designed for the recording of results, taking into account the number of sample assigned by GHL (Annex 1). In this table the quarantine and saprophytic fungi that sometimes occur are recorded. If any of the seeds were infected by a quarantine fungus the accession is rejected (Fig. 28).

Figure 28. Observation and reading of the presence of fungi.

Sometimes the germination of seeds of legumes is difficult and it is necessary to make a cut or scratch to induce the germination and to make the diagnosis of fungi properly (Fig. 29).

Figure 29. Process of seed scarification.

Health hazard: In using the knife to scratch the seed there is a risk of injury, the blade must be move cautiously out of where hand or fingers are.

Health hazard: It is very important to perform proper maintenance and calibration of the pHmeter to assure quality in the preparation of media and solutions.

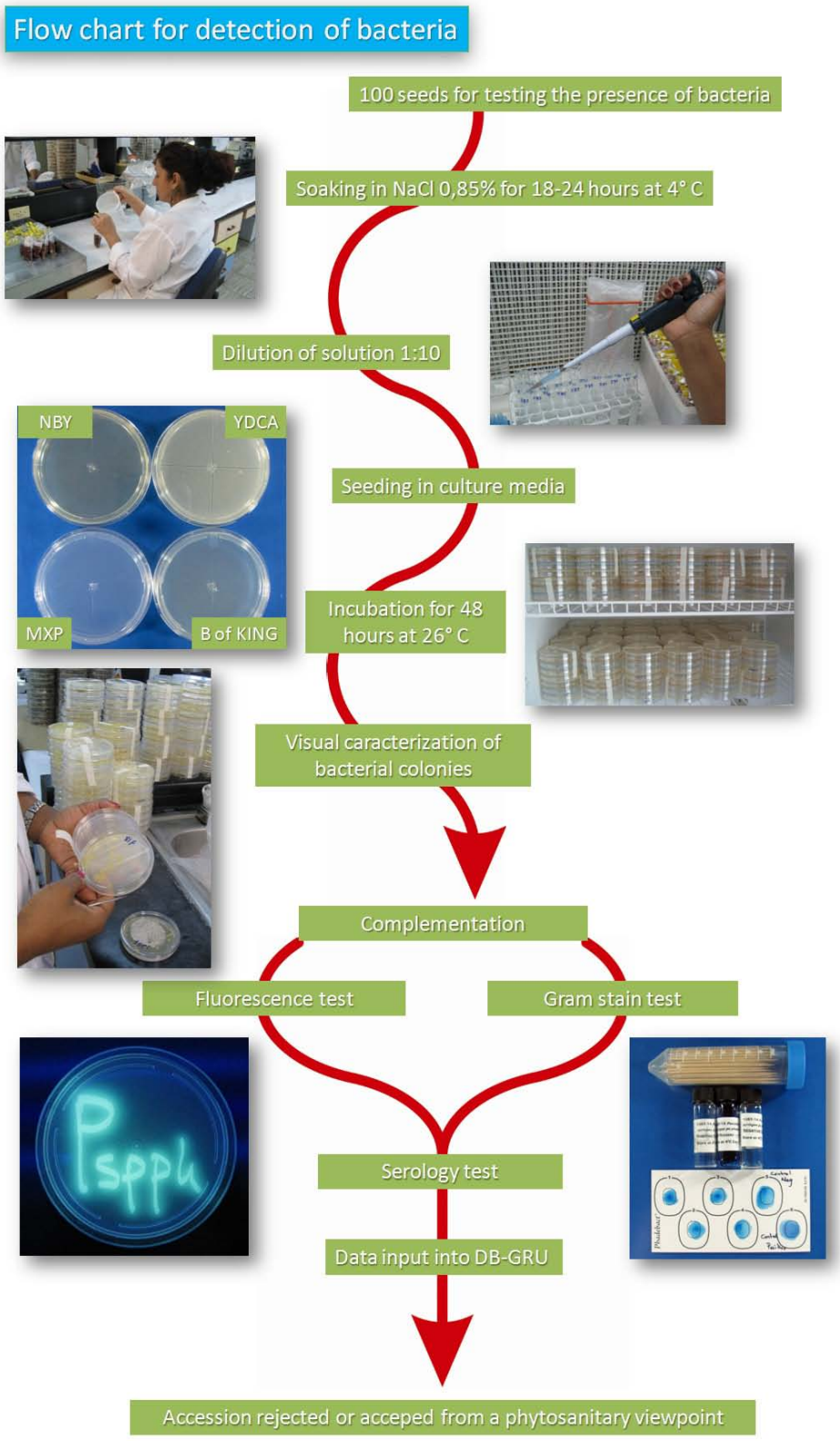
4.2 Detection of bacteria

Bacteria are haploid organisms, unicellular, mobile, with their own cell wall and membrane, but lack of nuclear membrane and reproduce by binary fission. Most pathogenic bacteria are rod-shaped (bacillus) and move in liquid media through flagella that are located at one end or around the entire cell.

The detection of bacteria in seeds is performed taking into account seed borne bacterial diseases that are considered of interest quarantine importance and affect beans, legumes and tropical grasses crops (Flowchart 4).

Plant pathogenic bacteria can survive in different ways under adverse environmental conditions and absence of host plants in the field. One of the most efficient ways of survival is through seed crops, infesting either (externally on the seed testa) or infecting the seed tissues (internally). They are expressed by vascular wilt, blights or leaf and fruit spots or formation of galls or tumors.

Because plant pathogenic bacteria are morphologically similar, the differentiation between species is mainly based on biochemical reactions produced in the culture media. All plant pathogenic bacteria grow in a nutrient media containing agar. However, the addition of some compounds or substances can inhibit the development of a particular genus or species of bacteria. Also, some genera of bacteria during growth produce pigments of defined colors that characterize them with relative good level of confidence [Castaño and Mendoza, 1997].



Flow chart 4. Diagnosis by GHL of bacteria induced diseases with quarantine impact.

4.2.1 Main bacterial diseases in the bean crop

There are three bacterial diseases in beans (*Phaseolus* spp.) that are considered of quarantine importance, as described below:

4.2.1.1 The bean common bacterial blight

This is a disease of major geographic distribution in the world. It has great economic importance where the bean crop coincides with the rainy season, warm weather and high humidity. In Latin America, it is endemic and causes losses in crop yield and grain quality. This disease is caused by the bacteria *Xanthomonas axonopodis* pv. *phaseoli* (E.F.Sm.) Dows. This bacteria can be present in the seed and can be transmitted internally or externally. The transmission of *X. axonopodis* pv *phaseoli* through the seed has been known since 1872. Viable and virulent bacteria have been recovered from bean seed after three, ten and fifteen years of storage [Cardona et al. 1995].

4.2.1.2 The halo blight

It is a common and serious disease in regions with cold or moderate temperatures. It is caused by *Pseudomonas syringae* pv. *phaseolicola* (Burk) Dows. These bacteria produce a toxin called phaseolotoxina, which is responsible for one of the characteristic symptoms, chlorotic halos surrounding the moist lesions. This toxin also causes systemic infection with extensive chlorosis and distortion on growth. The bacteria penetrate directly the pod to the seed. *P. syringae* pv. *phaseolicola* survives on infected seeds and plant debris on the soil surface until environmental conditions are conducive to the development of infection [Cardona et al. 1995].

4.2.1.3 The bacterial wilt

This disease is caused by the bacteria *Curtobacterium flaccumfaciens* (Hedges) Dows. Zaumeyer and Thomas. Its development is favored by temperatures above 32 °C and dry conditions. The bacteria are systemic and the development of the disease is very fast. In Colombia, it has been reported only once [Lenné et al. 1985; Cardona et al. 1995].

C. flaccumfaciens survives five to 24 years in infected seed, which may have yellow, orange or blue discolorations. The bacteria do not survive in the soil during the winter, but can survive from one planting season to another in crop residues or weeds. The most virulent strains are better adapted to survive [Cardona et al. 1995].

4.2.2 Major bacteria in forage legumes and tropical grasses

There are four diseases caused by bacteria affecting legumes and tropical grasses that are of quarantine importance as described below:

4.2.2.1 Pod blight, kilt and dieback

This disease is caused by the bacteria *Pseudomonas fluorescens* biotype II, causes dieback and wilt in young plants. It has been reported in Colombia, Costa Rica, Belize, Brazil, Mexico, Panama and Guatemala [Lenné, 1981; Lenné et al. 1990; Guevara-Gómez et al. 1983]. Its hosts are: *Centrosema* sp. (*C. acutifolium, C. pubescens, C. brasilianum, C. macrocarpum, C. schiedeanum and C. virginianum*), *Allium, Brassica, Phaseolus* sp., *Solanum* spp., *Leucaena leucocephala, Leucaena esculenta, L.*

pulverulenta, L. diversifolia and *L. shannoni*. It is distinct from other fluorescent *Pseudomonas* because it has more than one polar flagellum, does not produce carotenoids, does not grow at 41 °C, and hydrolyzes the agar but not the starch. The disease is characterized by water-soaked lesions in the growing parts that progress to cause wilt, rot, necrosis, dieback and defoliation. This bacteria are transmitted by seed; infection levels up to 32% have been reported in some seed lots [Guevara Gómez, 1982].

4.2.2.2 The halo blight

This disease is caused by the bacteria *Pseudomonas syringae* pv. *phaseolicola*. Symptoms appear as dark spots on the leaves surrounded by a yellow halo. This bacterium is transmitted by seed. Its hosts are: *Cajanus cajan, Centrosema pubescens, Lablab purpureus, Macroptilium* spp., *Phaseolus coccineus, P. lunatus, P. vulgaris, Pueraria* spp., *Vigna angularis, V. radiata Neonotonia wightii*. It is distributed worldwide [Lenné & Ordoñez, 1991].

4.2.2.3 Bacteriosis

This disease is caused by the bacteria *Xanthomonas axonopodis* pv. *phaseoli*. The main host is *Phaseolus vulgaris*, but also occurs in other species such as: *Brachiaria* sp., *P. lunatus*, *P. coccineus, P. acutifolius, Vigna aconitifolia, V. radiata, Lablab purpureus, Mucuna deeringiana,* and *Lupinus polyphyllus*. It develops symptoms on the leaves causing lesions with yellow rings, which can grow up to causing total necrosis. Symptoms may also occur on stems and pods. Infected seeds appear wrinkled and with some discoloration. It is a disease that is distributed worldwide.

4.2.2.4 Bacterial wilt

This disease is caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. The disease is characterized by wilting of leaves or parts of them during the hottest hours of the day and a later recovery as evening approaches and temperatures drop. As a result of bacterial shutter of the bundles, the supply of water is stopped and the leaves turn brown and fall. The pods of infected plants may appear yellow. The bacteria can be transmitted within and on the seed. This pathogen has been reported in many parts of the world including North and South America, Europe and Australia. The first report of this disease in Colombia was mentioned in 1981 in *Zornia brasiliensis* [Torres et al. 1982]. Among its hosts are *Lablab purpureus, Phaseolus coccineus, Phaseolus lunatus, Phaseolus vulgaris, Vigna angularis, Vigna unguiculata, Zornia* spp., and possibly *Glycine max*.

4.2.3 General procedures for the diagnosis of bacteria in bean seeds (Phaseolus spp.), forage legumes and tropical grasses

Because plant pathogenic bacteria are morphologically similar, the differentiation between species is mainly based on biochemical reactions produced in culture media. All plant pathogenic bacteria grow in a media containing agar as a nutrient. However, the addition of some compounds or substances can inhibit the development of a particular genus or species of bacteria. Furthermore, some genera of bacteria, when growing produce pigments of defined colors that characterize them with a relative good level of confidence [Castaño and Mendoza, 1997].

In general the methodology used for the diagnosis of bacteria is the same for beans and tropical forages. The steps to follow are described below and will focus on some specific differences for the detection of different bacteria. All solutions used in these procedures can be found in Annex 2.

4.2.3.1 Extraction and planting

The first step is to suspend 100 seeds per accession in sterile saline solution 0.85% (prepared with NaCL in distilled water), in sterile bags specially made for sampling and carefully marked with the GHL sample number (4-ounce bags for forage legumes and tropical grasses and 7-ounce bags for beans), for a period of 18 to 24 hours at 4 °C (Fig. 30).

Figure 30. Suspension of the seeds in 0.85% saline solution.

Once this extraction step is completed, a dilution of 1/10 per sample is made with saline solution at a volume of 5 ml in glass tubes marked with the GHL sample number. From this dilution 100 µl are poured in each of the petri dishes with different growth medium specific for the diagnosing bacterial marked with their respective GHL number (MXP Medium, King B, YDCA, NBY)(Fig. 31).

Figure 31. Preparation of the bacterial dilution 1/10.

Incubate for 48 hours at 26 °C and make identification and visual differentiation from the morphological characteristics of colonies according to the different media used as described below (Fig. 32). Subsequently, pending on the presence of dubious colonies specific tests are carried out for each genera of bacteria.

Figure 32. Observation and morphological identification of bacteria.

4.2.3.2 Identification and differentiation of bacterial colonies

4.2.3.2.1 Xanthomonas axonopodis pv. *phaseoli*

For the identification of bacteria of this genus specific MXP medium [Claflin et al. 1987] and the YDC medium [Wilson et al. 1967] are used. In the MXP colonies look creamy, round, with regular and transparent edges; they have the ability to grow in the presence of different antibiotics present in this medium (kasugamycin, gentamicin, and cephalexin) and also to hydrolyze the potato starch causing the presence of a halo around the colonies. When colonies are present with the respective halo they are reisolated in YDCA medium (Yeast Extract, Dextrose and Calcium Carbonate, Agar). Since this genus produces a pigment called xanthomonadina, which gives a yellow color to the colonies growing in this media, the colonies appear yellow, bright, round, and with sharp edges. Only with colonies that show these characteristics the identification is complemented using the serological agglutination technique with a specific commercial antiserum produced for *X. axonopodis* pv. *phaseoli*.

4.2.3.2.2 Pseudomonas syringae pv. *phaseolicola*

To identify this group of bacteria one uses the King B medium (King et al. 1954) which is specific for the detection of bacteria associated to this genus. Typical bacteria *P. syringae* pv. *phaseolicola* grow into transparent colonies, creamy, of regular edges, which produce a green pigment that diffuses into the medium and that looks fluorescent when it is observed under ultraviolet light. Similarly, the identification is completed using a specific commercial antiserum produced for *Pseudomonas syringae* pv. *phaseolicola*.

4.2.3.2.3 Pseudomonas fluorescens biotype II

To identify this group of bacteria one uses the King B medium too (King et al. 1954). The bacteria are incubated at 27 °C for 2 to 3 days and then examined in terms of the morphology of the colonies and for the presence of fluorescent pigments under black light (Fig. 33).

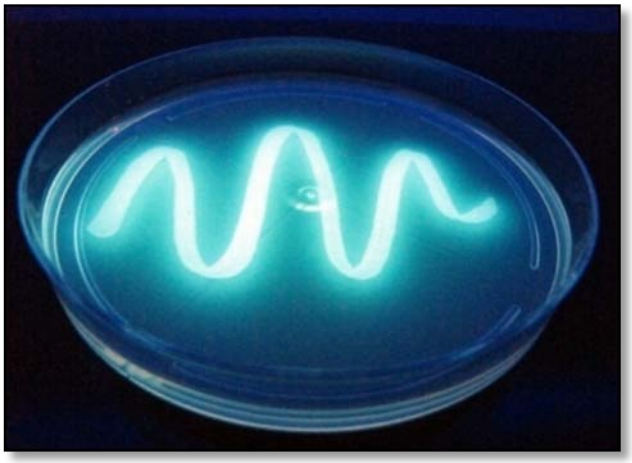


Figure 33. Observation of fluorescent pigments under black light .

4.2.3.2.4 *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*

To identify this group of bacteria often associated with genus *Curtobacterium* one uses the NBY medium (Nutrient Broth Yeast extract). The colors of typical colonies of *Curtobacterium flaccumfaciens* range from creamy white to the purple. The colonies are round, small, entire and with bright edges. Those colonies that look suspicious are selected and marked with the Gram stain (Crystal violet-1minute, lugo1-1minute, bleaching-(ethanol-acetone) 30 seconds, safranin-1 minute). Any colony isolated from Gram positive microorganisms (purple color at the end of staining) and that has a bacilliform shape are reisolated again in NBY medium at 37 °C (ideal temperature for growth of this bacteria), and then proceeds with the characterization with the serological kit. For the identification of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* a commercial kit PTA ELISA is used.

4.2.3.3 Description of serology used in the identification of bacteria

Commercial kits are used for the detection of *Xanthomonas campestris* pv. *phaseoli*, *Pseudomonas syringae* pv. *phaseolicola* and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*.

For the first two species of bacteria the following methodology is used:

- Samples are prepared by removing a small amount of bacteria of a typical isolated colony, using a special applicator that is provided with the kit.
- Prepare a suspension with the suspect colony from the corresponding average in sterile tubes with 0.5ml of sterile distilled water.
- Homogenize the mixture in a vortex.
- Homogenize well each kit reagents and add a drop of specific antiserum of each bacterium to detect in one of the circular holes of the plate that provides the kit.
- Add a drop of negative control to one of the circular holes of the plate that provides the kit and mix with the applicator.

- Add a drop of the positive control in other circles, mixing with the applicator.
- Add one drop of the dilution of the bacteria with sterile distilled water, mixed with the applicator.
- Shake the plate gently by rotation for 1 minute.
- Read the reaction after 60 seconds, if agglutination reaction is not obtained one can wait up to 3 minutes.
- The interpretation of the controls show the following (Fig. 34):

  In a positive reaction, granular or agglutination presence.
  In a negative reaction, no presence of agglutination.

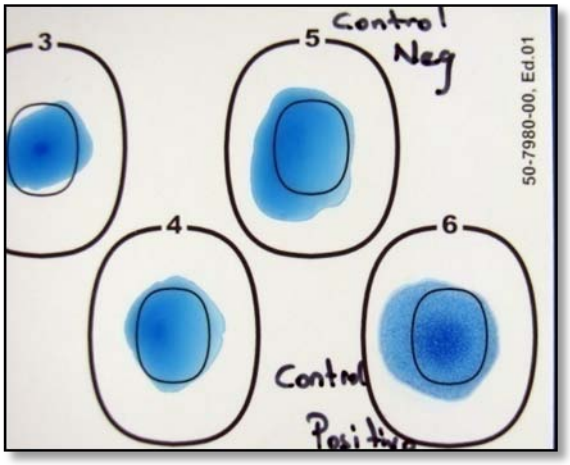


Figure 34. Serological reaction used for the diagnosis of *Xanthomonas campestris* pv. *phaseoli*, and *Pseudomonas syringae* pv. *phaseolicola*.

For the diagnosis of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* one applies the following methodology proposed by the commercial firm of the diagnostic kit Neogen Europe:

- Add 100 µl of each sample, including positive and negative controls per duplicate to the wells. Cover the ELISA plate, or place it in a plastic box. Incubate overnight at 4 °C.
- Wash the wells of the plate with washing buffer (materials included in the kit).
- Add block buffer that has been previously prepared, and incubate the ELISA plate at least 1 hour at 37 °C.
- Wash the wells as described above.
- Add 100µl of the probe (included in the kit) in each of the wells. Cover the ELISA plate and incubate it at 37 °C for 1 hour.
- Add 550µl of the conjugate buffer (included in the kit). Ensure the content of the vial is well mixed. Place the plate at 37 °C for 1 hour.

- Wash the wells again as described above.
- Add 100µl of the conjugate in each well, cover the plate and incubate again at 37 °C for 1 hour.
- Wash again as described above.
- Add 100 µl of substrate (included in the kit) in each of the wells. Cover the ELISA plate and incubate it in the dark at room temperature for 1 hour.
- Read the absorbance at 405 nm. A sample is considered positive when it shows an absorbance greater than the negative control, and is considered as negative if its absorbance is equal or below the negative control.

After the completion of all relevant tests to give a proper diagnosis one proceeds with the recording of results in an Excel table specially designed for this purpose, taking into account the GHL sample number (Annex 1). In this table the presence or absence of bacteria is noted, in the event that the result is positive for bacteria, the specific bacteria found in a given sample are registered.

Health hazard: When using lighters one must be careful to avoid fires.

Health hazard: When analyzing bacteria by ultraviolet light one should use the necessary protective elements such as safety glasses to avoid damage to health.

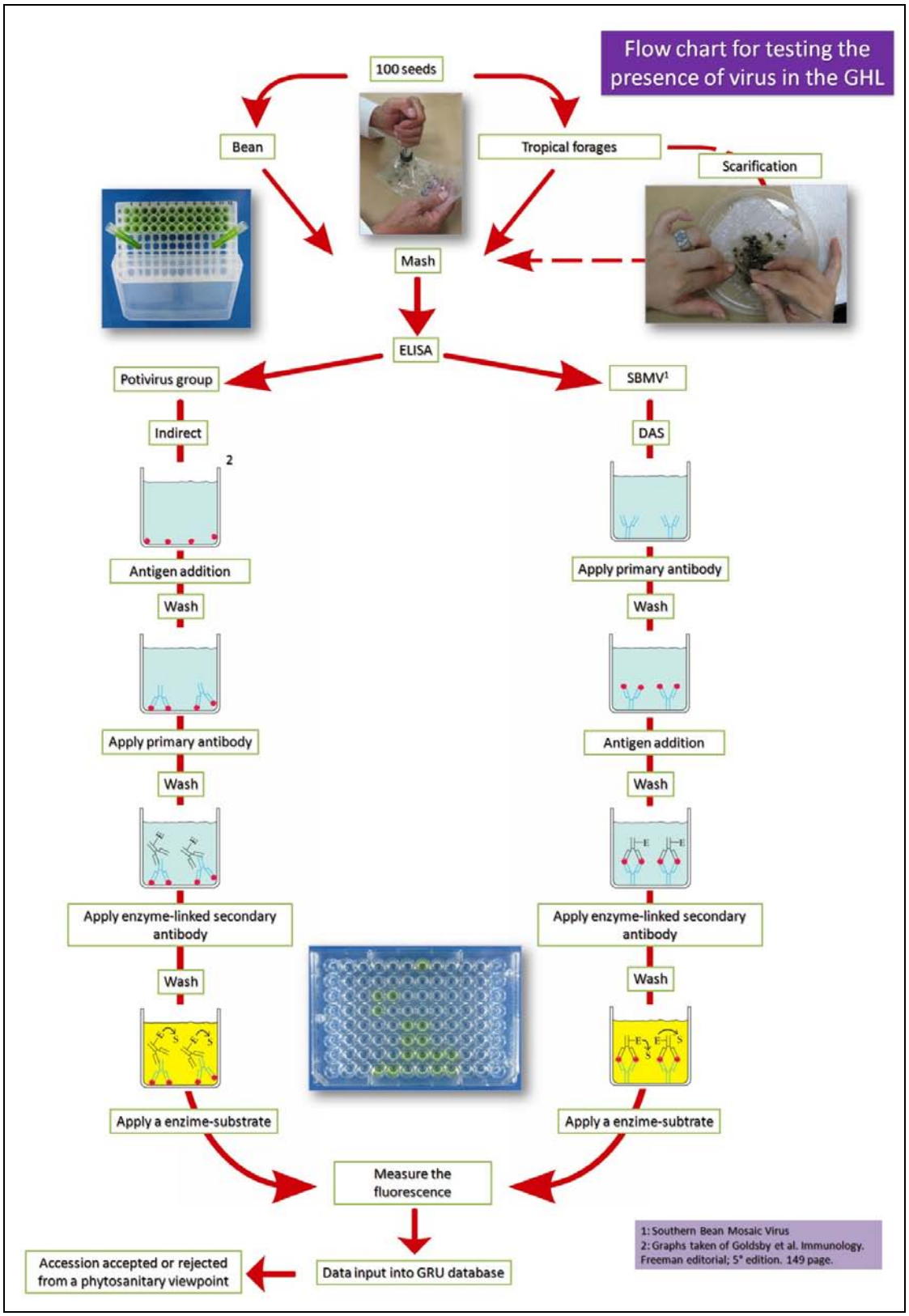
Health hazard: All materials used in the tests must be disinfected or sterilized properly, following specific procedures.

4.3 Virus detection

The viruses are ubiquitous pathogens of grasses and leguminous species worldwide. Legumes are particularly susceptible to virus, and 533 plant viruses cause natural infection in this group, while only 53 different viruses attack grasses [Morales, 1994].

Through the establishment of pastures, either by seed or by vegetative propagation, virus in grasses and legumes either seed-borne or not, can be widely distributed through the exchange of germplasm [Morales, 1994].

The diagnosis of seed-borne virus of quarantine importance affecting bean crops, forage legumes and tropical grasses is done in the GHL (Flowchart 5).



Flow chart 5. Diagnosis of virus in the GHL.

4.3.1 Major virus in the bean crop

4.3.1.1 Bean Common Mosaic Virus

This disease is caused by the Bean Common Mosaic Virus (BCMV) and the Bean Necrosis Mosaic Virus (BCNMV). The bean mosaic virus is the viral disease most widespread worldwide. This disease is caused by two groups of viral strains, those belonging to BCMV and those of the *Potyvirus* genus BCNMV family *Potyviridae* [Morales & Castaño, 2008].

Its symptoms depend on the variety of beans, the strain of the virus and environmental conditions, but usually light and dark green areas are seen in the affected leaves, and the leaves usually roll toward the lower surface (Fig. 35). Plants affected by the virus usually do not reach their normal size. The number of pods per plant is the yield component most affected [Cardona et al. 1995].

Figure 35. Foliar symptoms of virus BCMV in bean leaves (left: light and dark spots; right: rolling towards the lower surface of bean leaves).

This viral disease is the most widespread worldwide, due to its ability of seed-borne transmission and its rapid spread by aphids [Morales & Castaño, 2008].

4.3.1.2 Southern bean mosaic

The Southern Bean Mosaic Virus (SBMV) belonging to the *Sobemovirus* genus, is the causal agent of this disease. Although the disease was first reported in the southern USA, it has now been distributed worldwide [Morales & Castaño, 2008].

The SBMV is very easily transmitted by contaminated seed, being very stable outside the plant or its natural vectors. This virus is widely distributed in producing areas, because it is seed-borne and produced by infected plants; it is also transmitted by tools and by beetles of the genus *Cerotoma*, *Diabrotica* and *Ephilachna* [Cardona et al. 1995].

The symptomatology is limited to slight changes in tone and texture of the leaves unaffected with appreciable deformation or chlorosis (Fig. 36). Usually, infected leaves turn gray-green or olive and its texture is slightly coriaceous [Cardona et al. 1995].

Figure 36. Foliar symptoms of SBMV. From: Virology Unit, CIAT, 2009.

4.3.2 Major virus in tropical grasses and legumes forages

4.3.2.1 The peanut mottle

This disease due to the Peanut Mottle Virus (PeMOV) belonging to the genus *Potyvirus*, family *Potyviridae* is present in *Amaranthus retroflexus, Arachis hypogaea, Arachis pintoi, Beta vulgaris, Brassica rapa, Cajanus cajan, Chenopodium murale, Chenopodium quinoa, Chenopodium album, Citrullus lanatus, Crotalaria spectabilis, Cucumis sativus, Cyamopsis tetragonoloba, Glycine max, Lupinus angustifolius, Lupinus albus, Macroptilium lathyroides, Medicago sativa, Melilotus alba, Melilotus officinalis, Phaseolus acutifolius, Phaseolus lunatus, Phaseolus vulgaris, Pisum sativum*, *Senna bicapsularis, Senna obtusifolia, Senna occidentalis, Senna tora, Trifolium incarnatum, Trifolium pratense, Trifolium repens, Trifolium subterraneum, Trifolium hybridum, Vicia villosa, Vigna unguiculata* and *Vigna subterranea* [Allen & Lenné, 1998].

The characteristic symptoms induced by PeMOV in *Arachis pintoi* as it can be seen in Figure 37, consist of leaf lesions in ring form and with different degrees of variegation [Morales, 1994]. The virus is transmitted mechanically and by aphids in a persistent manner. This virus is seed-borne, but usually at percentages below 3% [Morales & Castaño, 2008].

Figure 37. Foliar symptoms of PeMOV in *Arachis pintoi* and *Macroptilium lathyroides* characterized by varying degrees of variegation.

4.3.2.2 Bean common mosaic

This disease is reported in: *Arachis hypogaea, Bauhinia purpurea, Cajanus cajan, Centrosema pubescens, Chenopodium quinoa, Cicer arietinum, Clitoria ternatea, Crotalaria incana, Crotalaria juncea, Crotalaria spectabilis, Cucumis sativus, Cyamopsis tetragonoloba*, *Glycine max, Lablab purpureus, Lupinus angustifolius, Lupinus luteus, Lupinus albus, Macroptilium atropurpureum, Macroptilium lathyroides, Medicago sativa, Melilotus alba, Phaseolus acutifolius, Phaseolus lunatus, Phaseolus vulgaris, Pisum sativum, Rhynchosia minima, Senna sophera, Senna tora, Sesbania herbacea, Trifolium incarnatum, Trifolium pratense, Trifolium repens, Trifolium subterraneum, Trifolium hybridum, Vicia sativa, Vicia villosa, Vigna radiata, Vigna unguiculata, Vigna vexillata, Vigna subterranea* [Allen & Lenné, 1998].

The pathology is produced by the Bean Common Mosaic Virus (BCMV) which was described previously (main bean crop viruses), which induces mosaics of dark and light green color, and malformation of leaves. The disease control is difficult due to the natural migration of most of its aphid vectors and the short time needed to transmit the virus [Morales, 1994].

Molecular taxonomic investigations have allowed the reclassification of several viruses that infect other legumes such as cowpea (*Vigna unguiculata*), *Vigna angularis*, *Cyamopsis tetragonoloba* and *Dendrobium* spp. into the list of BCMV strains increasing its wide host range but also making its symptoms more diverse [Morales & Castaño, 2008].

4.3.2.3 Southern bean mosaic

This disease caused by Southern Bean Mosaic Virus (see main bean crop virus) has been reported in the following legumes: *Cassia tora, Cicer arietinum, Cyamopsis tetragonoloba*, *Glycine max, Lupinus albus, Melilotus albus, Pisum sativum, Vigna mungo, V. radiata, V. subterranea, V. unguiculata,* and *V. unguiculata var. sesquipedalis* [Allen & Lenné, 1998].

Its symptoms include mosaic or leaf mottled light green or olive color, deformity, decreased size and curvature of leaves. The virus can be transmitted by mechanical inoculation, grafting, seeds, pollen and arthropods of the order Coleoptera family *Crysomelidae* [Allen & Lenné, 1998].

4.3.2.4 Centrosema mosaic

This disease caused by a Potyvirus isolated and characterized in Colombia as a strain of soybean mosaic virus. The hosts were: *Centrosema pubescens, Crotalaria anagyroides, C. retusa*, *C. goreensis, C. mucronata, Desmodium distortum.* It is transmitted by aphids and by seed at low levels [Lenné et al., 1990; Morales et al. 1990].

The disease symptoms are chlorosis, mosaic, leaf deformation, stunting and plant death. The virus caused serious damage in places where there is a high density of aphids [Lenné et al. 1990].

4.3.3 General procedures for the diagnosis of viruses in beans, legumes and tropical pastures seeds

For the diagnosis of virus for bean and tropical pastures and forages we use the same methodology. For the virus described previously the diagnosis is realized by means of the serological technology known as ELISA.

The Enzyme Linked Immune Sorbent Assay, also called ELISA, it is a enzyme immunoassay test, that is based on the recognition of an antigen (virus) by an antibody and the later union of this complex with one enzyme (Zavala, 1985), that forms a conjugate that retains simultaneously the immunological and enzymatic activity of the components. If the specific antibodies join with its respective antigen they produce a reaction, which can be visible with the presence of a yellow color according to type of enzyme used. This test has variants, which are used according to the purpose of the analysis being most currently used the indirect method and the method Sandwich of double antibody. This test is a sensitive method, of much precision and rapid detection.

For SBMV one uses the DAS ELISA method (double-antibody sandwich) by means of a commercial kit. For the virus of the BCMV, PeMoV, SMV-CE one uses the Indirect ELISA by means of a commercial Kit that detects a wide range of virus of the Potyvirus group (Annex 3).

The germinated seed, without sanitary problems in the detection of fungi, is used for the detection of virus. In the detection for bean one uses the hypocotyl and the primary leaves, in case of legumes and tropical pastures the whole germinated seed is used (Fig. 38).

Figure 38. Extraction of tissue from germinating bean seeds.

The seeds of bean are ground in water by hammering with masticator mixers (Fig. 39), whereas the germinated seeds of legumes and tropical pastures are ground in water manually or mechanically depending on the size (Fig. 40).

Figure 39. Grind of beans seeds.

Figure 40. Grinding of legumes seeds.

After grinding the samples are added to eppendorf tubes of 1.5 ml that contain the respective extraction buffer, depending on the type of ELISA to be done (Annex 4). They are very well mixed with help of plastic disposable pipettes (Fig. 41).

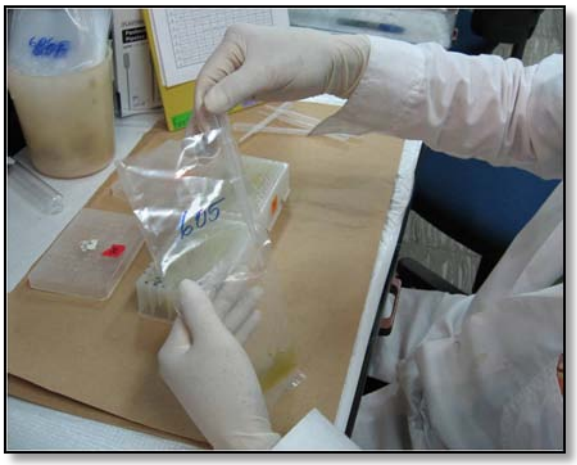


Figure 41. Addition of samples to tubes.

4.3.3.1 Double sandwich antibody ELISA (DAS-ELISA) for SBMV diagnosis

Before realizing the evaluation it is necessary to realize a scheme where the exact position of accession to evaluate inside the ELISA plate is described; hereby the respective result for each accession will be located easily (Annex 5). The preparation of solutions can be found in the annexes (Annex 4). This methodology is based on the report by Clark and Adams of 1977, which is described next:

1. The capture antibody or gammaglobulin is provided in a concentrated solution and must be diluted in the coating buffer before use. Prepare the capture antibody in dilution 1:1000 with the coating buffer. Mix the prepared capture antibody thoroughly and use it immediately. Dispense 100 µl of this solution into each test well. Incubate the plate in a humid box for 3-4 hours at 37 ° C.

2. Wash the plate; with the buffer PBS-Tween four times. For the initial wash, rinse the wells and reject the buffer at once. This step is done mechanically in an automatic microplate washer that has been programmed before to realize 3 washes of three minutes each (Fig. 42). Hold the plate upside down and tap firmly on a folded paper towel to remove excess liquid.

Figure 42. Automatic microplate washer (Every cycle of washing in this machine lasts approximately 6 minutes).

3. Add 100 µl of the prepared samples (Fig. 43). Incubate the plate in a humid box overnight at 4 - 6 ° C. Include samples of known controls: negative control, positive control, and neutral control. The samples are added according to the test diagram where every accession to evaluate is specifically located (Annex 5).

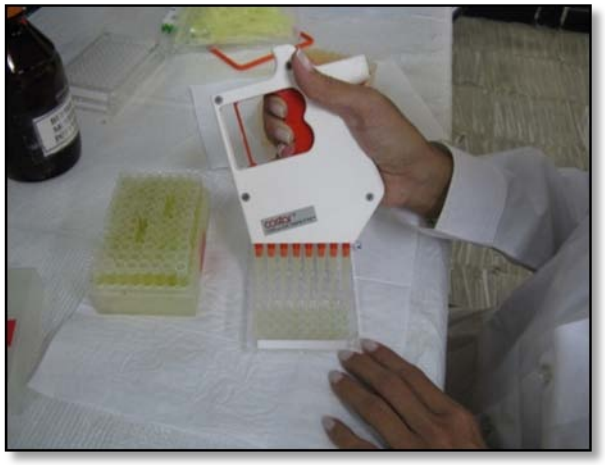


Figure 43. Adding of samples.

4. Wash again the plate; in the same way as in step 2, taking care of removing any residue of the samples.

5. The alkaline phosphatase enzyme conjugate is concentrated and must be diluted with the conjugate buffer before use. Prepare the enzyme-labeled specific antibody in dilution 1:1000 with the conjugate buffer. Mix the prepared conjugate thoroughly and use it immediately. Dispense 100 µl of this solution into each testwell. Incubate the plate in a humid box for 3-4 hours at 37 ° C.

6. Wash again the plate; in the same way as in step 2.

7. Add 50 µl of yellow substrate pNPP (Solution of p nitrofenil phosphate) into each test well. Read from 15 minutes onwards after the addition.

8. The reaction can be stopped after some time by adding 50 µl of 3M sodium hydroxide. This step is optional.

The reading of the results can be realized visually by observing the wells that should give a yellowish coloration (Fig. 44) or by measuring absorbance on a plate reader at 405 nm (Fig. 45). Test results are valid only if the positive control wells give a positive result and the neutral control wells remain colorless.

One considers to be "positive" any value bigger than 2 times the value of the negative control at least. Two readings by plate are realized in different times. All records of the readings are kept in a physical file.

Figure 44. Results of ELISA Test.

Figure 45. Reading the results in a Plate reader.

4.3.3.2 Indirect Antibody ELISA performed for the diagnosis of viruses of the genus Potyvirus

As described in the point 4.3.3.1 before realizing the evaluation it is necessary to draw a scheme where the exact position of accession to evaluate inside the ELISA plate is described; hereby the respective result for each accession will be located easily (Annex 5). The preparation of solutions can be found in the annexes (Annex 4). This methodology is described next:

1. Add 100 µl of the ground samples in the sample extraction buffer (Annex 4). Incubate the plate in a humid box for 2 hours at room temperature. Include samples of known controls: negative or healthy control, positive control and neutral control. The samples are added according to test diagram where every accession to evaluate is specifically located (Annex 5).
2. Wash the plate with the buffer PBS-Tween four times. For the initial wash, rinse the wells and discard the buffer at once. This step is done mechanically in an automatic microplate washer that has been programmed before to realize 3 washes in three minutes each (Fig, 42).
3. Capture antibody or gammaglobulin is provided in a concentrated solution and must be diluted with coating buffer before use. Prepare the capture antibody in dilution 1:200 with the coating buffer. Mix the prepared capture antibody thoroughly and use it immediately. Dispense 100 µl of this solution into each testwell. Incubate the plate in a humid box overnight at 4 -6 ° C.
4. Wash again the plate; in the same way as in step 2.
5. The alkaline phosphatase enzyme conjugate (Anti mouse lgG whole molecule) is concentrated and must be diluted with the conjugate buffer before use. Prepare the enzyme-labeled specific antibody in dilution 1:8000 with the conjugate buffer. Mix the prepared conjugate thoroughly and use it immediately. Dispense 100 µl of this solution into each testwell. Incubate the plate in a humid box for 1 hour at room temperature.
6. Wash again the plate; in the same way as in step 2.
7. Add 50 µl of yellow substrate pNPP (Solution of p nitrofenil phosphate) into each testwell. Read from 15 minutes onwards after the addition.
8. The reaction can be stopped after some time, adding 50 µl of 3M sodium hydroxide. This step is optional.

The reading of the results can be realized by visually observing the wells that should give a yellowish coloration (Fig. 44) or by measuring absorbance on a plate reader at 405 nm (Fig. 45). Test results are valid only if the positive control wells give a positive result and the neutral control wells remain colorless.

One considers to be "positive" any value bigger than 2 times the value of the negative control at least. Two readings by plate are realized in different times. All records of the readings are kept in a physical file.

After the accomplishment of these methodologies and of the corresponding readings one proceeds to register the results obtained in the table of Excel designed for the record of the results, bearing in mind the number of sample GHL (8.1 Annex 1).

Health hazard: Sample macerators should be handled with care to avoid labor accidents.

5. Documentation of bean and tropical grasses health results

The data obtained from the various tests are then filed in Excel tables designed for the storage of results by groups according to different pathogens evaluated, taking into account the number of accession and no longer the GHL sample number. Once these Excel tables are completed, they are stored in physical and electronic files (Annex 6).

In the case of data entry for many accessions, Excel files are synchronized into the database and recorded. In the case of data entry for one or few accessions, they are recorded as follows:

- Enter into the GRP database by giving a Username and Personal Password, for the respective staff; select the option BEANS or FORAGE depending on the tested species. Then select the HEALTH icon to enter the data about the results obtained in the indexing process (Fig. 46).

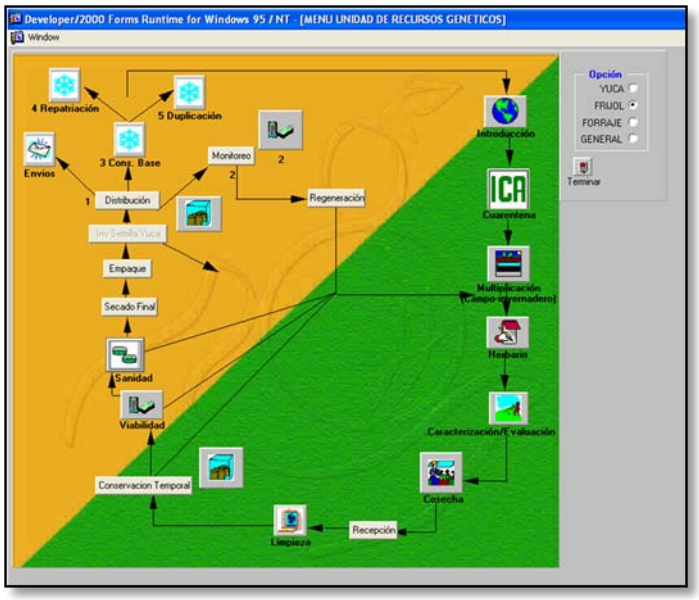


Figure 46. Interface to the GRP database for data entry.

- Enter the number of the analyzed accession.

- Enter each of the data that are in the application: Accession name, source, date of entry in GHL, test date, results for fungi, bacteria and virus, responsible Staff, phytosanitary quality, observations where it shows the name of the pathogen, namely in case the accession has resulted rejected, and tests conducted. In the results for the pathogen one uses: A: Accepted, and R: Rejected. In case of a positive result for any of the pathogens, the accession is automatically rejected (Fig. 47).

- Save the stored data.

- Send a copy of the obtained results to the staff in the Genebank of the GRP through an email notifying that health results were entered.

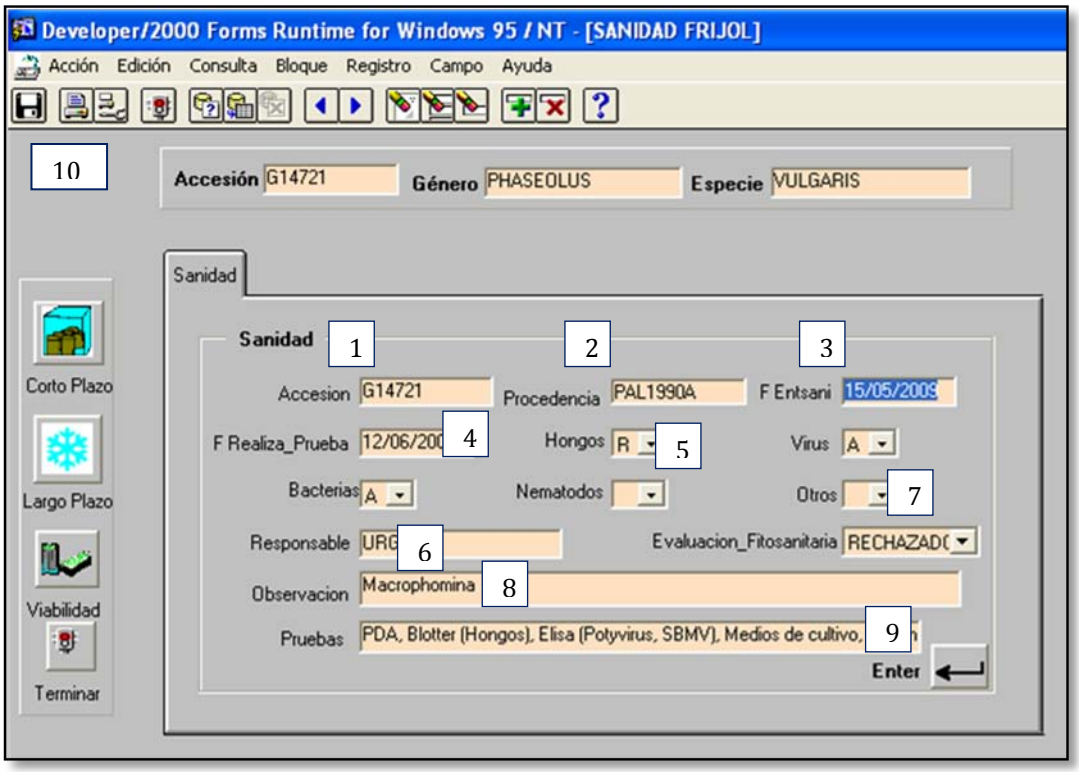


Figure 47. HEALTH Interface. Entry process of results in the database, data to be stored 1) Accession number, 2) Origin, 3) Date of entry of samples to GHL, 3) Date when results were obtained, 4) Results obtained (A/R), 5) Staff responsible for the testing, 6) Phytosanitary Evaluation (Accepted/Rejected), 7) Observations: where you type the specific name of the pathogen, 8) Testing conducted: methodology used, and finally 9) Save.

6. Laboratory safety regulations

In GHL we have rules that must be followed in order to prevent health risks for the workers, maintain the facilities and laboratory equipment safe, and ensure the quality and integrity of all data obtained during the different processes. All staff working in the laboratory must know and follow the established rules.

Some of the possible risks and their control mechanisms are listed below:

- The laboratory should be kept clean, orderly and free of materials not related to work.
- Laboratory surfaces are disinfected at the beginning and at the end of each working day with a solution of sodium hypochlorite at 5%.
- All laboratory equipment should be in good functioning and preventive maintenance should be performed.
- No food, drink or smoke is allowed in the laboratory.
- All workers must wear appropriate clothing and personal protective equipment such as laboratory coats, safety glasses and gloves, routinely. In case of manipulating infectious agents respiratory protective equipment will be used. When leaving the laboratory these items must be removed and stored in appropriate closets.
- Each one should be responsible for personal hygiene, washing hands before and after staying in the lab with antiseptic soap and water.
- When handling volatile solutions and organic solvents, these operations should be performed in the extraction chamber to minimize exposure to fumes, mists and vapors in the laboratory.
- "Mouth pipetting" is prohibited. The pipetting is carried out with devices designed for that purpose, and the staff must be properly trained for its proper use.
- The staff should work under ideal ergonomics.
- All materials such as pipette tips, pipettes, and glass items must be sterilized before use.
- In the preparation of solutions and culture media cautions such as the use of an analytical balance for weighing components, and use of clean materials should be taken. The agitation of the mixture should be done with the help of a magnetic stirrer. The container in which the solution is stored must be labeled indicating the name of the solution, concentration, preparation date, and finally a proper sterilization if relevant.
- All procedures for handling fungi and bacteria should be performed in a laminar flow chamber, following all established standards of biosafety.
- Materials, samples and seed contaminated must be decontaminated before being eliminated. The steam autoclaving is the method of choice for all decontamination processes. The material for the decontamination and disposal should be placed in special plastic bags that allow sterilization by heat in autoclave. All material is autoclaved for 20 minutes at 121 º C (250 F) and a pressure of 15 pounds per square inch. The time starts when the manometer reaches the

desired temperature and pressure. When the autoclave reaches the time required, the material are removed and discarded without risk of contamination (Fig. 48).

Figure 48. Sterilization of waste.

- Keep an adequate environment in the lab, which is controlled by the periodical monitoring for the presence of fungi and bacteria that can cause contamination to the samples or affect the health of workers.

- Fire prevention is very important, knowing all ignition sources that are in the laboratory: flames, heat sources, electrical equipment. Equally, flammable reagents must be purchased and stored in quantities as small as possible. You need to know exactly the place of the security features available such as fire extinguishers or emergency kit.

- The extinguisher must be light weighted so that it can be used easily when needed by anyone.

- In case of occurrence of any accident it should be reported immediately to the head of the research laboratory and the Work Health Office of CIAT.

7. Bibliography

Abawi, G. S. 1994. Pudriciones radicales. In: Pastor Corrales, M. A.; Schwartz, H. F. (eds.). Problemas de producción del fríjol en los trópicos. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Alexopoulos, C. J & Mims, C. W. 1979. Introductory mycology. New York. John Wiley and Sons, New York, USA.

Allen, David J; Lenné, Jillian M. (eds.). 1998. The pathology of food and pasture legumes . CAB International, Oxon, UK.

Argawal V. K., Sinclair J. B. 1987. Principles of Seed Pathology. CRC Press Inc, Boca Raton, Florida, USA.

Barnett, H. L., Hunter, B. B. 1998. Illustrated genera of imperfect fungi. 4. ed. Press, St. Paul, Minnesota, USA.

Cardona Mejía, C., Flor Montoya, C. A., Morales, F. J., Pastor Corrales, M. A. 1995. Problemas de campo en los cultivos de fríjol en el trópico. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Castaño Zapata, J., Del Río Mendoza, L. 1997. Manual para el diagnóstico de hongos, bacterias, virus y nemátodos fitopatógenos. Zamorano Academic Press, Manizales, Colombia: Universidad de Caldas, Facultad de Ciencias Agropecuarias, Zamorano, Honduras.

Claflin, L.E., Vidaver, A.K., Sasser, M. 1987. MXP, a semi-selective medium for *Xanthomonas campestris* pv. *phaseoli*. Phytopathology 77(5):730-734.

Correa Victoria, F. J., Pastor Corrales, M. A., Saettler, A.W. 1994. Mancha angular de la hoja. In: Pastor Corrales, M. A., Schwartz, H. F. (eds.). Problemas de producción del fríjol en los trópicos. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Ellis, M. B. 1976. More Dematiaceous hyphomycetes . Commonwealth Mycological Institute, Kew, United Kingdom.

Frison, E.A., Bos, L., Hamilton, R.I., Mathur, S.B., Taylor, J.D. (eds.). 1990. FAO/IBPGR Technical Guidelines for the Safe Movement of Legume Germplasm. Food and Agriculture Organization of the United Nations, Rome, Italy/International Board for Plant Genetic Resources, Rome, Italy.

Gálvez G. E., Mora B., Pastor Corrales, M. A. 1994. Mustia hilachosa. In: Pastor Corrales, M A., Schwartz, H. F. (eds.). Problemas de producción del fríjol en los trópicos. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Gilman, J. C. 1957. A manual of soil fungi. Rev. 2. ed. Iowa State College Press, Ames, Iowa, USA.

Guevara Gómez C. L. 1982. Etiología de la muerte descendente de *Centrosema* spp. y el efecto del patógeno en su rendimiento y calidad durante el tiempo del establecimiento de la leguminosa. Tesis (Ingeniero Agrónomo). Universidad Nacional de Colombia, Facultad de Ciencias Agropecuarias, Cali, Colombia.

Guevara Gómez, C. L., Lenné, J. M., Torres González, C. 1983. Etiology of dieback of *Centrosema* spp. and the effect of pathogen on yield and quality during the period of establishment of the legume [abstract]. Phytopathology. USA 73(1):122.

Hanlin, R. T. 1990. Illustrated genera of Ascomycetes. APS Press, St. Paul, Minnesota, USA.

Hanlin, R. T. 1998. Combined keys to illustrated genera of Ascomycetes, volumes I & II APS Press, St. Paul, MN, USA.

Kameswara Rao N., Hanson J., Ehsan Dulloo M., Ghosh K., Nowell D., Larinde M. 2006. Handbooks for genebanks No. 8 Manual of seed handling in genebanks. Biodiversity International, Rome, Italy.

King, E.O., Ward M.K., Raney D.E. 1954. Two simple media for demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.

Lenné, J. M. 1981. Evaluation of diseases of indigenous forage legumes in Central and South America. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Lenné, J. M., Chavarro, A., López, C. 1985. Effect of *Corynebacterium flaccumfaciens* on yield of *Zornia glabra* and *Phaseolus vulgaris* in Colombia. Phytopathology (USA) 75(11):1288.

Lenné J.M., Sonoda R.M., Lapointe, S.L. 1990. Diseases and pests of *Centrosema* . In: Schultze-Kraft, R., Clements, R.J. (eds.). *Centrosema*: Biology, agronomy, and utilization. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Lenné J.M., Ordoñez J.H. 1991. Enfermedades de las pasturas en su establecimiento y posibles estrategias de control. In: Lascano C.E., Spain J.M. (eds.). Reunión del Comité Asesor Red Internacional de Evaluación de Pastos Tropicales (6, 1988, Veracruz, México). Establecimiento y renovación de pasturas: Conceptos, experiencias y enfoque de la investigación. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Lenné, J. M., Trutmann, P. (eds.). 1994. Diseases of tropical pasture plants. CAB International; Centro Internacional de Agricultura Tropical (CIAT); Natural Resources Institute (NRI), Oxon, United Kingdom.

Morales, F. J. 1994. Viral diseases. In: Lenné, Jillian M.; Trutmann, Peter (eds.). Diseases of tropical pasture plants. CAB International; Centro Internacional de Agricultura Tropical (CIAT); Natural Resources Institute (NRI), Oxon, United Kingdom.

Morales, F. J., Castaño J. M. 2008. Enfermedades virales del fríjol común en América Latina. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Morales, F. J., Niessen, A.I., Castano, M., Calvert, L. 1990. Detection of a strain of soybean mosaic virus affecting tropical forage species of *Centrosema*. Plant Disease 74(9):648-651.

Neergaard, Paul. 1977. Seed pathology. John Wiley, New York, USA.

Nelson, P. E., Toussoun, T. A., Marasas, W. F. O. 1983. Fusarium species: An illustrated manual for identification. Pennsylvania State University Press, University Park, Pennsylvania, USA.

Pastor Corrales, M. A., Tu, J. C. 1994. Antracnosis. In: Pastor Corrales, M. A., Schwartz, H. F. (eds.). Problemas de producción del fríjol en los trópicos. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia. p. 87-119.

Schwartz, H. F. 1994. Hongos patógenos adicionales. In: Pastor Corrales, M. A., Schwartz, H. F. (eds.). Problemas de producción del fríjol en los trópicos. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Schwartz, H. F., Morales, F. J. 1994. Patología de la semilla. In: Pastor Corrales, M. A. Schwartz, H. F. (eds.). Problemas de producción del fríjol en los trópicos. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Schwartz, H. F., Steadman, J. R. 1994. Moho blanco. In: Pastor Corrales, M. A., Schwartz, H. F. (eds.). Problemas de producción del fríjol en los trópicos. Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia.

Torres C., Lenné, J. M., Victoria, J. I. 1982. Bacterial wilt of *Zornia* spp. caused by *Corynebacterium flaccumfaciens*. In: Lozano J. C., Gwin, P. H. (eds.). International Conference on Plant Pathogenic Bacteria (1982, Cali, Colombia). Centro Internacional de Agricultura Tropical (CIAT); Agricultural Editor's Office, College of Agriculture, University of Missouri, USA.

Wilson, E. E., Zeitoun, F. M., Fredrickson, D.L. 1967. Bacterial phloem canker, a new disease of Persian walnut trees. Phytopathology 57: 618-621.

Zavala, T.E. 1985. El uso de la técnica ELISA para detectar virus en el Programa Nacional de Papa del INIA. Toluca, México.

Zillinsky, F. J. 1983. Common diseases of small grain cereals: a guide to identification. International Maize and Wheat Improvement Center (CIMMYT), Mexico D. F., Mexico.

8. Annexes

8.1 Annex 1

Table of germplasm input to GHL for its analysis.

GHL #	Accession	Origin	Entry date	Test date	Fungi	Xspp.	Psfl.	Curt	Poty	SBMV	Observ.	Test	Genus	Specie
151	245	QUI2009A	24/06/2009									PDA, Blotter (Fungi), Elisa (Potyvirus, SBMV), Culture media, Gram staining, serology (Bacteria: *Xanthomonas campestris* pv *phaseolicola*, *Pseudomonas syringae* pv *phaseolicola*, Bacillus Gram +)	Zornia	sp.
152	252	QUI2009A	24/06/2009									PDA, Blotter (Fungi), Elisa (Potyvirus, SBMV), Culture media, Gram staining, serology (Bacteria: *Xanthomonas campestris* pv *phaseolicola*, *Pseudomonas syringae* pv *phaseolicola*, Bacillus Gram +)	Zornia	sp.
153	3674	QUI2009A	24/06/2009									PDA, Blotter (Fungi), Elisa (Potyvirus, SBMV), Culture media, Gram staining, serology (Bacteria: *Xanthomonas campestris* pv *phaseolicola*, *Pseudomonas syringae* pv *phaseolicola*, Bacillus Gram +)	Desmodium	heterocarpon
154	3843	QUI2009A	24/06/2009									PDA, Blotter (Fungi), Elisa (Potyvirus, SBMV), Culture media, Gram staining, serology (Bacteria: *Xanthomonas campestris* pv *phaseolicola*, *Pseudomonas syringae* pv *phaseolicola*, Bacillus Gram +)	Desmodium	heterocarpon

8.2 Annex 2

Preparation of culture medium used for the diagnosis of fungi and bacteria.

Every culture medium is prepared with distillated water and after preparation it is autoclaved.

PDA medium (Potato Dextrose Agar)

Potato dextrose agar	39 g
Granulated agar	10 g
Final volume with distillated water	1 l

YDC medium

Yeast extract	10 g
Dextrose	20 g
Calcium carbonate	20 g
Agar	20 g
Final volume with distillated water	1 l

MXP medium

Dipotassium acid phosphate	0.8 g
Potassium acid phosphate	0.6 g
Potassium bromide	10.0 g
Yeast extract	0.7 g
Potato starch	8.0 g
Glucose	1 .0 g
Agar	20.0 g
Final volume with distillated water	1 l

King B medium

Peptone protease	20.0 g
Dipotassium acid phosphate tetrahydrated	1.5 g
Magnesium sulfate heptahydrate	1.5 g
Agar	20.0 g
Glycerol	10.0 ml
Final volume with distillated water	1 l
pH	7.2

NBY medium

Nutrient broth	8.0 g
Yeast extract	2.0 g
KH2HPO4	2.0 g
KH2PO4	0.5 g
Glucose	2.5 g
Agar	15.0 g
MgSO4.7H2O	0.2 g
Final volume with distillated water	1 l

8.3 Annex 3

Virus of Potyvirus genus detected throughout commercial antiserum.

Potyvirus group detected by commercial antiserum	
Alstroemeria Mosaic Virus	Narcissus Degeneration Virus
Amaranthus Leaf Mottle Virus	Narcissus Yellow Stripe Virus
Araujia Mosaic Virus	Onion Yellow Stripe Virus
Asparagus Virus 1	Ornithogalum Mosaic Virus
Bean Common Mosaic Virus	Papaya Ringspot Virus-P
Bean Yellow Mosaic Virus	Papaya Ringspot Virus-W
Bearded Iris Mosaic Virus	Parsnip Mosaic Virus
Beet Mosaic Virus	Passion fruit Woodiness Virus
Bidens Mottle Virus	Pea Mosaic Virus
Blackeye Cowpea Mosaic Virus	Pea Seedborne Mosaic Virus
Cardamon Mosaic Virus	Peanut Mottle Virus
Carnation Vein Mottle Virus	Peanut Stripe Virus
Carrot Thin Leaf Virus	Pepper Mottle Virus
Celery Mosaic Virus	Pepper Severe Mosaic Virus
Centrosema Mosaic Virus	Pepper Veinal Mottle Virus
Clover Yellow Vein Virus	Plum Pox Virus
Cockstoot Streak Virus	Pokeweed Mosaic Virus
Colombian Datura Virus	Potato Virus A
Commelina Mosaic Virus	Potato Virus V
Cowpea Aphid-Borne Mosaic Virus	Potato Virus Y
Daphne Y Virus	Soybean Mosaic Virus
Dasheen Mosaic Virus	Statice Virus Y
Datura Shoestring Virus	Sugarcane Mosaic Virus
Freesia Mosaic Virus	Sweet Potato Feathery Mottle Virus
Garlic Mosaic Virus	Sweet Potato Latent Virus
Gloriosa Stripe Mosaic Virus	Sweet Potato mild mottle virus
Groundnut Eyespot Virus	Tamarillo Mosaic Virus
Guinea Grass Mosaic Virus	Tobacco Vein Mottling Virus
Helenium Y Virus	Tobbaco Etch Virus
Henbane Mosaic Virus	Tulip Breaking Virus
Hippeastrum Mosaic Virus	Tulip Chlorotic Blotch Virus
Hyacinth Mosaic Virus	Turnip Mosaic Virus
Iris Fulva Mosaic Virus	Vallota Mosaic Virus
Iris Mild Mosaic Virus	Watermelon Mosaic Virus 1
Iris Severe Mosaic Virus	Watermelon Mosaic Virus 2
Johnsongrass Mosaic virus	White Lupin Mosaic Virus
Leek Yellow Stripe Virus	Wisteria Vein Mosaic Virus
Lettuce Mosaic Virus	Yam Mosaic Virus
Maize Dwarf Mosaic Virus	Zucchini Yellow Fleck Virus
Malva Vein Clearing Virus	Zucchini Yellow Mosaic Virus

8.4 Annex 4

Solutions used for ELISA test.

All solutions must be stored between 4-6° C.

PBS buffer (pH 7.4)

Sodium chloride	8.0 g
Monobasic potassium phosphate	0.2 g
Dibasic sodium phosphate 12 hydrated	2.9 g
Potassium chloride	0.2 g
Final volume with distillated water	1 l

PBS-Tween buffer

PBS buffer + 0.5 ml Tween-20 in each liter.

Covered buffer used in DAS ELISA (pH 9.6)

Anhydrous sodium carbonate	0.318 g
Sodium bicarbonate	0.586 g
Final volume with distillated water	200 ml
This buffer must be prepared every week	

Sample buffer for DAS ELISA (antigen)

Polivinil-pirrolidona, PVP 40.000 MW	10 g
Sodium sulfate anhydrous	0.65 g
Ovoalbumina	1 g
Final volume with PBS-Tween buffer	500 ml

Conjugate buffer for DAS ELISA

Bovine Seroalbumin	0.5 g
Final volume with PBS-Tween buffer	250 ml

Sample buffer for Indirect ELISA (pH 9.6)

Anhydrous sodium carbonate	0.795 g
Sodium bicarbonate	1.465 g
Polivinil-pirrolidona, PVP 40.000 MW	10.0 g
Final volume with distillated water	500 ml

Covered and conjugate buffer for Indirect ELISA

Bovine Seroalbumin	0.5 g
Polivinil-pirrolidona, PVP 40.000 MW	5.0 g
Final volume with PBS-Tween buffer	250 ml

8.5 Annex 5

Table used in GHL for sample distribution in ELISA test.

Date ELISA_____

Plate No._____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D				Control (+)	Clean	Control (-)						
E												
F							Control (+)	Clean	Control (-)			
G												
H												

8.6 Annex 6

Table designed for diagnosis pathogen input.

GHL No	Accession	Origin	Entry date	Test date	Fungi	Vir	Bact.	Other	Phytosanitary test	Observ.	Test done	Genus	Specie
219	G25109	PAL2008A	1/30/2008	3/17/2009	A	A	A		ACCEPTED		PDA, Blotter (Fungi), Elisa (Potyvirus, SBMV), Culture media, Gram staining, serology (Bacteria: *Xanthomonas campestris* pv *phaseolicola*, *Pseudomonas syringae* pv *phaseolicola*, Bacillus Gram +)	*Phaseolus*	*lunatus*
220	G25110	PAL2008A	1/30/2008	3/17/2009	A	A	A		ACCEPTED		PDA, Blotter (Fungi), Elisa (Potyvirus, SBMV), Culture media, Gram staining, serology (Bacteria: *Xanthomonas campestris* pv *phaseolicola*, *Pseudomonas syringae* pv *phaseolicola*, Bacillus Gram +)	*Phaseolus*	*lunatus*
221	G25113B	PAL2008A	1/30/2008	3/17/2009	A	R	A		REJECTED	Potyvirus	PDA, Blotter (Hongos), Elisa (Potyvirus, SBMV), Culture media, Gram staining, serology (Bacteria: Xanthomonas campestris pv phaseolicola, Pseudomonas syringae pv phaseolicola, Bacilos Gram +)	*Phaseolus*	*lunatus*