Lesson 1 Basic Concepts of Phytosanitary Quality

Objectives

To review:

- The importance of evaluating the phytosanitary quality of germplasm
- The basic criteria for such evaluation
- The relationship between the phytosanitary quality control and different stages of *ex situ* conservation

Introduction

One essential component of the *ex situ* conservation of germplasm is its phytosanitary quality. This component, as previously defined in *Module 3, Submodule B, Lesson 2*, refers to the absence of pathogens associated with planting materials and/or micro-organisms that cause deterioration during multiplication and storage. To obtain germplasm that meets this requirement, it is essential that, throughout the different stages of the *ex situ* management cycle, control measures are applied that ensure the materials are subjected to as little risk as possible. Consequently, plant health quality control attempts to:

- Reduce the risks involved in transferring germplasm from one country or region to another.
- Contribute to maintaining the material free of pathogens of quarantine interest and/or of those that imply risk for conservation in terms of causing total or partial losses in the production of planting materials (seeds or propagules), genetic erosion, poor quality, or deterioration during storage.
- Facilitate the availability of germplasm without plant health restrictions for the users.
- Facilitate decision-making for saving materials affected by damaging micro-organisms or of quarantine interest.
- Contribute to compliance with international standards.

The application of control measures involves the verification of results. To this end, different procedures and methodologies are used, some of which will be mentioned as we develop the theme. First, we will consider some concepts to better understand the theme 'verification of plant health quality'.

Basic Concepts and Criteria for Evaluating the Phytosanitary Quality

Phytosanitary quality refers to the concept whereby germplasm undergoing *ex situ* conservation is found free of pathogens of quarantine interest and/or those associated with the planting materials (whether seeds or propagules) and which may cause deterioration or contribute to genetic erosion.

When considering pathogens of quarantine interest, the definitions of the International Plant Protection Convention (IPPC) should be taken into account. Under the rubric 'Pest' (Box 1), pathogens (Figure 1) include micro-organisms (bacteria, spiroplasms, phytoplasmas, *Phytomonas* spp., fungi, and nematodes) and biotic agents (viruses and viroids) that can cause disease in plants (Agarwal and Sinclair 1987; González 1976; Neergaard 1977).

Box 1

IPPC definitions for pests

Pest: any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products.

Regulated non-quarantine pest: a non-quarantine pest whose presence in plants for planting affects the intended use of those plants with an economically unacceptable impact and which is therefore regulated within the territory of the importing contracting party.

Regulated pest: a quarantine pest or a regulated non-quarantine pest.

Quarantine pest: a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

SOURCE: FAO (1997).

Diseases caused by pathogens can sometimes lead, ultimately, to a plant's death, without its being able to produce the necessary reproductive structures and thereby causing loss of genotype (or genetic erosion). In other plants, pathogens cause the loss or reduction of the conservable product or reduce its quality by infecting and remaining within the tissues, so that when the germplasm is transported, so are the pathogens. Similarly, when infected materials are used for improvement programmes or multiplication, results are severely affected. To evaluate the plant health quality of germplasm and plan suitable management measures, several aspects should be taken into account, as discussed below.

Origin of materials

This must be considered as each collection site (country or region where the material was collected) has a level of risk associated with the availability of information on its pathogens and the activities carried out for their control (Table 1).

Pathogen inventory and quarantine classification, according to region or country

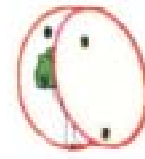
With this information, suitable measures for control can be implemented to guarantee the safe transfer and management of germplasm. For example, for the purposes of management, pathogens are classified in three groups:

- *Group A:* Dangerous pathogens that have a high epidemic potential but are not found in the region of introduction (exotic pathogens).
- *Group B:* Pathogens possessing moderate epidemic potential but not found in the regions of introduction or are occurring in restricted areas under effective control.
- *Group C:* Pathogens that are not considered to be of quarantine importance, but which affect the quality of planting materials.

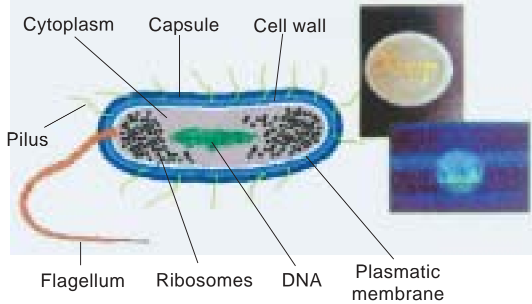
When germplasm comes from high-risk sites (i.e., categories 1 to 7, Table 1), it may carry pathogens of category A, B, or C. Given the characteristics of the pathogens included in groups A and B, the material should be filtered through post-quarantine control (closed quarantine). The procedure includes the production of seeds or propagules from the introduced germplasm and release of the same, if their health is duly verified. When information is available for

Types of pathogens

- Bacteria
- Spiroplasms
- Phytoplasmas
- *Phytomonas* spp.
- Fungi
- Nematodes
- Viruses
- Viroids



Bacteria



Prokaryotic organisms that lack chlorophyll and multiply by binary fission. Their colonies usually have a gelatinous aspect. More than 200 species cause diseases in plants.

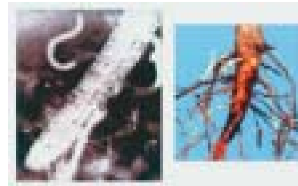
Fungi

Non-vascular, heterotrophic organisms that lack chlorophyll and other photosynthesizing pigments. Usually filamentous, they reproduce by means of sexual or asexual spores. More than 100,000 species exist, 8000 of which are pathogenic on plants.

Viruses

Obligate molecular parasites that possess RNA or DNA and have similar structures to the normal macromolecules found in cells. They depend absolutely on the mechanisms of the host's live cells to synthesize proteins and generate energy.

Nematodes



Cylindrical elongated worms that may be macro- or microscopic in size. They live in the soil or water and can parasitize animals or plants. In the case of the latter, they attack underground or aerial organs, using stylets to extract sap. Most of the species that attack plants belong to the phylum Nematoda, class Secernentea, order Tylenchida.

Figure 1. Pathogens associated with planting materials (diagrams adapted by B Pineda from different sources used in a personal lecture, 2004).

Table 1. Germplasm collection sites and associated risk.

Collection site	Risk level[a]
Collected from any site but plant identity at least to genus was unknown at the time of collection (e.g., grasses collected vegetatively)	1
Collected in a centre of plant and/or pest diversity	2
Collected in the wild, far removed from agricultural areas; wilderness	3
Tubers, roots, seeds, etc., collected in the market place	4
Farms	5
Orchards, plantations	6
Experimental fields (except those where plants are screened for resistance)	7
Experimental plots isolated from commercial plantings or located where certain pests are not present	8
Commercial greenhouses with floor beds	9
Commercial greenhouses with raised benches	10
Research greenhouses with raised benches	11
Approved certification	12
Plant tissue cultures, aseptic plantlet cultures, etc., particularly when derived from pathogen-tested mother plants	13
Collected from other containment facilities/quarantine stations provided the plants are pathogen-tested and grown under a high level of phytosanitation	14

a. ‘1’ denotes the highest hazard/risk site; ‘14’, the lowest.

SOURCE: Kahn (1999).

pathogens of groups B and C, then the planting materials can be analyzed through suitable sampling and released if found free of infection or contamination.

Levels of risk that germplasm movement implies

The movement of germplasm inevitably implies a level of significant risk. Planting materials (seeds, propagules, or plant parts) that are transported or transferred may well harbour pathogens within their tissues and thus carry them without their being noticed (Brunt et al. 1990; Frison and Feliu 1991; FAO and IPGRI 2004). Risk may be greater or lower, depending on:

- The plant genus or species;
- Presence of pathogens of quarantine importance transmitted through seed or planting material, or in association with the planting material in the exporting country or region of origin;
- Geographical distribution of such pathogens, and their life cycle and type of parasitism;
- The volume and frequency of international exchange of plants and planting materials; and
- The favourability of environmental factors.

Not all agents found in germplasm necessarily take on economic or quarantine importance. Many are ubiquitous and are established in the importing country; others are economically important and may be of quarantine significance. Even so, despite their ubiquity, pathogens have races that vary in aggressiveness according to the regions or countries where they are registered. Hence, race may become a risk factor in germplasm transfer.

Plant Health and its Relationship with Different Stages of Germplasm Management

Plant introduction

This stage—the transfer of germplasm from one country or region to another—should be understood as involving plant health risks and that, therefore, the procedure is subject to legislation (IPPC). Each party interested in moving the germplasm should agree on the terms of transfer, assuring the other parties of the legality of transport and of compliance with established plant health requirements (Barton and Siebeck 1994; COSAVE 2003; EPPO 2004; FAO 1997; OIRSA 2004).

The principal risk in moving germplasm is the transfer of pests and pathogens, which must be detected during quarantine, a procedure that includes inspection to detect pests and pathogens, treatment or cleaning of samples, and certification and release of material where no danger is presented, or its destruction if it is heavily contaminated or no technology is available to clean it (Nath 1993).

Germplasm that propagates through seed is planted under greenhouse conditions (closed quarantine) so that it germinates and develops seedlings (Figure 2). During establishment, the germplasm is inspected by plant health authorities, and, where necessary, samples are made and analyzed in the laboratory to intercept possible pathogens of quarantine interest.

When materials of vegetative reproduction (propagules or plant fragments) are introduced, procedures require more care, as each material taken from the mother plant may contain pathogens that are usually found in association with it. In this case, the initial selection of an adequate source of propagation is essential. Individual plants should be examined with all care to discover possible genetic disorders, bud variations, and symptoms of viruses or other disease pathogens. If a ‘clean’ plant is not found, then the pathogen must be eliminated by sanitizing a small part of the plant such as a stake, bulb layer, growth bud, or meristem that can constitute adequate initial material for propagation.

To obtain ‘clean’ planting material, various techniques are used, as not all have necessarily the same effectiveness for all plants or for all pathogens. An example of a cleaning technique is the system applied by CIAT to sanitize cassava (*Manihot esculenta*), a species that propagates vegetatively and is conserved *in vitro* (CIAT 1980, 1982; Mafla et al. 1992; Roca et al. 1991). The procedures are:

- Select mother plants, from which stakes, 15–20 cm long with vigorous buds, are taken. They are then surface-disinfected by submersion for 5 min in a solution of the insecticide dimethoate (Sistemin® at 0.3%), left to dry for 1–2 h under shade, and planted in pots that contain a sterilized substrate (e.g., soil to sand at 1:2).
- Apply thermotherapy for 3 weeks. That is, the pots with the seedlings are placed in a thermotherapy chamber, with a temperature range of 40°C (day) to 35°C (night),

illumination at 3000–4000 lux, and high relative humidity.
- Three or 4 weeks after thermotherapy, cut the buds to be used to extract meristems. The blade must be disinfected with detergent before each cut.
- Sterilize the tissue by placing the cut buds in a beaker with a mesh that facilitates their management in the laboratory. In a laminar flow chamber, they are disinfected by rapid submersion in 70% alcohol, rinsed with sterilized water, placed into a solution of sodium hypochlorite at 0.5% for 5 min, and finally rinsed three times with sterilized bi-distilled water.
- Isolate and plant the meristems (tissue structures of 0.3–0.5 mm, which include the meristematic dome with one or two leaf primordia). Under aseptic conditions in a laminar flow chamber and under the visual field of a stereomicroscope (10X–40X), the apical bud is caught with forceps and the appendages (leaves and stipules) covering the apex removed with a scalpel until a brilliant structure, 0.3–0.5 mm long with 1 or 2 leaf primordia, appears. Then a fine cut is made. This operation should be carried out very quickly and carefully to prevent excessive dehydration and possible death of the meristem. The explant is placed in a culture medium suitable for growth and development, ensuring that the basal part remains on the medium's surface.
- Incubate the culture. That is, the planted meristems are placed in test-tube racks and taken to a growth chamber with temperatures at 26°–28°C; illumination at 1000 lux, using fluorescent lamps, type daylight; and a 12-h day length.
- Leave seedlings to develop. This stage usually lasts 3 to 4 weeks, after which the explants must be transferred to a medium suitable for growth and development, with a prior cut in their bases to prevent any possible callus from forming. Three weeks after having been transferred to the new medium, the explants begin growing. Roots emerge, and a completely developed plant becomes available for continuing with the indexing tests (*Module 3, Submodule D, Lesson 2*).

Figure 2. Quarantine. Entrance to the quarantine greenhouse for introductions, seeds being planted, and developing seedlings (photos by B Pineda, GRU, CIAT).

Multiplication or regeneration

In this stage, germplasm produced in the open field runs the inherent risk that material may be lost or may deteriorate on being exposed to various adverse environmental or biological agents. Disease from pathogen attack can sometimes cause the eventual death of plants without their succeeding in producing the reproductive structures needed for conservation, thus resulting in the loss of genotype (i.e., genetic erosion). In other plants, conservable products may be lost or reduced in number, or their quality lost as pathogens infect tissues

and remain within them in such a way that transporting the germplasm would also mean transporting the pathogens.

During production, agronomic principles are applied and, hence, include all the practices needed to obtain the quantity and quality of propagules required for the different purposes of conservation. A major agronomic practice is the control of diseases caused by different types of pathogens. To apply control methods to achieve success in multiplication or increase, we must not only know the type of agronomic requirements of a given species, but also have information on the diseases that can affect the targeted germplasm, including causal agents, life cycle, mechanisms of dissemination, and factors favouring their development.

Harvest

Practices of plant health control also play an important role in this stage. Timely harvesting and good management of the germplasm obtained are fundamental to maintaining the germplasm's health, provided that, during production, adequate control measures had been applied. Seeds should also be harvested, preferably during the dry season, as harvesting in rainy or humid periods increases the risk of deterioration by micro-organisms that normally associate with the seeds. It is essential that harvested materials are placed in paper or cloth bags and duly identified before being transported to the sites for predrying and later conditioning.

Conditioning

During this stage, rigorous measures should be applied when cleaning equipment, tables, and places where activities are carried out, as seeds can be easily contaminated with micro-organisms normally present in residues from fruits or harvesting. Contaminating micro-organisms can contribute to the germplasm's deterioration during the subsequent steps of conservation if these precautions are not taken.

Testing the biological status

Essentially, test conditions must be guaranteed to permit the germination of materials without risk of contamination. In particular, the plant health quality of soil, water, trays or pots, and other implements used in evaluations must be checked to minimize the risk of contaminating seedlings and obtaining erroneous data on the germplasm's biological status.

Evaluating the Lesson

After this lesson, you should be familiar with the most important aspects of plant health quality control, the criteria to consider for the respective evaluations, and the relationship of plant health control with different stages of *ex situ* conservation.

Before going on to the next lesson, briefly discuss the importance of plant health quality control in the context of *ex situ* conservation of materials with which you are familiar.

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Next Lesson

In the next lesson, you will become familiar with the principal procedures for controlling the plant health quality of germplasm.

Lesson 2 Procedures for Verifying Phytosanitary Quality

Objective

To describe the general procedures for verifying phytosanitary quality

Introduction

To speak about phytosanitary quality without referring to health seems senseless. As human beings, we pay attention to our health every day, to maintain us active, vigorous, and with high levels of productivity. With plants, however, we do not check their health in the way they deserve. If we are to conserve germplasm (whether as propagules or seeds), then maintaining its health in the broad sense of the word is as essential for it as it is for us.

Ex situ conservation, especially those activities dealing with introduction, multiplication, and regeneration, requires considerable care in maintaining the germplasm's health for the length of its biological cycle. Being attentive during a plant's cycle so that it is not affected by pests and diseases (pathogens) should translate into a healthy plant whose products (seeds and propagules) are equally healthy, that is, they also possess the desired attributes for phytosanitary quality.

The verification of germplasm health, which results from the above-mentioned activities, should be carefully carried out. Likewise, information obtained for decision-making on germplasm management should be generated and collected according to the germplasm bank's objectives. Hence, procedures and techniques for diagnosing plant diseases should be used. Some of these will be mentioned in this lesson.

General Procedures

To verify the plant health status (i.e., quality) of a given germplasm, whether during plant establishment and development or in its products, we use procedures commonly employed in phytopathology to identify diseases and apply control measures (Agarwal and Sinclair 1987; Gerard 1984; FAO and IPGRI 2004; ISTA 1999; Neergaard 1977). These procedures can be conducted, either (1) directly at the site of confinement (quarantine) or production (field, greenhouses, and laboratories) through periodic inspections, or (2) through specialized procedures, some of which are described below.

Plant health inspection

As materials develop in the sites of quarantine, multiplication, regeneration, and determination of the biological quality, periodic inspections and observations should be practised to determine the presence of symptoms that would permit identification of diseases. Severity and incidence of the observed diseases should then be evaluated and the causal agents identified.

Where direct identification is not possible during inspection, then representative samples for specialized diagnosis should be collected. The samples can initially be examined in the laboratory under a stereomicroscope to find signs that may indicate the pathogen's nature. If such inspection proves the presumed presence of pathogens, whether of a fungal, bacterial,

viral, or other nature, then methodologies suitable for isolating and identifying them must be applied. These methodologies include the use of humidity chambers; isolation on specialized culture media; observation under light or electron microscope; use of identification keys; and verification, using Koch's postulates (Box 1), in healthy materials of the same species.

Box 1

Koch's Postulates*

1. The micro-organism should be always associated with the disease, and the disease, in its turn, should not appear without the micro-organism being or having been present.
2. The micro-organism should be isolated under pure culture and its specific characters should be studied.
3. When a healthy host is inoculated with the pure culture under favourable conditions, it should produce symptoms of the disease.
4. The micro-organism should be re-isolated from the inoculated host and should show the same characteristics under culture as that previously isolated.

* Adaptation from those originally recommended by Koch in 1881 for similar studies with humans and animals. They apply only to facultative parasites (in the saprophytic phase). The postulates must be reformulated for obligate parasites.

SOURCE: González (1976).

We point out that, in diseased tissue, not only is the casual micro-organism found but, often, other micro-organisms are also found, living as saprophytes and even growing in culture. Pure cultures must therefore be established for each organism separately and its pathogenicity determined, as according to Koch's postulates (Box 1).

Seed analysis. Seed analysis should be carried out in the laboratory on representative samples. This activity is usually executed by expert personnel, who use equipment and verification procedures already established for the type of pathogen being examined (Langerak et al. 1988). Before conducting seed analysis, personnel should clearly understand that **obligate** (those requiring live tissue for survival) and **facultative parasites** (having a saprophytic phase) exist in association with seeds and that their detection requires specific procedures. Obligate parasites do not grow on culture media, whereas facultative parasites do.

Also important to consider are the types of association that tend to occur between pathogens and seeds (Figure 1). Sometimes, they associate internally; other times, externally (Figure 1). When they are found active and embedded in seed tissues, then they are parasitic and cause **infections**. However, no relationship of parasitism exists if they are external and are being passively carried on seed coats, harvest residues, or particles of contaminated soil (**infestation**); or are mixed with seed (**concomitant contamination**) (Neergaard 1977).

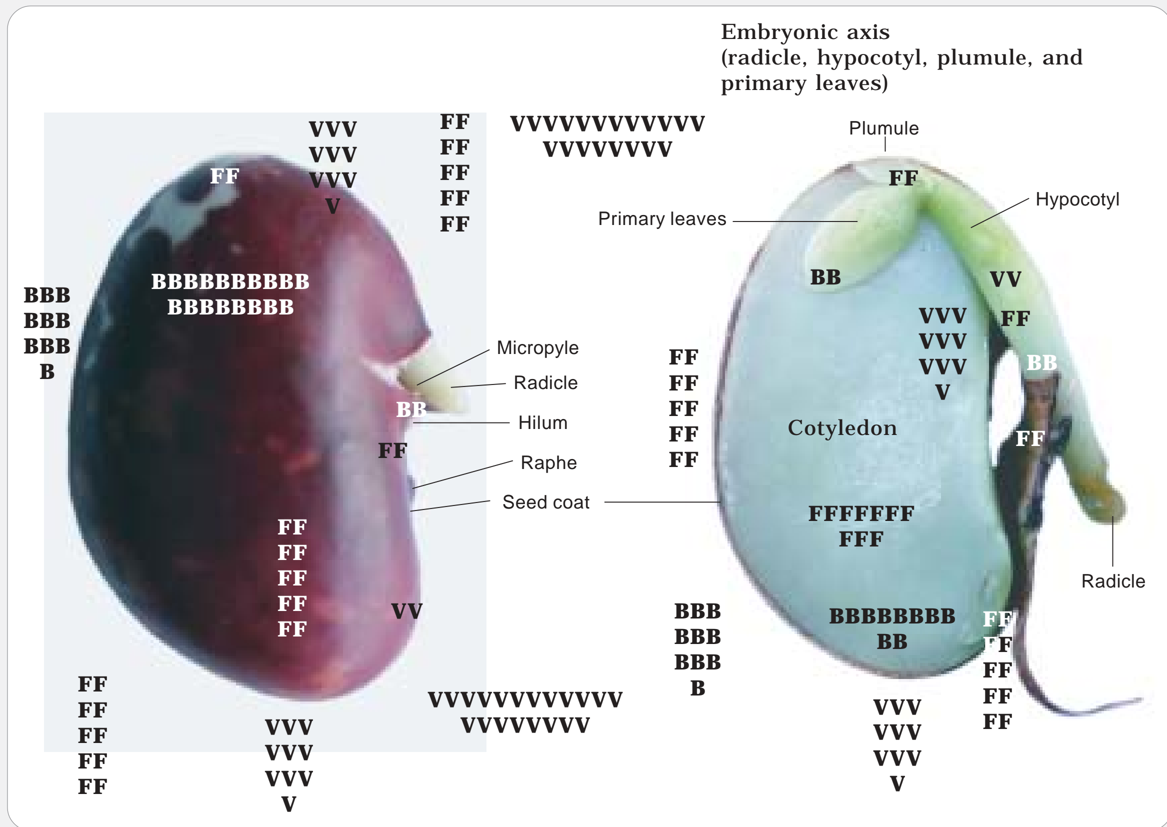

Figure 1. Association between pathogens and seeds. Fungi (F), bacteria (B), and viruses (V) may be found internally, causing infection in the tissues, or externally as contaminants, being either mixed or in external contact with tissues (graphic design by B Pineda, GRU, CIAT).

The basic procedures for verifying the plant health quality of seeds include preliminary activities (sampling, reception, registration, and storage), preparatory work, and analysis, duly organized to fulfil the entrusted function (Figure 2). Overall, these procedures include:

- Sampling, identification, packaging, and dispatch of materials to the laboratory.
- Reception of samples, and verification of identity and size.
- Division of samples for diagnosis according to pathogen type (samples separated for fungi, bacteria, viruses, and other pathogens, as according to requirements).
- Preparation of elements needed to verify pathogens according to type (i.e., separate elements for fungi, bacteria, viruses, and other pathogens, as according to requirements).
- Visual inspection of samples to detect mixtures and abnormalities in seeds.
- Processing and planting of samples according to pathogen type (procedures for fungi, bacteria, viruses, and other pathogens, as according to requirements).
- Incubation.
- Observation and analysis.
- Reports and documentation.

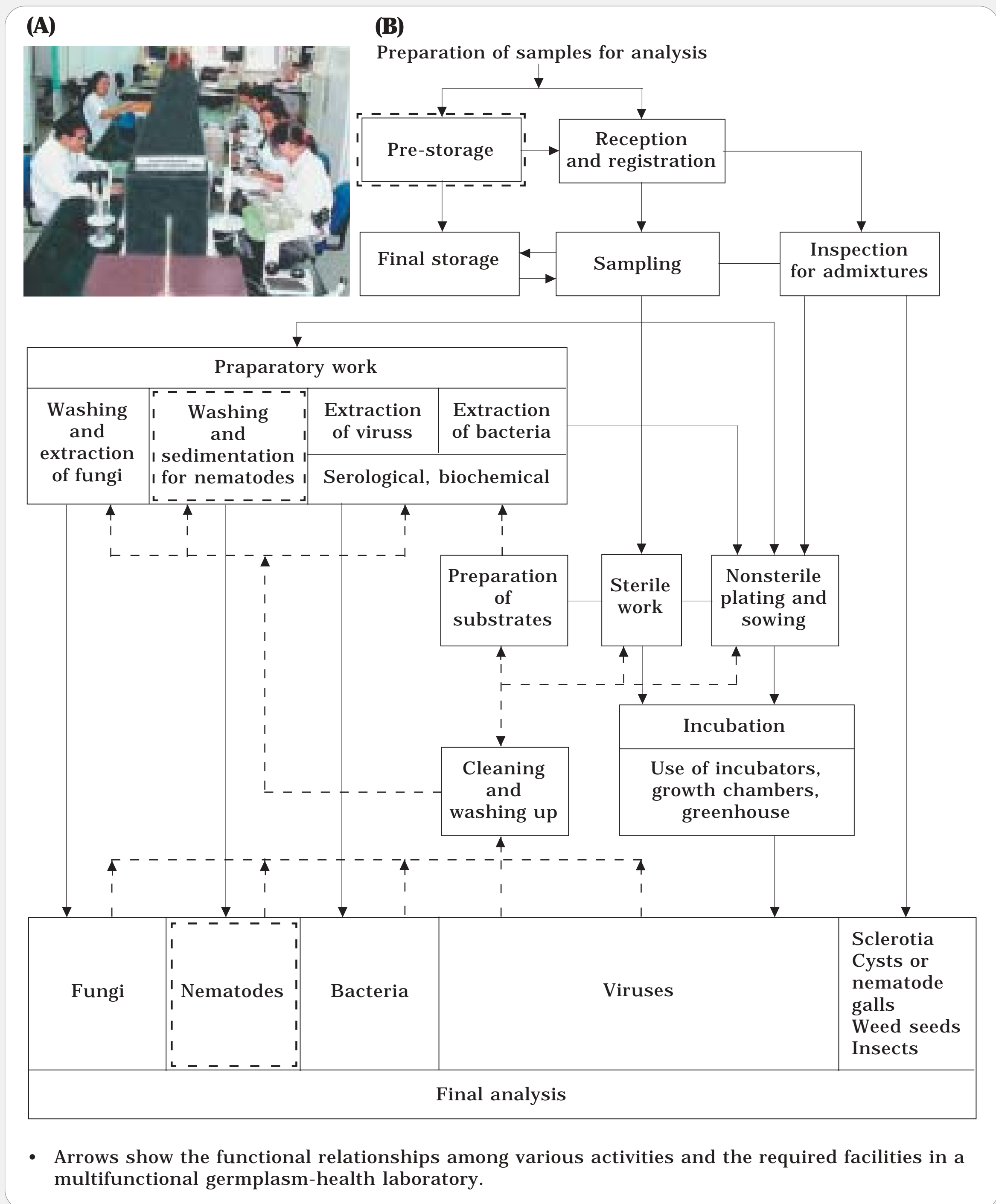

Figure 2. A multifunctional germplasm-health laboratory. **(A)** The area for the microscopic analysis of seeds. Example of the Germplasm Health Laboratory of the Genetic Resources Unit at CIAT. **(B)** Flow chart of activities. (Chart is adapted from Langerak et al. 1988.)

The methodology to determine the seed health status uses different techniques to detect pathogens, depending on the class and type of micro-organism (e.g., obligate or facultative parasite, or saprophytes), the characteristic relationship it has with seeds (infection, infestation, or concomitance), or level of precision required. A given technique is selected according to the test's purpose and the objectives for analysing seed health (Tables 1, 2, and 3).

Once the results of the analyses are obtained with respect to the germplasm's quality (Figure 3), decisions can be made and management carried out accordingly. When standards for the quality demanded are met, the germplasm can be conserved according to the bank's goals. Any affected materials found may be subjected to cleaning procedures that will help rescue, reintroduce, multiply, or regenerate them.

Analysing materials for vegetative propagation

The procedures and methodology for analysing the phytosanitary quality of propagules or plant fragments used for vegetative propagation are based on the same principles as for seeds. Normally, in the field, germplasm can be infected by pathogens to a greater or lesser extent and, hence, needs its plant health to be monitored according to circumstances. Evaluations and analyses must be conducted with special care, as any plant part (e.g., stem, root, leaf, cutting, root cutting, stake or stem cutting, meristem, or callus) or organ (e.g., bulb, corm, rhizome, cutting, or tuber) used for propagation contains pathogens such as fungi, bacteria, viruses, viroids, or nematodes. Materials for vegetative propagation are at greater risk and require stricter plant health procedures. In fact, for analysis, observations can be carried out directly on the organs or plant parts to be used or the material can be planted and germinated and the results assessed in the seedlings with the naked eye, as described for seeds.

Plant health inspection is especially relevant for selecting materials for use in propagation. Usually, the selected clone comes from a single plant from which several propagative units (which should be as disease-free as possible) are taken. Selection starts with observing and evaluating in detail the material that is to be multiplied for conservation. All aerial (e.g., leaves, branches, stems, flowers, and fruits) and subterranean organs (e.g., roots, tubers, and bulbs) should be inspected to ensure they are in suitable condition. That is, they do not present spots, mosaics, or malformations in leaves and stems; stems do not present fissures, spots, or aqueous wounds, but do present a healthy and brilliant epidermis; and, overall, the material does not present necrosis, cankers, or rot in any organ; or wilt, dwarfism, or abnormal growth.

Even if the planting material is at very high risk with respect to plant health, the current tendency is to use *in vitro* culture techniques and thermotherapy as an alternative to safely maintain the germplasm free of pests and pathogens. That is, to guarantee the acquisition of materials of high plant health quality, the above-mentioned techniques are combined and verifications are carried out, applying several diagnostic techniques (indexing), according to case (Figure 4).

Table 1. Methods used to detect fungi and bacteria in analyses of seed health.

Method	Type of observation	Verifications	
		Fungi	Bacteria
Examination of dry seeds	With the naked eye, lenses of low magnification, and/or stereomicroscope	Presence of discoloration, morphological abnormalities, and pathogenic fructifications mixed with the seeds	Discoloration, presence of morphological abnormalities, and bacterial growth
Examination of seeds immersed in water	With stereomicroscope, light close to ultraviolet	Presence of fungal fructifications or characteristic fluorescence	Bacterial growth, presence of characteristic fluorescent pigments
Rinse from seeds	With microscope, of sediments obtained after centrifuging	Presence of spores, remains of fructifications, chlamydospores, and microsclerotia	–
Incubation of seeds (blotter test)	With stereomicroscope and microscope, after incubation, under conditions of light, temperature, and humidity suitable for seeds to germinate and fungi to grow and sporulate	Presence of fructifications, spores, mycelia, sclerotia, and microsclerotia	Bacterial growth, presence of characteristic fluorescent pigments
Incubation of seeds (agar-plate test)	With stereomicroscope and microscope, after incubation, under conditions of light and temperature suitable for seeds to germinate and fungi to grow and sporulate; Visual appraisal of bacterial colonies	Presence of fungal colonies with fructifications, spores, mycelia, sclerotia, and microsclerotia	Presence of colonies typical of the bacterial species being followed up
Biological tests (seedling-symptom test)	Visual appraisal, after seeds germinate (whether in soil, or on paper towelling or filter paper; or grown in test tubes on culture medium) under controlled conditions, i.e., suitable confinement, light, humidity, and temperature	Symptoms in seedlings (absence of symptoms does not necessarily indicate that seedlings are free of fungi, as latent infections can occur)	Symptoms in seedlings and presence of the bacterium when isolated (Koch's postulates must be applied)
Biological tests (pathogenicity tests)	Visual appraisal, of symptoms on adapted hosts, induced by inoculation with colonies of fungi or bacterial associated with the seeds	Symptoms in seedlings as a consequence of the inoculations; presence of fructifications, spores, and mycelia (Koch's postulates)	Symptoms in seedlings as a consequence of the inoculations; presence of the bacterium for re-isolation (Koch's postulates)

(Continued)

Table 1. (Continued.)

Method	Type of observation	Verifications	
		Fungi	Bacteria
Biological tests (using bacteriophages)	Visual appraisal, of colonies affected by specific bacteriophages	–	Presence of lytic zones around the bacterial colonies
Observations of fluorescence	Observations under light close to ultraviolet (340 nm)	Pale blue, yellow, or greenish fluorescence, according to pathogen or organism present in the seeds	Pale blue, yellow, or greenish fluorescence, according to pathogen or organism present in the seeds
Histopathological methods	With microscope, of seed tissues duly processed and stained	Presence of hyphae and, in some cases, fructifications within tissues	Presence of bacteria within the tissues
Embryo counts	With microscope, of embryos duly processed and stained	Presence of fungal structures (e.g., *Ustilago* spp. and *Sclerospora graminicola*)	–
Immersion in sodium hydroxide method	With stereomicroscope, of seeds treated with NaOH at 0.2% for 24 h at 18°–25°C	Presence of fungal structures (e.g., *Tilletia barclayana* or *Neovossia horrida*)	–
Biochemical tests (serology, ELISA, microprecipitin, and immunofluorescence)	Observation of precipitation reactions, and coloration or fluorescence, according to the type of test used	Presence of precipitates, and coloration or fluorescence, according to the fungus intercepted	Presence of precipitates, and coloration or fluorescence, according to the bacterium intercepted
Molecular and biochemical methods	Observations of gels or colour reactions, according to the pathogen or type of test used	Presence of coloured bands or colorimetric reactions, according to the pathogen intercepted	Presence of coloured bands or colorimetric reactions, according to the pathogen intercepted

Table 2. Methods used to detect viruses and viroids by analysing seed health.

Method	Type of observation	Verifications
Examination of dry seeds	With naked eye, lenses of low magnification, and/or stereomicroscope	Discoloration, reduced size, presence of morphological abnormalities
Biological tests (seedling-symptom test)	Visual appraisal, after seeds germinate (whether in soil, or on paper towelling or filter paper; or grown in test tubes on culture medium) under controlled conditions, i.e., suitable confinement, light, humidity, and temperature	Observation of symptoms in seedlings
Biological tests (pathogenicity tests)	Visual appraisal, of symptoms on suitable hosts, induced by inoculation of seed extracts or suspect seedlings	Symptoms in seedlings or in indicator plants as a consequence of inoculations
Histopathological methods	With electron or optic microscope, of seed tissues duly processed and stained	Presence of viruses or viral inclusions within the tissues
Observations with electron microscope: negative staining, immunosorbent electron microscopy (ISEM and ornamentation)	With electron microscope, of seed tissues duly processed and stained	Presence of viruses or viral inclusions within the tissues
Biochemical tests (serology, ELISA, agglutination [chloroplast and latex], tagged antibodies, and immunofluorescence)	Observation of precipitation reactions, and coloration or fluorescence, according to the type of test used	Presence of precipitates, and coloration or fluorescence, according to the virus intercepted
Molecular techniques	Observation of gels or colour reactions according to the pathogen or type of test used	Presence of coloured bands or colorimetric reactions, according to the virus intercepted

Table 3. Methods used to detect nematodes by analysing seed health.

Method	Type of observation	Verifications
Examination of dry seeds	With naked eye, lenses of low magnification, and/or stereomicroscope	Discoloration, presence of morphological abnormalities, or nematode cysts mixed with seeds
Examination of seeds immersed in water	With stereomicroscope	Presence of nematodes, eggs, larvae, and cysts
Extraction of nematodes from affected tissues or soil mixed with seeds	With stereomicroscope, after extraction	Presence of nematodes, eggs, larvae and cysts

One example is the procedure employed by the Genetic Resources Unit at CIAT for cassava (*Manihot esculenta*), a plant that propagates vegetatively. The process begins with selecting mother plants, from which stakes are taken and subjected to thermotherapy. Meristems are then cut, cultured, and micropropagated *in vitro* (see *Module 3, Submodule D, Lesson 1*). They are then propagated in specialized culture medium for 4–5 weeks in a growth chamber until they are large enough to transplant to the greenhouse.

Once transplanted to pots, they are given appropriate care to obtain stems and leaf tissue for applying screening tests, which are applied according to available facilities and to the viruses being targeted for detection (e.g., common cassava mosaic virus or CCMV, cassava X virus or CsXV, and frogskin disease or FSD). The techniques applied may include diagnoses based on visible symptoms, grafts, serological methods of diffusion, precipitation, enzyme immunoassays (ELISA), and PCR (Figure 3). The materials that show negative results for the different indexing tests should be maintained or propagated under conditions that prevent re-infection. If materials show positive results to the indexing tests, then thermotherapy is re-initiated, followed by meristem culture, and subjected again to indexing tests (CIAT 1982; Roca and Mroginski 1991; Roca et al. 1991a, b).

Evaluating the Lesson

After this lesson, you should be familiar with the general procedures for verifying plant health quality.

Before going on to *Submodule E*, briefly comment on the importance of verifying plant health quality. If you have had some experience in this regard, indicate what procedures were applied.

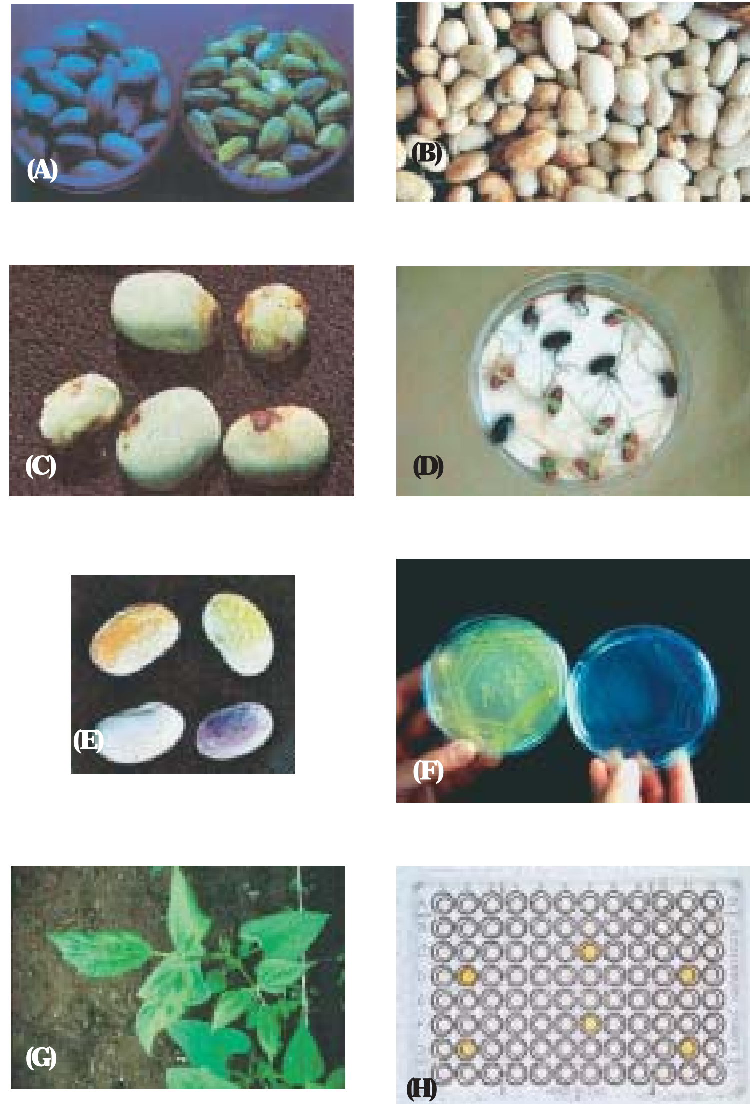

Figure 3. Results of applying diagnostic techniques in the control of plant health quality in seeds. **(A)** Peanut seeds examined under ultraviolet light: left, healthy seeds; right, seeds infected by *Fusarium oxysporum*. **(B)** Bean seeds infected by various pathogens, mixed with healthy seeds. **(C)** Beans seeds infected by anthracnose. **(D)** Bean seeds infected by *Macrophomina phaseoli*, mixed with healthy seeds. **(E)** Seeds of beans infected by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens.* **(F)** Bacterial colonies of *Xanthomonas campestris* pv. *phaseoli* in YDCA and MXP. **(G)** Bean seedling infected by bean common mosaic virus (BCMV). **(H)** ELISA plate: coloured wells correspond to samples with viruses and colourless wells to healthy seeds. (From Ahmed and Ravindener Reddy 1993 [A]; Schwartz and Pastor-Corrales 1989 [C and E]; photographs by B Pineda, GRU, CIAT [B, D, F, G, and H].)

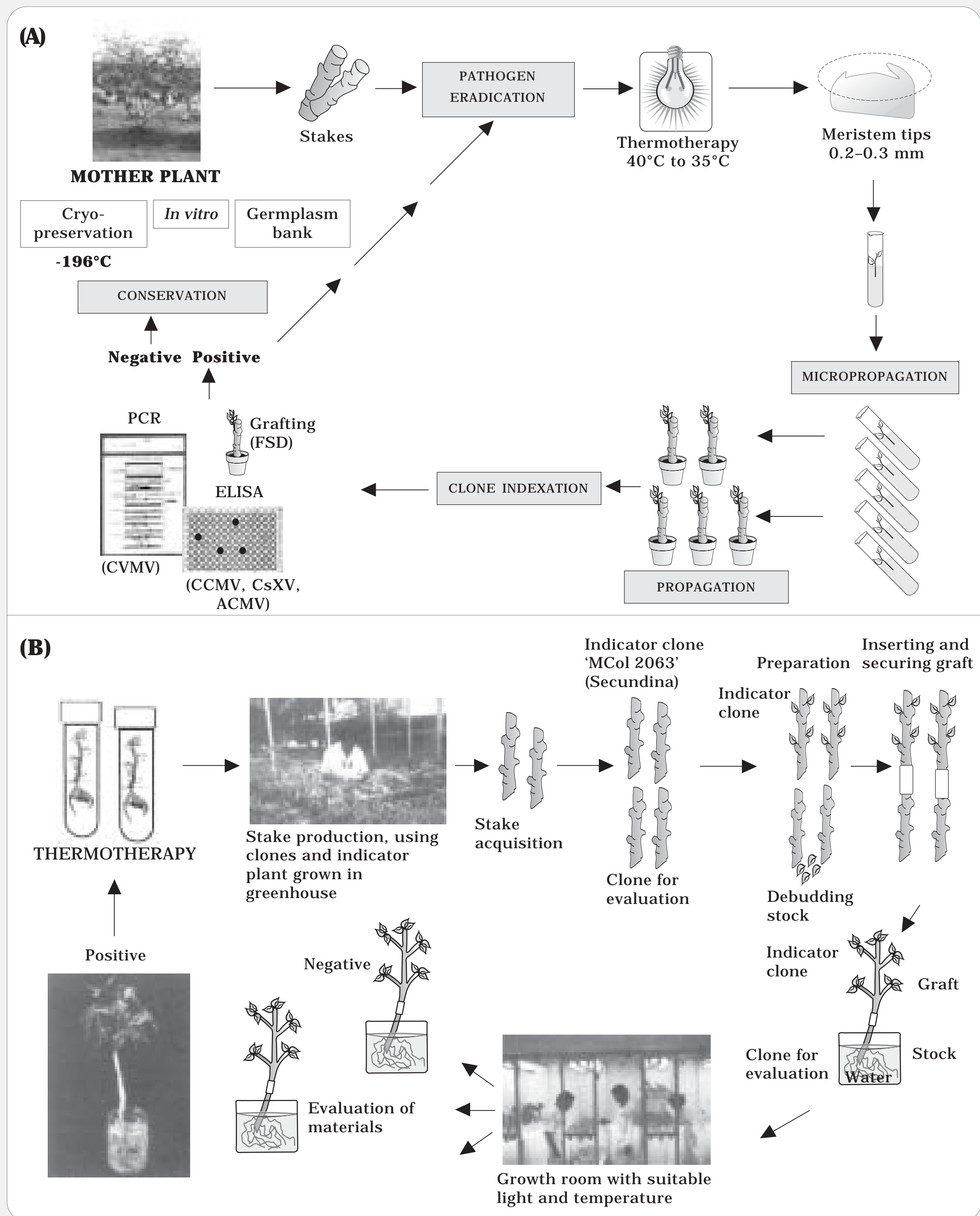

Figure 4. System for eradicating pathogens **(A)** and indexing cassava germplasm **(B)**. (Diagrams redesigned by B Pineda from the *Flow Chart for the* In vitro *Management of* Manihot *Germplasm at CIAT* by Flor et al. nd.)

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Next Lesson

In the next *Submodule E*, you will study the principal aspects of storing (conserving) germplasm.