PROCEDURES USED BY THE GERMPLASM HEALTH LABORATORY (GHL) OF CIAT

INTRODUCTION

The main responsibility of the Germplasm Health Laboratory (GHL) is to test the health status of germplasm (beans, tropical pastures and cassava) intended for international and national export.

To minimize the phytosanitary risks associated with the movement of germplasm, especially concerning the inadvertent transport of pathogens and pests of quarantine significance, CIAT is following a regulatory and quarantine program, in close cooperation with the plant quarantine authority of its host country "Instituto Colombiano Agropecuario (ICA)". The objectives of the program are: 1) to prevent the spread of seed borne diseases and to minimize the risk of accidentally introducing exotic pests and pathogens into Colombia, 2) to inspect screenhouses and glasshouses where imported germplasm is increased, 3) to inspect field and greenhouses where the germplasm intended for international export is increased, and 4) to test the seed health status of germplasm for international export.

The ICA Plant Quarantine Officer, stationed at CIAT, carries out field and greenhouses inspections and issues "ICA's Phytosanitary Certificate" (which accompanies all out-going germplasm from Colombia) based on that inspection and results obtained by the GHL.

FACILITIES

CIAT's facilities for germplasm health testing and procedures are designed as a multifunctional laboratory to test seeds and tissues for fungi, bacteria, viruses, and occasionally nematodes. Facilities for seed health testing include the GHL of 136 m^2.

Seed health testing activities include: 1) reception, registration, sampling and storage of submitted samples; 2) preparation of working samples for testing, and 3) analysis. Figure 1.

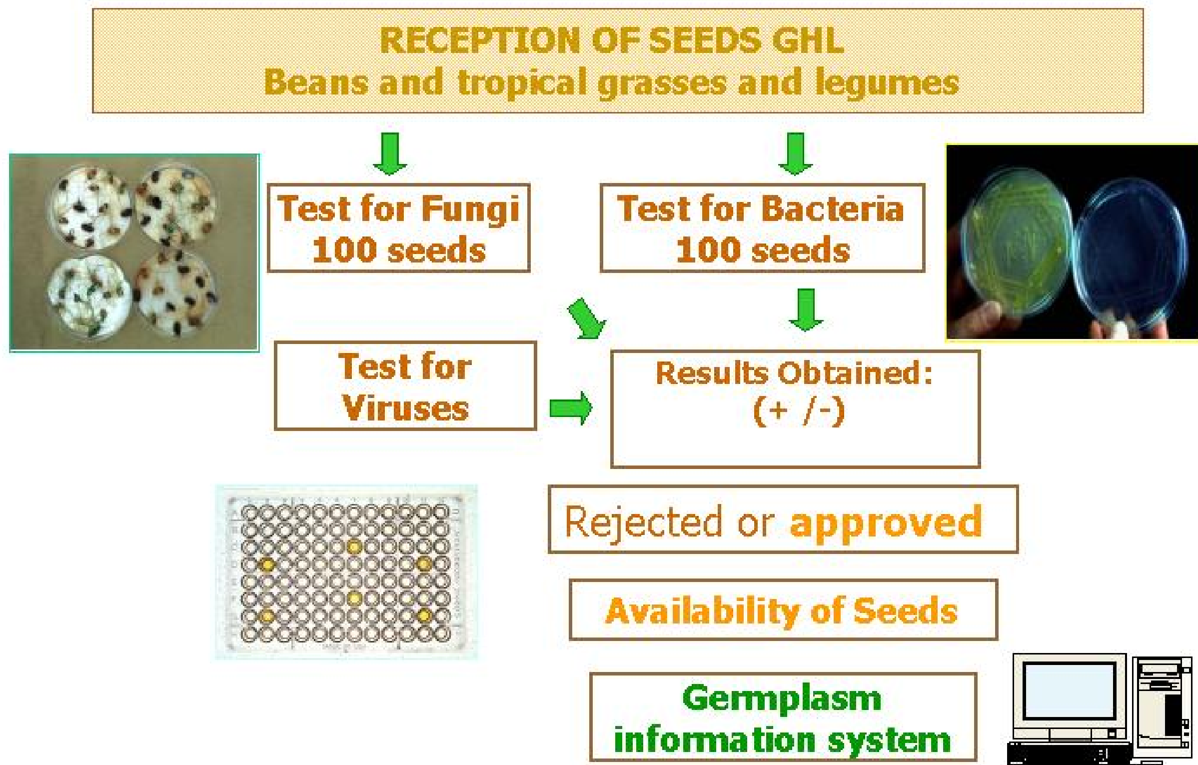


Fig 1. Flow chart GHL

TESTING PROCEDURES

The seed health testing methods used at CIAT for beans, cassava and tropical pastures are summarized in Table 1.

Bean germplasm and Tropical Pastures

Bean germplasm leaving CIAT and the one used for exchange, conservation, and characterization in the GRU are multiplied in isolate fields under favorable ecological conditions for bean growth with supervision by ICA quarantine officers. The harvested seeds are analyzed by GHL prior to shipment abroad or long-term conservation.

To detect pathogens of quarantine significance, the GHL uses the methodologies recommended by CIAT's pathologists and virologists. When a recipient country has additional requirements, the GHL executes additional tests whenever possible to comply with the specific quarantine regulations of the recipient country.

For common beans (*Phaseolus vulgaris*) and other species of *Phaseolus* (*P. lunatus, P. acutifolius*)

seeds aimed at international germplasm exchange and conservation are tested in the GHL to intercept seed-borne pathogens that are recorded in the bean seed producing areas. Testing seed samples of at least 100 seeds for each pathogens group to check fungi, bacteria and viruses uses routine methods.

Fungi detection

For fungi two incubation methods are used: blotter test and agar plate. The basic principle in these methods is to provide a high level of relative humidity and optimum light and temperature conducive to fungal development. The blotter test is a combination of the **in vitro** and the **in vivo** biological techniques where the seeds are sown in petri dishes with moistened filter paper. When the agar plate is used, seeds treated with 75% ethanol and 1% sodium hypochlorite, are plated in petri dishes on a suitable agar media, generally potato dextrose agar with lactic acid 25%, 100 microliters/Lt of media. After plating both procedures include the placing of seeds in culture plates and then incubation under near ultraviolet light (NUV) and fluorescent light with a 12 hr. cycle light and dark at +20 - 27°C. After eight days of incubation the seeds are examined using a stereobinocular microscope. The fungi are identified by morphology. The figure 2 showed the flow chart for fungi detection.

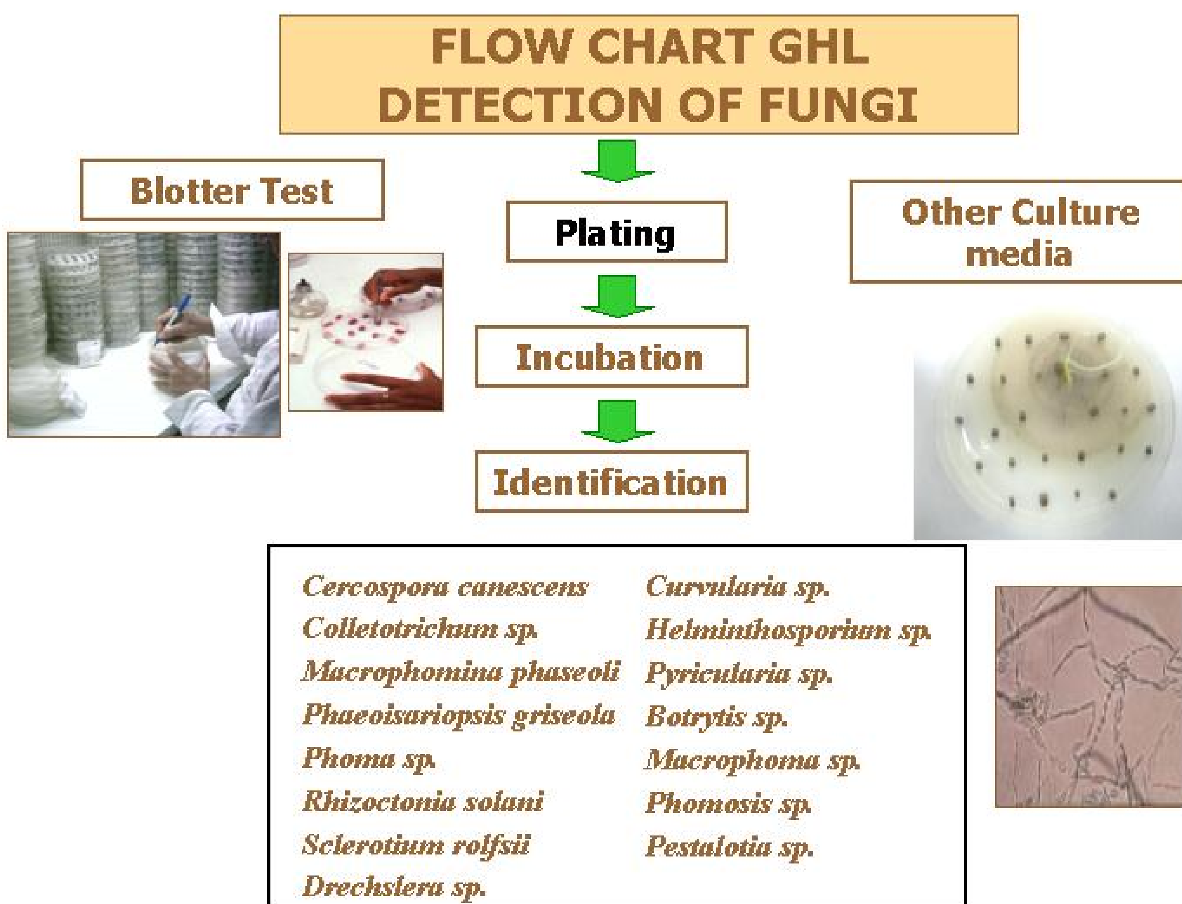


Fig 2. Flow chart GHL for detection of fungi

Bacteria detection

For the detection of seedborne bacteria (*Xanthomonas campestris* pv. *phaseoli* and *Pseudomonas syringae* pv. *phaseolicola*), the GHL uses methods such as isolation in culture media and serology. The method used in the detection of *Xanthomonas campestris* pv. *phaseoli* is the agar plate dilution technique. The seeds are washed thoroughly in running tap water and then for extraction they are placed in a bag with sterilized saline solution. The samples in the plastic bag are incubated overnight at +6°C.

Aliquotes of the seed leachate are removed and serially diluted 10 fold to 1:100; microlitres from each dilution are spread onto the surface of YDC Agar, plates are incubated at +27°C and after 3 days examined for colonies. Also SLH uses the MXP semiselective culture medium using a technique described by Claflin et al. (1987). MXP is a semi-selective medium for *X. campestris* pv. *phaseoli* where *X. campestris* pv. *phaseoli* hydrolyzes starch around the colonies. A first identification uses color and morphology of the colonies, and the confirmation is done with serological reactions with a specific antiserum.

The detection of *P. syringae* pv *phaseolicola* has an extraction phase like *X. campestris* but the spreading is onto the surface of King B medium (Saettler et al. 1989). The *P. syringae* pv *phaseolicola* is identified by presence of the fluorescent pigment and with the serologic agglutination test.

The detection of *Curtobacterium flaccumfasciens* pv. *flaccumfasciens* (*Corynebacerium flaccumfasciens* fsp *flaccumfasciens*, has the same extraction phase. Then the bacterias are identified using color and morphology of the colonies and the gram coloration. There are only gram positive bacteria of quarantine importance. The GHL considers information in relation to the presence of this kind of pathogens around the production areas and makes decisions as to use specific methods of detection. The figure 3 showed the flow chart for bacteria detection.

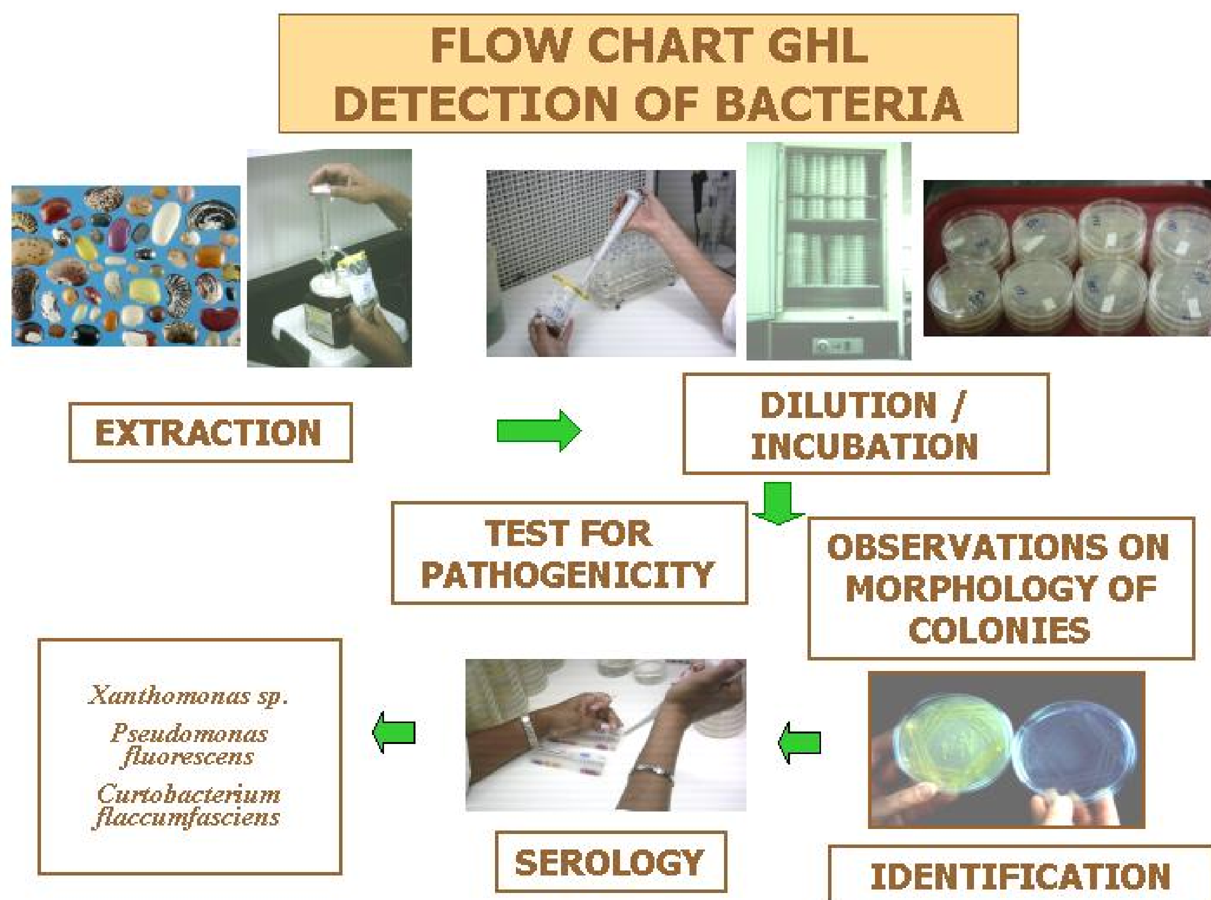


Figure 3. Flow chart GHL for detection of bacteria.

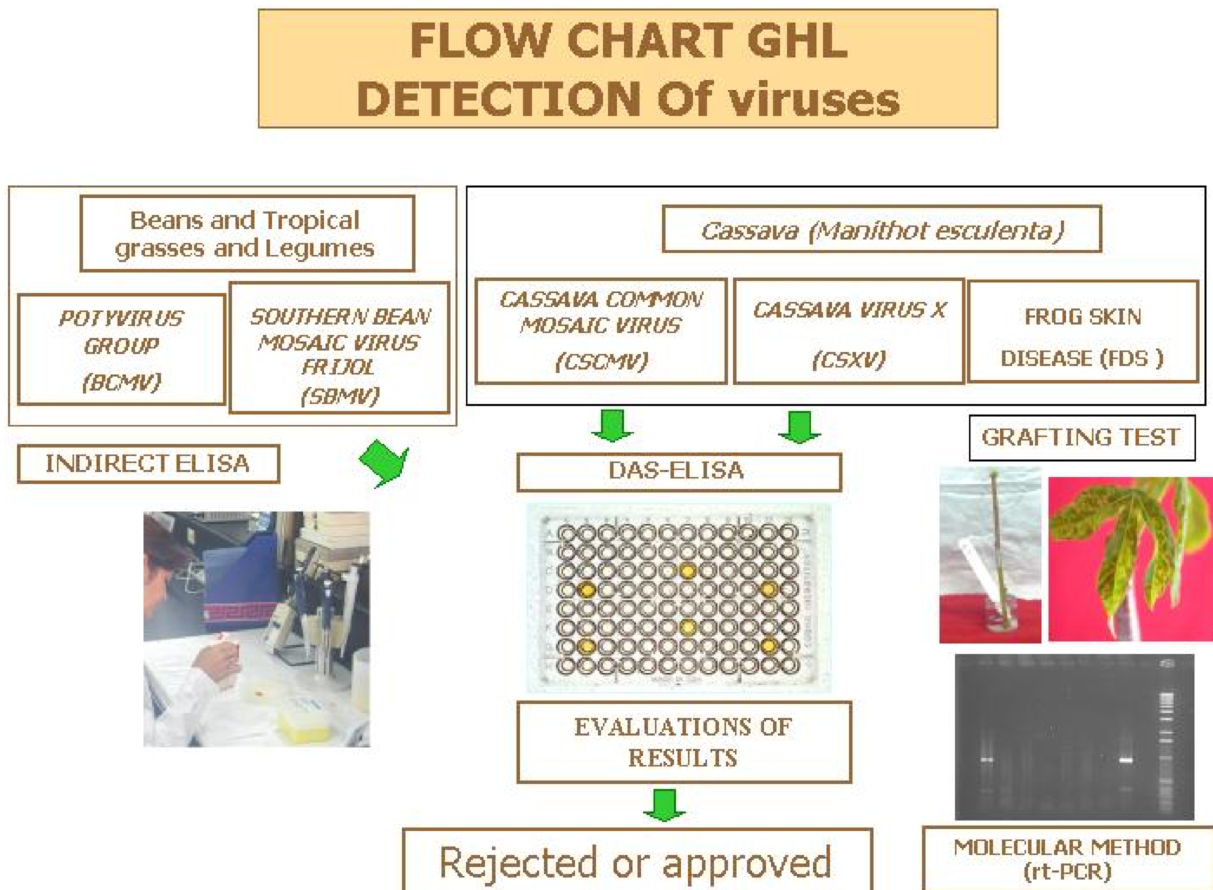
Virus detection

The ELISA test is used for the examination of bean seeds in order to check absence of the seedborne viruses: bean common mosaic virus (BCMV) and bean southern mosaic virus (BSMV) (Clark et al. 1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses are given elsewhere. For the detection of common mosaic virus we use the protocol with a Monoclonal antiserum against the potyvirus group. In order to check the samples against BSMV we use the DAS Elisa Reagent set.

When a country is concerned by a specific virus we need to make another testing with the required specific probes, for example the detection of the tobacco streak virus (TSV).

When introduction of tropical pastures germplasm (legumes and grasses) is required, an importation permit and a phytosanitary certificate are required for such introduction. Untreated seed samples are sent to the plant quarantine office at CIAT headquarters for visual inspection. They are then treated with pesticides, pregerminated under lab conditions, and monitored for their health status and performance. Selected seedlings are transplanted in pots of sterilized soil and maintained under closed post-quarantine glasshouse conditions. Late flowering species are monitored for three months

while early flowering species are observed for one vegetative cycle. Seedlings that are rejected are incinerated. Plants with disease symptoms are rouged and incinerated. The germplasm is released only if there are no quarantine problems. The figure 4 showed the flow chart for viruses detection.



Cassava (*Manihot* spp.)

For cassava, the import and export procedures are as follows:

1) For stakes (unrooted stem cuttings): these are grown in greenhouses in the foreign country of origin. This material is treated by thermotherapy (+40°C day /35°C night) for 3 weeks and then a meristem culture on synthetic media is made in the laboratory. The plants once developed are tested for cassava virus diseases (CCMV, CsXV) using ELISA. If one plant from one shoot tip shows the presence of the virus, all plants are destroyed. The virus-tested plants are transplanted into sterilized soil and retested for the Frogskin disease (FSD) by grafting to a healthy hypersensitive clone and analysis by RT-PCR.

2) For in vitro material: the Staff of CIAT or National Program culture the shoot tips in synthetic media in laboratory facilities provided by national programs of the foreign country. When the shoot tips in test tubes arrive at CIAT, they are transferred to new media and micropropagated. Once new tips are produced in vitro, they are harvested, transferred to new

media, and treated by thermotherapy (+37°C day /35°C night) for 12 days. Newly developed shoot tips go through a total of 3 cycles of thermotherapy, after which they are grown under normal conditions, at +26-28 °C (Mafla G. et. al., 1984). The plants developed in vitro are tested for cassava virus diseases (CCMV, CsXV) using ELISA. If one plant from one shoot shows presence of the virus, all plants are destroyed. Virus-tested plants are transplanted into sterilized soil and retested for the Frogskin disease by grafting to a healthy hypersensitive clone and analysis by RT-PCR (Cuervo, 2006). Plants that are proven negative in all tests are released.

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Table 1. Seed health testing methods applied at CIAT to detect Bacteria (B), Fungi (F), Viruses (V), Nematodes (N), Insects (I), and Weeds (W).

SEED HEALTH TEST	CROP SPECIES		
	Bean	Cassava	Trop. Pastures
Seedling symptom test	F,B,V	F,B,V	F,B,V
Blotter test	F	F	F
Agar test	F	F	F
Indicator test	B, V	V	V
Washing test	F	F	F
Washing sedimentation test	N	-	N
Serological test (tube agglutination)	B	-	B
Immunoenzymatic test (ELISA)	V	V	V
Dilution plating test	B	B	B
Direct visual inspection	F,N,I,W	F,N,I,W	F,N,I,W
Polymerase Chain reaction (PCR)		V	

Table 2. Pathogens of quarantine significance of Bean germplasm and Tropical Pastures tested by the Germplasm Health Laboratory.

Pathogens	CROP SPECIES
Fungi	
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Cercospora canescens	Tropical Pastures
Colletotrichum sp.	Tropical Pastures and Bean
Macrophomina phaseoli	Tropical Pastures and Bean
Phaeoisariopsis griseola	Bean
Phoma sp.	Tropical Pastures and Bean
Rhizoctonia solani	Tropical Pastures and Bean
Sclerotium rolfsii	Tropical Pastures and Bean
Drechslera sp.	Tropical Pastures
Curvularia sp.	Tropical Pastures and Bean
Helminthosporium sp.	Tropical Pastures
Pyricularia sp.	Tropical Pastures
Botrytis sp.	Tropical Pastures and Bean
Macrophoma sp.	Tropical Pastures and Bean
Phomosis sp.	Tropical Pastures and Bean
Pestalotia sp.	Bean
Ascochyta sp.	Bean
Bacteria	
Xanthomonas campestris pv. *phaseoli*	Bean
Pseudomonas syringae pv. *Phaseolicola*	Bean
Curtobacterium flaccumfasciens	Tropical Pastures and Bean
Xanthomonas sp.	Tropical Pastures
Pseudomonas fluorescens	Tropical Pastures
Viruses	
Potyvirus Group	Tropical Pastures and Bean
Bean common mosaic virus (BCMV)	Tropical Pastures and Bean
Bean southern mosaic virus (BSMV)	Tropical Pastures and Bean