LABORATORY MANUAL
ISOLATION AND PRESERVATION OF FUNGI AND BACTERIA WITH POTENTIAL ANTAGONISM AGAINST *Fusarium oxysporum* f.sp. *cubense* COLECTED FROM SOIL SAMPLES

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Introduction

In the soil, there are numerous bacteria and fungi that act as natural antagonists of phytopathogens, meaning they have the ability to inhibit the growth and activity of organisms that cause diseases in plants. Among these, some species of bacteria of the genus Pseudomonas are reported, known for their ability to produce a wide variety of antimicrobial antibiotic compounds and hydrolytic enzymes that can inhibit the growth of phytopathogens. Bacteria of the genus Bacillus are also recognized for their antagonistic activity against phytopathogens by producing antibiotics, chitinolytic enzymes, and antimicrobial peptides that inhibit the growth of fungi and phytopathogenic bacteria. Also fungi like Trichoderma or Gliocladium present that characteristics.

Table 1 briefly presents a description of all the processes to be carried out to obtain antagonistic microorganisms (fungi and bacteria) from the soil for the control of *Fusarium oxysporum f. sp. cubense*, starting from field selection to the development of commercial formulations for use in the field crops.

The objective of this manual is to describe the laboratory procedures for isolating and purifying antagonistic fungal and bacterial colonies from soil against phytopathogens, as well as their preservation for subsequent use in in vitro confrontation tests within the project "Establishment of an alert system for *Fusarium oxysporum f. sp. cubense* in banana cultivation and disease mitigation strategies." The procedure is described:

Procedure for Isolation and Preservation of Soil Fungi and Bacteria in the Laboratory.

Soil samples obtained from the field and the area of interest should preferably be processed immediately after sampling. Otherwise, they should be stored in refrigeration at 4 to 6 °C. It is important to note that longer storage times will decrease the likelihood of obtaining the desired microorganisms.

In any laboratory work, prioritizing asepsis in the process of isolating microorganisms to avoid possible contaminations is essential. It is recommended to always wear aprons and gloves, and the laboratory materials used should be sterilized. It is also preferable to work near a lit Bunsen burner to reduce potential contamination risks.

Table 1: Brief Description of the Development to Obtaining Antagonistic Bacteria and Fungi against *Fusarium oxysporum* f. sp. Cubense (FOC)

1. Preparing the Soil Sample

Homogenize the collected samples and weigh 5 grams for subsequent isolation.

Depending on the availability of laboratory materials, a smaller or larger amount of soil can be considered for processing.

Petri dishes of larger size (9 cm) require more availability of culture media and space in incubators but they can obtain more colonies of microorganisms. Smaller-sized plates (4 cm) allow savings in culture media and require less space at the expense of processing a smaller amount of soil samples.

2. Isolation of Microorganisms

This procedure should preferably be performed within the biological safety cabinet.

We can divide this process into 3 stages: Serial dilutions, in vitro streak plating, and incubation.

2.1. Serial Dilutions for the Isolation of Bacteria and Fungi from Soil samples

Serial dilutions are carried out with the aim of reducing the microbial load in each dilution, so that we can avoid plates with a high microbial load where colonies cannot be isolated, separated, and, above all, purified.

For each processed sample, the goal is to find the best dilution that allows for the correct isolation and purification of bacterial colonies from the soil. Picture in Annex 2 shows part of the procedure followed in the serial dilutions.

The procedure is described as follows:

- Place the 5 grams of soil in a sterile 50 ml Falcon tube, add sterile water or saline solution to reach a volume of 50 ml. This initial soil solution is called the "Stock Solution". Shake the “Stock Solution” using a Vortex mixer to obtain the suspension to start the serial dilutions.
- Transfer 1 ml (milliliter) of the previously shaken stock solution to a first sterile tube containing nine (9) ml of sterile distilled water, resulting in a total volume of 10 ml, which is referred to as the "first suspension". Place this first suspension on the Vortex mixer for proper mixing to obtain the "first Vortex dilution" of 1/10 or 10^{-1}.
- With the 10^{-1} dilution, repeat the previous procedure. Place 1 ml of the first dilution into a second sterile Falcon tube containing 9 ml of sterile distilled water. Shake the resulting 10 ml suspension for proper mixing to obtain the "second dilution” at 1/100 or 10^{-2}.
- Repeat the procedure described above with the "second dilution 10^{-2}". Place 10 ml of the "second dilution" into a Falcon tube containing 9 ml of sterile distilled water. Shake the resulting 10 ml suspension in the Vortex mixer for proper mixing to obtain the third dilution.

- To obtain the fourth, fifth, and subsequent dilutions, repeat this procedure successively until all desired dilutions are achieved. For the purposes of the project, dilutions were obtained up to 1/100000 or 10^{-5}.

It is recommended to avoid manual contact or contact of implements with the edges of tubes or the inside of caps, as this can lead to contamination in subsequent dilutions. Additionally, it is important not to neglect labeling the tubes with correct, easily interpretable, and identifiable names using permanent ink.

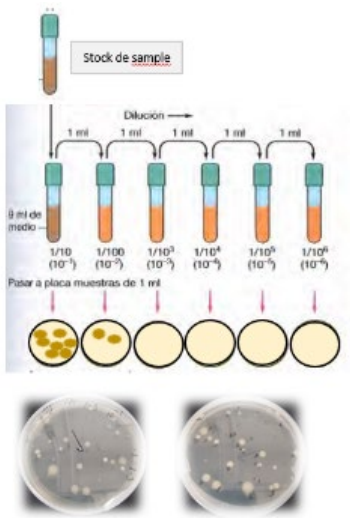


Diagram 1. Serial dilutions of soil samples

2.2. In vitro Streak Plating

Inoculate all previously obtained dilutions onto Petri dishes containing sterile Potato Dextrose Agar (PDA) medium, with the aim of identifying the dilution that allows for the best growth of colonies and microorganisms in the appropriate quantity for subsequent isolation and purification. Too many colonies on a plate do not allow for proper isolation and purification. Few colonies on a plate suggest strain loss due to excessive dilution.

The procedure is as follows:

- Start with the Falcon tube of highest dilution (in our case, 10^{-5}), agitating it on the Vortex to keep the organisms in suspension.
- Immediately thereafter, take 20 µl (microliters) from the highest dilution tube and deposit it in the center of the surface of a Petri dish containing sterilized PDA medium.
- Using a Drigalsky spatula, perform sweeps over the surface of the culture medium, spreading the 20 µl diluted sample previously deposited across the entire plate.

- Stop the sweeping process when the Drigalsky spatula no longer slides easily. After this process, the plates are closed, sealed with parafilm, and labeled correctly.
- Repeat the procedure described above with the next lower dilution, agitating on the Vortex, depositing 20 µl on the plate containing sterilized PDA medium, sweeping with the Drigalsky spatula, closing, sealing, and labeling the plates.
- Repeat the same process until all dilutions have been plated.

It is recommended to flame the Drigalsky spatula using a Bunsen burner between each plating process for each dilution to prevent possible contaminations.

2.3. Incubation

Incubate the plated dishes at 26°C for 24 hours, then observe possible colony growth.

If colony growth is slow, continue to incubate them for up to 48 or 72 hours to achieve better colony development and the appearance of new colonies. Plates containing higher dilutions require more incubation time.

3. Bacteria Selection, Isolation, and Purification

After plating and incubating for 24 hours, monitor and evaluate the plates. Inspection is visual and macroscopic:

- The observed colonies should be isolated and should not show contact, merging, or overlap with other bacterial colonies or organisms such as fungi.
- Observe the morphology of the growing colony (Annex 3) and detail its characteristics, including:
  - Shape and appearance (wrinkled, compact, "cottony", smooth edges, wavy, lobed, etc.), size (large or small).
  - Color (whitish, creamy, transparent, or various pigments).
  - Elevation (flat, convex), etc.
- Group similar colonies for subsequent selection and isolation, labeling on the back of the Petri dish.

Once the colonies have been grouped, the exhaustion technique is followed for their isolation and purification.

- Using the tip of a toothpick (see Annex 4c), previously sterilized, take small portions of bacterial colonies and then streak them onto the surface of Petri dishes containing solid and sterilized LB culture medium. This process is called colony streaking for subsequent isolation and purification.
- After streaking and seeding the plates, they should be labeled according to the selected groupings and incubated for 24-48 hours at 26°C.
- At the end of the incubation period, axenic colonies identical or similar to the initial colony of each grouping are obtained. This process results in the isolation of colonies.

- From isolated colonies on Petri dishes, repeat the process by taking small portions of bacterial colonies with a toothpick and streaking them onto new Petri dishes containing sterilized solid LB medium, which will be incubated again at 26°C for 24-48 hours to obtain isolated and purified colonies.
- Evaluate the plates containing purified colonies, ensuring the uniformity of all developing colonies. If a colony is found to be non-uniform compared to the others, repeat the purification process from this plate by selecting, replicating, and streaking the desired homogeneous colony onto a new Petri dish. If necessary, repeat the process until all colonies are homogeneous and pure.
- After incubation and obtaining purified colonies on the Petri dish, they should be stored at 6°C for preservation.

4. Selection, Isolation, and Purification of Fungi

After streak plating on Petri dishes containing PDA medium, and following incubation, the plates should be monitored 48 to 96 hours later to observe the development of fungal colonies. Fungal growth on PDA medium takes longer than bacterial development.

After the plates containing fungi have been incubated, macroscopic observation of fungal colonies, considering their rapid growth, color, or development halos, are suggested as possible indicators of antagonism. The development of homogeneous colonies should be grouped and labeled for identification.

If only fungal colonies (and not bacterial colonies) are desired, the culture media for streak plating should contain antibiotics to prevent or reduce bacterial growth.

The procedure followed is described:

- Petri dishes containing sterilized PDA medium with antibiotics will be used.
- From incubated streak-plated dishes, select pure colonies that do not have growth, contact, or proximity to bacterial or other fungal colonies. Fungal colonies smaller than 0.5 mm are suggested for isolation.
- Using the tip of a previously sterilized toothpick, take fragments or portions of mycelium from the selected pure colonies to transfer them to Petri dishes containing sterilized PDA medium with antibiotics. The seeding points should be marked.
- Using a toothpick, take portions of mycelium from the colony and transfer them to various seeding points on the new PDA plates containing antibiotics. The seeding points of the mycelium fragments or portions should be identified.
- Incubate the plates containing the fungal seeding points at 25°C for 3 to 5 days and subsequently observe their pure growth.
- If contaminants arise during incubation, repeat the procedure of transferring fragments or portions of mycelium to new Petri dishes containing PDA medium with antibiotics from these contaminated plates until pure isolates are obtained.

5. Conservation and Maintenance of Pure Bacteria

After the bacterial colonies have been purified, they need to be preserved. The procedure is described below:

- From already purified axenic colonies, and using sterile forceps, hold a sterilized toothpick and collect a portion of the bacterial colony with its tip. Transfer this portion to a 10 ml Falcon tube containing 4 ml of sterile liquid LB medium. Place the toothpick, along with the bacterial colony, into the Falcon tube, which should then be capped and shaken using a Vortex mixer (see Annex 4 a).
- The tube containing the toothpick with portions of bacterial colony is then incubated in an incubator with a reciprocal shaker at a temperature of 37°C and 150 rpm (revolutions per minute) for 18 hours (16-20 hours) to multiply the bacterial cells (see Annex 4b). The turbidity of the LB medium (see Annex 5) indicates bacterial growth and reproduction. During incubation, an additional Falcon tube containing only liquid LB medium and without bacterial inoculation (no bacterial portion on the toothpick) should be placed in the incubator as a control to compare turbidity and to ensure the aseptic process.
- 50% glycerol (50 ml glycerol mixed with 50 ml distilled water), previously prepared, homogenized by agitation, and sterilized twice consecutively, should be used for preservation.
- In sterile 2 ml cryovials, deposit 0.5 ml of 50% glycerol, then add 0.5 ml of the bacterial suspension after 18 hours of incubation.
- Once closed, labeled, and sealed with parafilm for preservation, the cryovials are refrigerated at 8°C for storage.

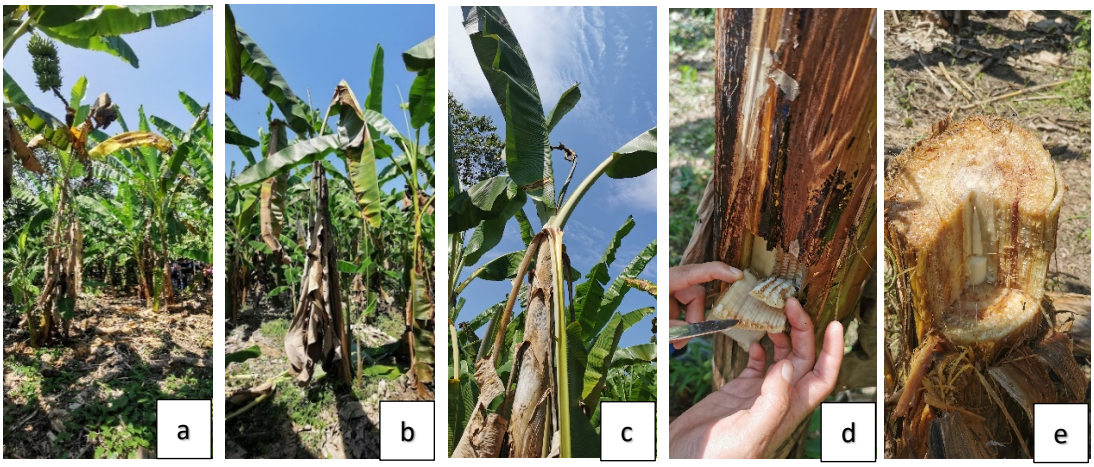
6. Conservation and Maintenance of Fungi

After fungal colonies have been purified, they need to be preserved. The process is described below:

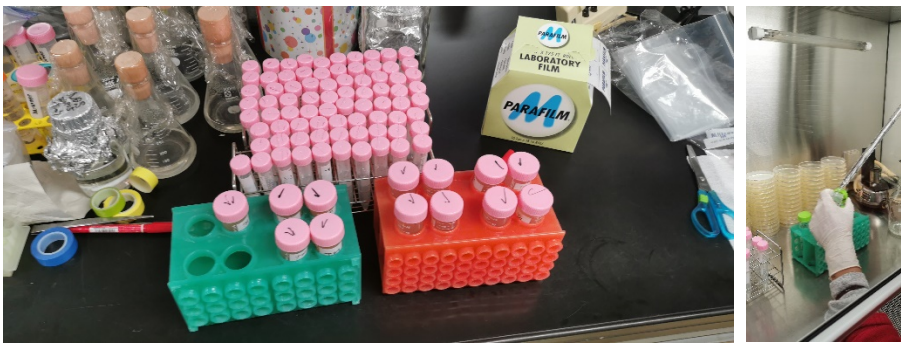
- In 2 ml cryovials, deposit 1 ml of sterilized PDA medium with antibiotics and wait for it to solidify.
- Purified fungi from Petri dishes containing sterilized PDA medium with antibiotics are used to obtain a portion of mycelium, which is then deposited onto the culture medium inside the prepared cryovials.
- The cryovials are closed, labeled, and sealed with parafilm for preservation at 8°C.

ANNEX

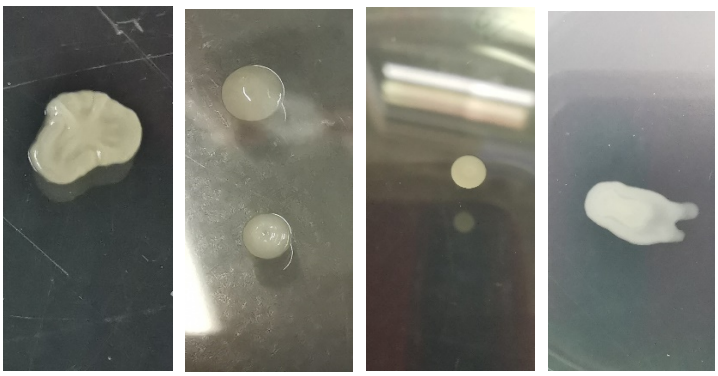
Annex 1: a. Banana plantation in Tingo María - Huánuco – Peru
b, c: Symptoms of wilt caused by *Fusarium oxysporum f.sp. cubense* in banana
d, e: Symptoms in Banana pseudosteam



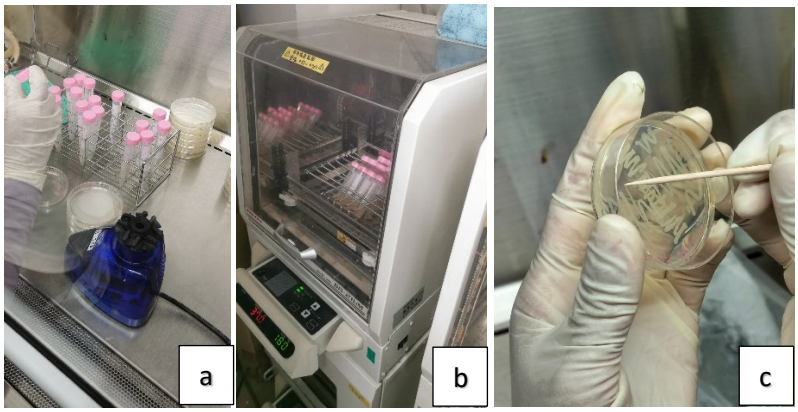
Annex 2: Serial Dilution Procedure in Falcon Tubes



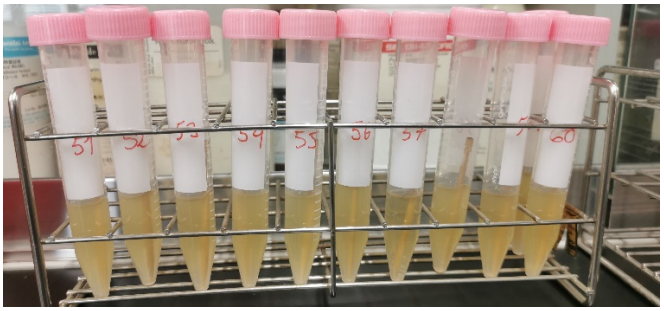
Annex 3: Various Morphologies of Bacterial Colonies



Annex 4: a. Bacterial Multiplication in Falcon Tubes for Subsequent Conservation
b. Shaking Incubator for Bacterial Multiplication
c. Collection of portions of bacterial colonies



Annex 5: Falcon Tubes after Incubation Showing Turbidity of the Liquid Medium



Annex 6: In vitro antagonism tests to select bacterial strains with antagonistic potential against *Fusarium oxysporum f. sp. cubense*