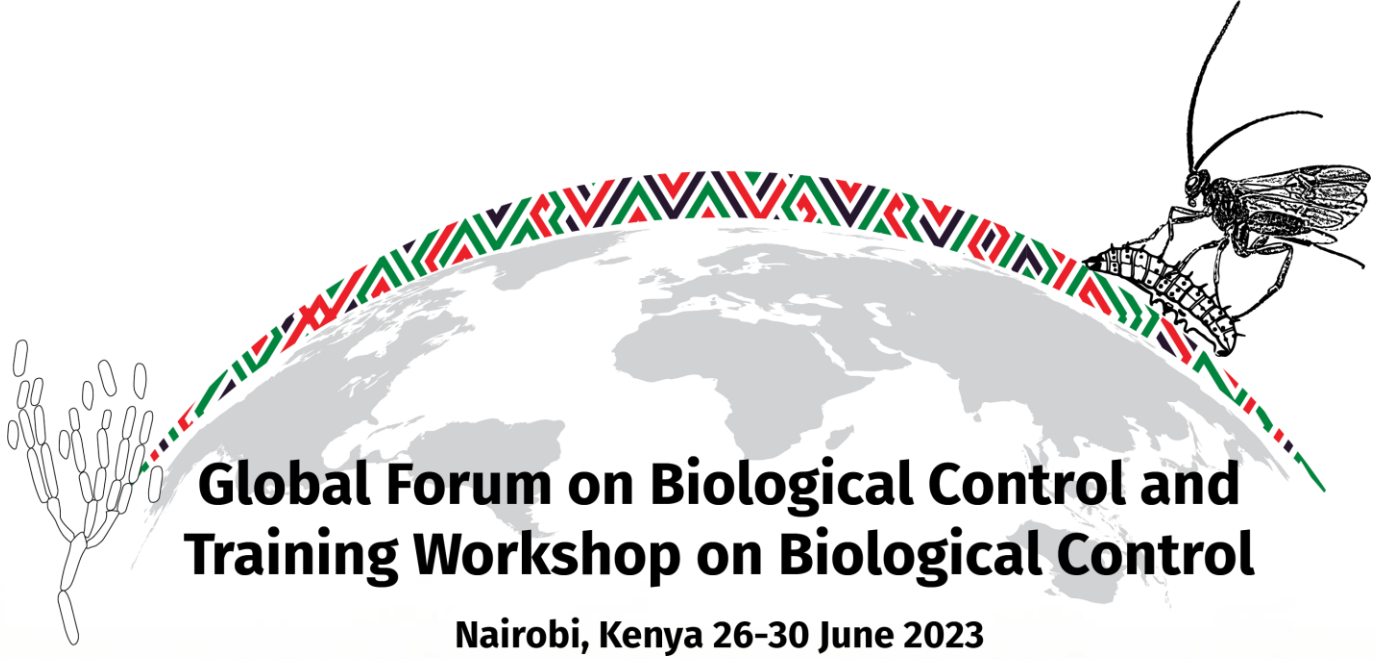

Global Forum on Biological Control and Training Workshop on Biological Control

Nairobi, Kenya 26-30 June 2023

Storage of fungal isolates, contamination management and key identification features

Levi Ombura

Arthropod Pathology Unit

Co-organized by

Food and Agriculture Organization of the United Nations

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Methods of preservation

- The aim of the preservation of isolates is to increase shelf life with minimal loss of viability to the microorganism
- There are three main categories; short-term, mid-term and long-term methods.

1) Short-term preservation methods

- Involves maintenance of cultures for up to one year. Examples: **Distilled water stasis** and **mineral oil**. The cultures can be either kept in the fridge or at room temperature.

Advantages

✓ Technologically simple.

✓ Inexpensive so widely used.

Mineral oil

Disadvantages

✓ For distilled water stasis, one must check and maintain the water level often.

✓ Must monitor for contaminants.

✓ Labour-intensive and time-consuming.

✓ Phenotypic characters may change

✓ Space intensive; tubes must be upright

Methods of preservation (… *cont'd*)

2) Mid-term preservation

- process by which spores are stored for periods longer than one year but below 3 years.
- Examples include **silica gel (at -20°C)** and **use of standard freezer (-20°C).**

Silica gel

Advantages

- ✓Cultures are stored for a long time hence not time-consuming.
- ✓Technologically simple.
- ✓Allows continuous monitoring of phenotypes.
- ✓Spores on silica gel can either be stored at -20°C or room temperature.

Disadvantages

- ✓ Long-term success depends on the security of screw cap closure
- ✓Subject to losses during power failures
- ✓Storage of spore at -20°C is not recommended for long (except for silica gel or cultures on filter paper)

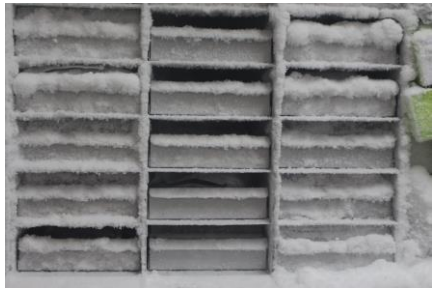
Methods of preservation (… *cont'd*)

3) Long-term preservation

- Process by which spores are stored for periods longer than five years and retain their viability and pathogenicity.
- Examples include use of **ultra-low freezer (-80°C)** and **cryopreservation in liquid nitrogen.**

Advantages

✓ Cultures are stored for a long time hence not time-consuming.
✓ Suitable for all cultured and nonculture fungi.
✓ Allows continuous monitoring of phenotypes.
✓ Long-term viability is maximized.



Glycerol stocks in -80 °C freezer

Disadvantages

✓ It is expensive when starting because it requires equipment like freezers, liquid nitrogen dewars etc.
✓ For the -80°C freezer, prolonged power failures may lead to losses.
✓ For cryopreservation, a continuous supply of LN_2 is required.
✓ For cryopreservation, there is no guarantee against leakage of LN_2 into cryovials.

Long-term preservation of fungi

- The viability of frozen cultures may be affected by (i) **the choices of medium from which preservation is attempted**, (ii) **Cryoprotectant**, (iii) **the rate of freezing** and, (iv) **temperature stability during storage**.

- Cryoprotectants act to favour intracellular water freezing as the glassy rather than icy state (where ice crystal growth can destroy membranes and, thereby, kill cells). Unfortunately, water can convert from a glassy to a crystalline state (with initiation and growth of ice crystals) even while remaining frozen. **This 'devitrification' is favoured by fluctuating temperatures**.

- Cryoprotectants can help prevent ice crystal formation during the freezing, storage, and thawing of cultures. The most widely used cryoprotectants for fungi include **glycerol**, **dimethylsulfoxide** (DMSO), **polyethylene glycol**, and **propylene glycol**.

- Most mycological laboratories that freeze fungi use **10% glycerol** because of its simplicity, convenience and reliable effectiveness.

- It is the comparatively large volume of cryoprotectant in a cryovial that fixes the temperature at which the vial contents freeze with a strongly exothermic release of the 'heat of fusion', ensuring the most uniform possible temperature drop rate (usually ca. **-1°C per min**) throughout a freezing process.

- Cryoprotectant solutions should be made and sterilized in small batches (e.g., 100 mL) to minimize contamination risks to every vial filled from that batch.

Long-term preservation of fungi (protocols)

Protocol A. For fungal cultures that do not sporulate or that produce mycelia that grow deep into the agar

- Sterilized 2-mL screwcap cryo-vials are filled with 0.5-1.0 mL sterile 10% glycerol.
- Plugs 4mm in diameter are cut from vigorously growing cultures using a sterilised scalpel blade.
- Several plugs are placed in the vial, the cap is tightened, and the tube is placed directly into boxes and then stored at -20 °C for 24 – 48hrs (to allow for uptake of the cryoprotectant). This is called 'curing'.
- After the curing process, transfer the vials to a – 80 °C freezer for long-term storage.

Protocol B. To make suspensions of spores or mycelial fragments from cultures growing on the surface of agar slants or plates

- The colony surface is flooded with 20% glycerol or 5% DMSO and gently scraped with a pipette or sterile scalpel blade.
- The fungal mycelial and spore suspension is pipetted in aliquots of 0.5 mL into sterile 2-mL screwcap cryo-vials.
- The vials are placed into cryo-boxes and then put into a -20 °C freezer for 48hr to allow sufficient uptake of the cryoprotectant (glycerol).
- After the 48hrs, the vials are transferred immediately to the ultra-low freezer and stored at – 80 °C.

Long-term preservation of fungi (protocols)

Protocol C. The mycelium of a fungus that grows only in liquid culture

- The broth culture is fragmented for a few seconds in a sterile mini-blender and mixed with equal parts of 20% glycerol or 10% DMSO to give a final concentration of 10% glycerol or 5% DMSO, respectively.
- The mixture is then treated as described in protocol B.

Culture recovery

- Cultures are thawed rapidly by placing vials in a warm water (37 °C) bath until the last trace of ice dissipates. The thawed culture samples/agar plugs are then transferred aseptically to appropriate growth media.

Checking the viability

- Always check the viability and purity of a preserved sample a few days after its preparation (2 – 7 days). If the sample is contaminated, inviable, or fails to meet any expectations, the rest of its lot is also unacceptable and should be discarded after a new lot is frozen and confirmed to be acceptable.

How to avoid contamination

1. Maintaining sterility

- As much as possible, preparation of specimens and fungal isolation should be carried out in a laminar flow hood.
- When such equipment is not available, a sterilized portable Plexiglas box (e.g., 25 x 40 x 40 cm) with holes for access on one side and a hinged lid has been used successfully.
- It is recommended that the surfaces be disinfected with 65 - 80% alcohol.
- All supplies and equipment used should be sterile.

2. Working rapidly

3. Be suspicious: Never trust that any growth that is out of the norm on your culture plates.

4. Be guarded: Never use culture plates you haven't prepared yourself or you're unsure of.

5. Be proactive: Check for contamination regularly.

6. Be diligent: Never leave your culture plates to someone who isn't fastidiously organized with impeccable technique.

7. Be pessimistic: Assume that contamination is a matter of time so always be on the lookout for possible weak points in your workbench set-up.

8. Be organized: It is best practice to use get fresh cultures from the germplasm every 3 months, have backups and maintain careful records of what you have.

9. Be thorough: When cleaning hoods/biosafety cabinets and incubators, take care to thoroughly remove any detergents.

10. Be careful: Do not leave your inoculated culture plates under fluorescent light for extended periods as this can provoke toxin production in response to photoactivation of certain ingredients in media.

Overcoming contamination

Contaminants include bacteria, opportunistic fungi, presence of mites

Fungal contaminants

- Fungal contaminants (e.g., Penicillium, Mucor, Cladosporium, etc.) can grow rapidly in entomophthoralean cultures.
- Often, very little can be done to overcome this situation once it has started, and the best course of action is simply to discard that culture and try again if possible.

Bacterial contaminants

- Bacterial contamination can occasionally be overcome because, initially, bacteria often grow slowly, and growth is localized. Once a bacterial colony or other contaminant is first detected, a piece of the mycelial mat should be removed sufficiently far from the contaminant and transferred to a fresh medium.

- In some cases, the surface of the culture can be contaminated, while entomophthoralean conidia are stuck to the inner wall of the container. Attempts to save the culture can be made by scraping conidia from the walls of the tube with a piece of sterile solid medium and transferring them to a fresh medium.

- A projection of conidia upwards to the inverted bottom of a Petri dish and then pouring liquid medium into the dish can also assist in saving the culture.

Acknowledgements

- Dr. Subramanian Sevgan, Dr. Akutse Komivi Senyo, Dr. Fathiya Khamis, Dr. Saliou Niassy and Dr. Samira Mohamed
- Technical team at Arthropod Pathology Unit – *icipe*
- All of you for listening

Thank you

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