Single spore isolation of fungi

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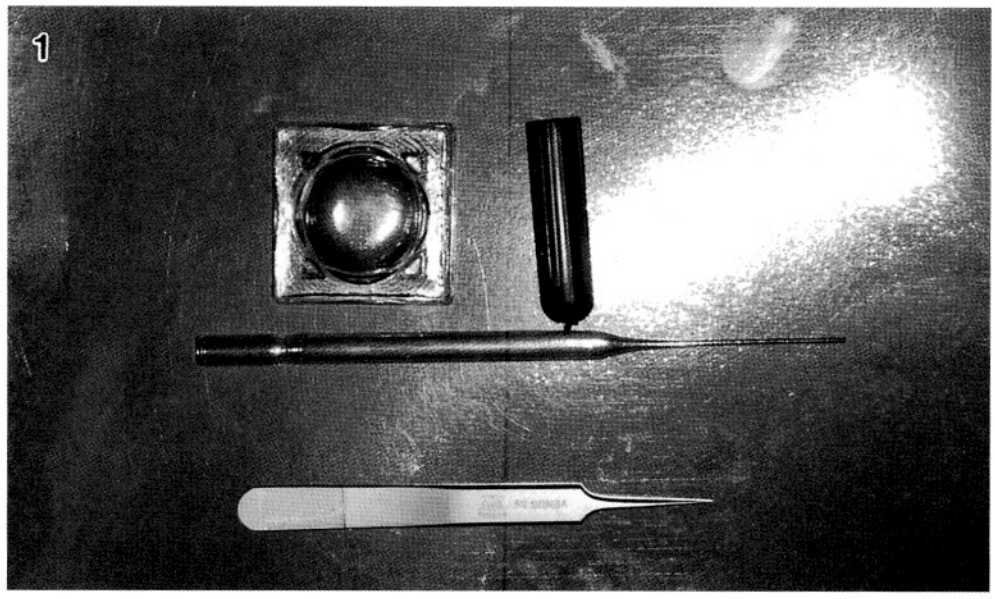
Methods to isolate fungi from single spores are outlined. These methods are specifically designed for mycological laboratories which are not necessarily well funded. Therefore, they involve a simple procedure, are relatively inexpensive, and most importantly effective. Furthermore, only basic equipment is required. By using these methods, most fungi, with the exception of those that do not germinate on artificial medium, can be isolated. Some approaches are suggested to prevent mite infestations and to reduce the risk of bacterial contamination.

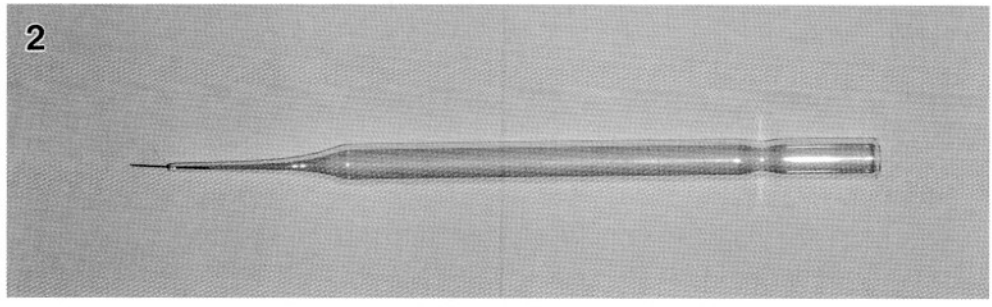
Key words: culture collection, fungal isolations

Introduction

The process of identifying fungi does not rely solely on morphological features for identification. The use of fungal cultures, obtained from single spore isolations are fundamental to the identification of many fungi. In terms of the morphological species concept, cultures can provide extra characters for identification, and anamorph-teleomorph connections. Cultural characteristics may also be useful and have traditionally been fundamental in the identification of certain taxa such as species of *Fusarium* and *Collectotrichum*. The phylogenetic species concept, which can include comparison of both morphological and molecular characters, in most cases require single spore cultures (Goh and Hanlin, 1997). The biological species concept has been tested by mating fungal cultures, thus isolates derived from single spores are fundamental for these studies. Cultures also allow repeatable testing of results by other researchers and may also be important in the discovery of novel metabolites (e.g. Strobel *et al.*, 1996).

The importance of careful identification of fungi and obtaining cultures that have been isolated from single spores cannot be over stressed (Smith, 1969). In order to study fungi more successfully, it is important that simplified keys are developed for identification, and simple effective methods are





Figs. 1-2. Some tools useful in fungal isolation. **1**. Extra fine forceps, Pasteur pipette, small glass container and pipette teat. **2**. Glass needle.

established in order to isolate fungi. Isolation methods must be simple to perform, even for those who do not have experience in isolation, relatively inexpensive and effective. There are many methods for isolating and storing fungi (Hilderbrand, 1938; Smith and Onions, 1994), however, some of these are expensive or difficult to carry out. In this paper, we describe a commonly used method for single spore isolation, which is inexpensive, easy to carry out, and effective. Only basic equipment is required and much of the equipment can be reused, therefore even poorly funded laboratories can carry out the procedures that are outlined.

The most common problems when isolating fungi are contamination by bacteria and yeasts, and the fact that the wrong species may inadvertently be isolated. To overcome these problems, dilution of spore masses can be used to

reduce the chances of yeast contamination and different antibiotics (e.g. penicillin, 0.5 g/L, streptomycin, 0.5 g/L) can be used to reduce bacterial contamination. There are several methods to ensure that the correct single spore is isolated (Hansen, 1926) and a commonly used method is reported here. Unfortunately, not all spores will germinate in artificial media, and other techniques may be required for successful isolation of these fungi. Fungal cultures stored in a high nutrient medium, such as Potato Dextrose Agar (PDA) may lose their ability to produce enzymes or metabolites, or perform other functions. Care is therefore needed in selecting a storage medium (Smith and Onions, 1994).

General material

For effective isolations to be carried out, an isolation room or clean bench with as little air movement as possible is desirable. A dissection microscope with illumination from both below and above, kept permanently in a lamina flow cabinet is desirable. A compound microscope can be kept near by to check spores and to observe germinating spores). This will reduce the likelihood of contamination. Alcohol (70%) is used to clean the working surface and some sort of burner is required for sterilizing utensils.

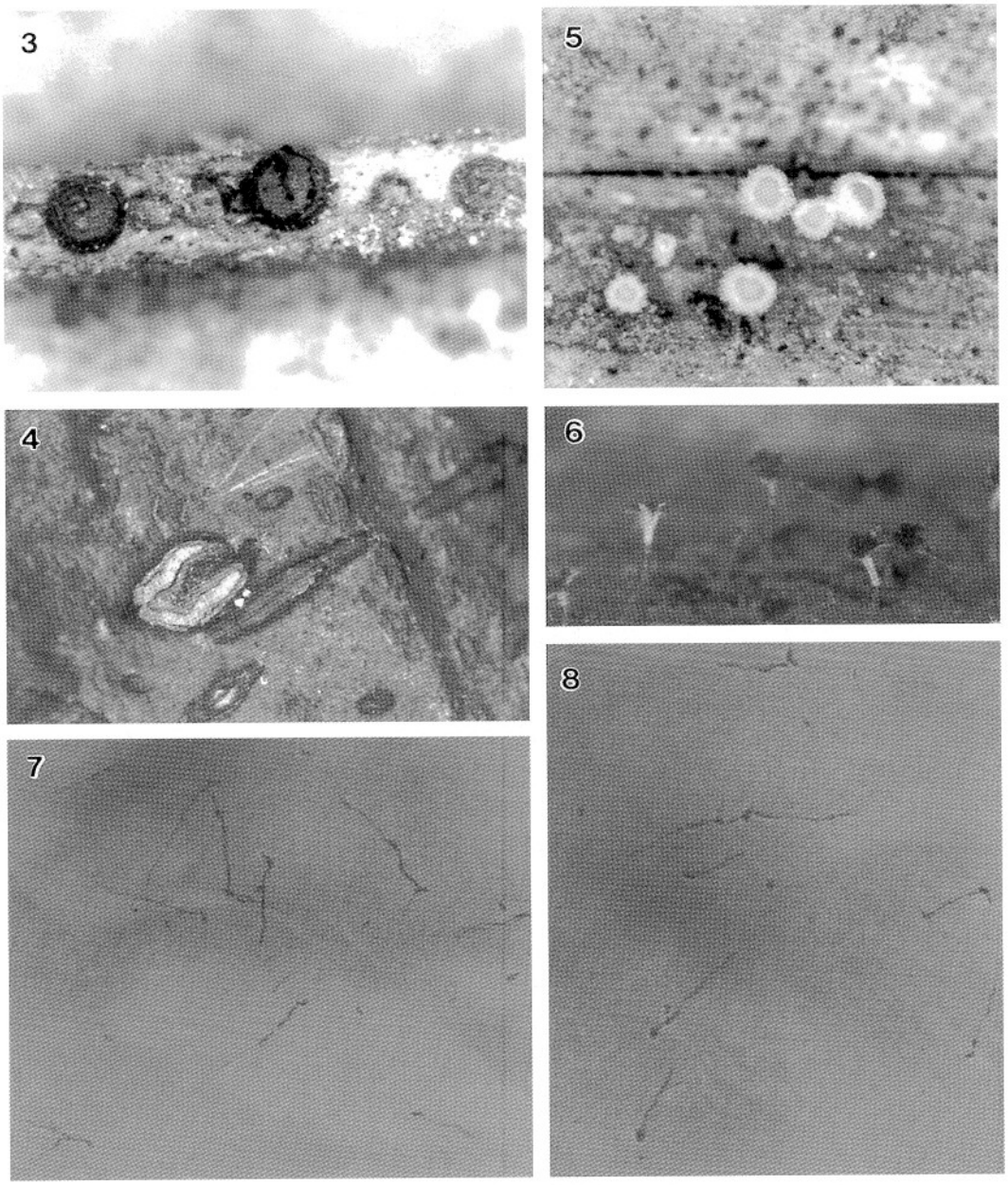
In the methods recommended below, water agar (WA) and PDA are routinely used as inexpensive media. Other media may be required for difficult fungi, such as V8 juice agar for lower fungi, or corn meal agar (CMA) when sporulation is required in lignicolous fungi. Sterilised water and sterilised Pasteur pipettes must also be prepared beforehand.

A dissecting microscope and a compound microscope are necessary for fungal identification. A small glass container or glass slide, sterilised water, sterilised Pasteur pipettes, and extra fine forceps are desirable for single spore isolation (Fig. 1). An alcohol lamp, insect needle and a Pasteur pipette are used to make a glass needle. A clean 25 C incubator is important for the storage of cultures.

Making the glass needle

The narrow end of the pipette is broken so that about 2 cm remains and an insect needle (metal needle for mounting insects) is placed inside the broken end and flamed until the glass melts and the needle is firmly fixed (Fig. 2). It is easier to use the needle if it is less than 1 cm outside the end of the glass pipette, otherwise it will break easily.

Other kinds of glass needles (Goh, 1999) can be used and are similar to the one mentioned here. It is more difficult to sterilize glass needles, therefore the metal one mentioned here is preferred. Special isolation needles, such as the



Figs. 3-8. Different types of fungi and germinated spores. **3, 4**. Ascomycetes with cleistothecia. **5**. Ascomycete with apothecia. **6**. Hyphomycete. **7, 8**. Germinated spores.

Microtool (T5340 Microtool needle and T5344 Microtool Handle, Agar Plano Scientific Ltd.) can be purchased, but they are very expensive and fragile and not suitable for beginners.

Addition of antibiotics or other inhibitory substances

Agar is prepared and autoclaved at 121 C for 20 minutes and antibiotics are added when the temperature of the agar is about 50 C, i.e. it can be held by hand. If the temperature is too high, the antibiotics will denature and become ineffective. As a general rule, 0.5 g antibiotics per L of agar will reduce bacterial contamination significantly, but this figure may need to be varied depending on the requirements. If the antibiotics are sterilized, a sterilized syringe should be used to transfer 2 to 4 ml sterilized water to dissolve the antibiotics and then suitable amount of antibiotics are added to the agar directly using the syringe. If the antibiotics are not sterilized, dissolve the antibiotics into 5 to 10 ml sterilized water and sterilize by passing through a sterilized membrane filter (0.2 μm or 0.45 μm pore size). The agar is poured into 90 mm or 60 mm Petri dishes inside a laminar flow cabinet (or on a sterilised bench) and allowed to solidify. They can be dried overnight in a safety cabinet. When preparing dissolved antibiotics and pouring agar, it is better to use a burner to keep the surrounding environment aseptic. In order to overcome the problem of overgrowth by other fast growing fungi, such as *Penicillium* and *Trichoderma*, growth inhibitors can be added to slow down the radial growth of the fungal colonies. Rose Bengal (0.3 g/L) is commonly used to inhibit growth. This can be autoclaved with the media, which is very convenient, as there is no additional likelihood of contamination.

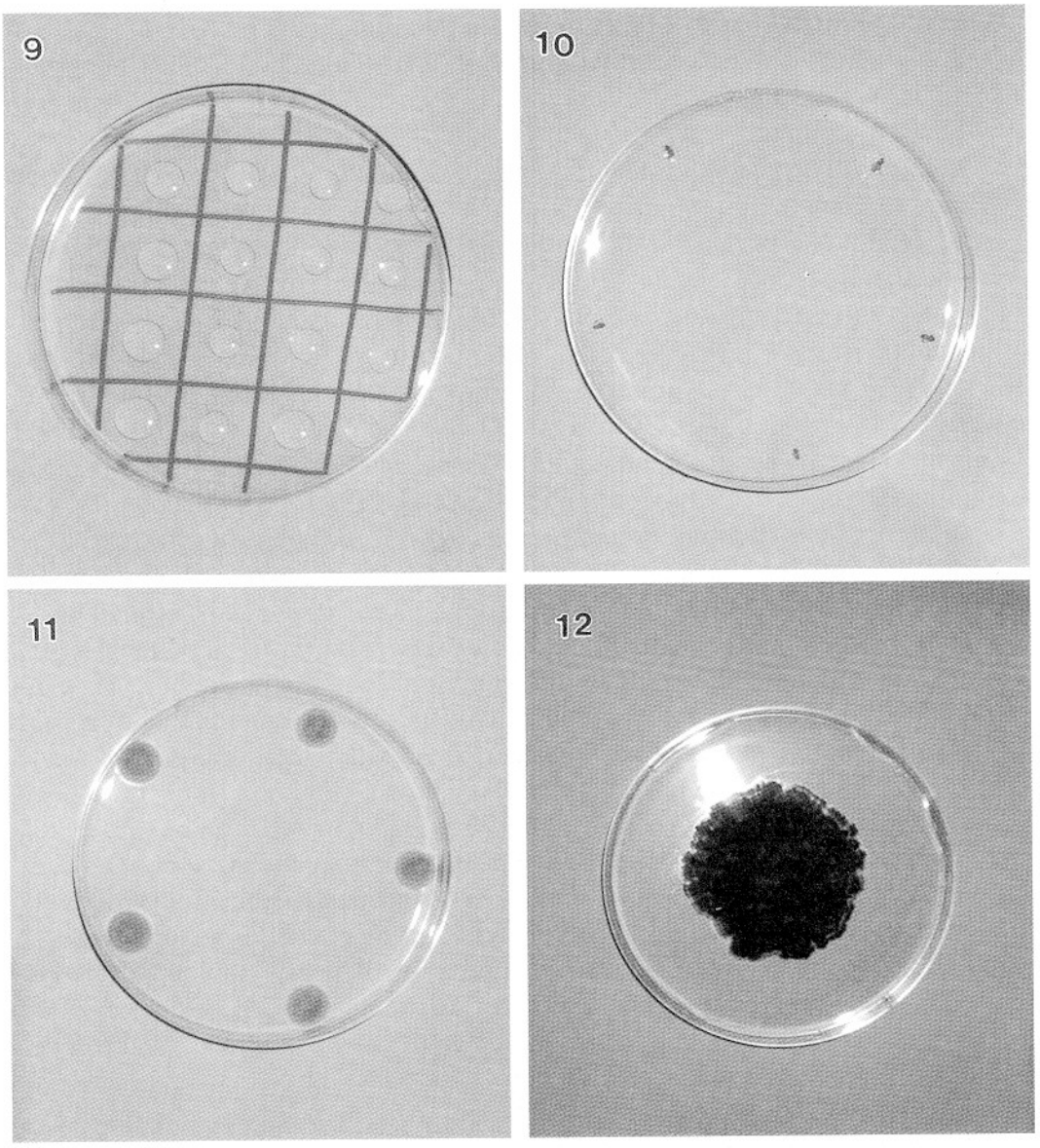
Addition of complex materials

In order to maintain the culture in good condition, various substrates can be used to promote better growth of mycelia or formation of fruiting structures (Guo *et al.*, 1998). This can include the addition of wood, leaves or cellulose filter paper to the surface of the media. Material, which is similar to, or the same as, the substrates on which the fungi originally grew, is desirable. This material should be autoclaved twice (121 C, 20 minutes), before use and can be placed on the agar surface aseptically.

Isolation from single spores suspensions

There are many different groups of fungi, e.g. ascomycetes, coelomycetes, basidiomycetes and hyphomycetes and each has different types of fruiting bodies. The methods to isolate each of them are therefore different.

Fungi with closed fruiting bodies (Figs. 3, 4; e.g. ascomycetes with cleistothecia: *Zopfiella* sp.; ascomycetes with perithecia: *Nectria* sp. and coelomycetes with pycnidia: *Phoma* sp.) can be removed from the substrate surface using fine forceps and broken open in sterilized water in order to provide a spore suspension. If the fruiting body is submerged in the substrate, a



Figs. 9-12. Fungal cultures. **9**. Spore suspension on 16 squares on water agar plate. **10**. Five pieces of agar containing germinated spores on PDA plate. **11**. Fungal colonies of about 1 cm diam. **12**. A pure fungal culture.

razor blade is used to cut the sporocarp open to expose the internal contents. The contents can then be transferred to a drop of sterilized water on a small glass container or slide in order to provide a spore suspension.

Fungi with cup-shaped fruiting bodies (ascomycetes with apothecia:

Rhytisma sp., Fig. 5, and coelomycetes with acervuli: *Chaetospermum* sp.), can be transferred directly, by removing the whole fruiting body. This can be placed in sterilized water and squashed with fine forceps, in order to provide a spore suspension.

Basidiomycetes with gills can produce numerous spores. A spore suspension can be obtained by removing a few segments from the gills and agitating them in sterilized water. Isolation can also be carried out from sterile tissue within the cap. A few pieces of sterile tissue can be aseptically torn from the split fruiting structure and placed on water agar.

Spores of hyphomycetes can be picked up directly from the substrate using fine forceps or a needle (Fig. 6). It is better to pick up spores only (not conidiophores), as this reduces the chance of contamination. The spores are placed in sterilized water and agitated in order to provide a spore suspension.

Treatment of spore suspensions

A glass container or glass slide is sterilised with ethanol and wiped with a towel on which ethanol (70%) has been sprayed. A sterilised pipette is then used to transfer about 6 drops of sterilised water into the glass container or onto the glass slide. The suspension is then prepared as above. Sixteen squares are marked on the bottom of the water agar plate (Fig. 9). The prepared homogenous spore suspension is then transferred with a sterilised pipette, onto the surface of the water agar plate, with a drop placed above each of the drawn squares. Alternatively about six drops of the suspension can be pipetted onto the centre of the agar plate and this can be carefully shaken to spread the suspension. If this method is followed it is a good practice to mark the outer edge of the suspension on the base of the Petri dish. Both of these methods may help to locate the germinating spores later. A small drop of the suspension should be used at this stage to make a permanent slide and to check that the correct fungus has been selected.

The unsealed plate is incubated at 25 C for 12-24 hours. It is not sealed as this allows some of the surface water to dry out. If the plate is sealed with parafilm, water will often accumulate on the surface of the agar and will increase the chance of contamination. The spores are checked within 12 hours and then every 24 hours to establish germination. Once the spores have germinated (Figs. 7, 8), a sterilised glass needle is used to pick up a small piece of agar containing a spore. In order to establish that the spore is the one desired, and maintain quality control, a slide is prepared and examined under the compound microscope. If the spores do not germinate after 12 hours, seal the plate with Parafilm and examine periodically. Ten germinated spores are transferred and distributed evenly onto two PDA plate (Fig. 10) and incubated

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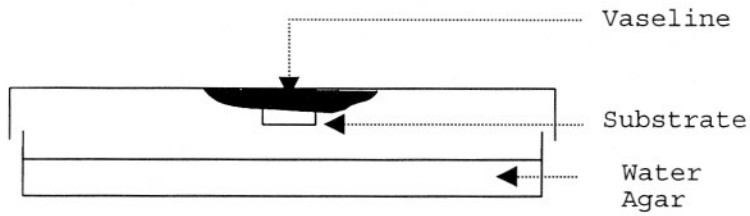

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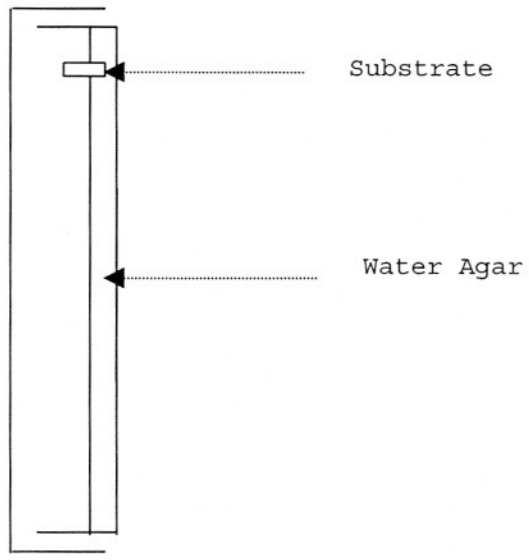

Figs. 13, 14. Spore drop/shooting methods. **13.** Basidiomycetes. **14.** Ascomycetes.

at 25 C until their colony diam. are about 1 to 2 cm (Fig. 11). A small piece of mycelium with agar can then be cut and transferred to another PDA plate and the culture (Fig. 12) is checked after few days, if there is no contamination, a pure culture has been obtained. Cultures can then be stored on the desired media.

Isolation using the spore shooting or drop methods

A suitable fungus is selected, e.g. an ascomycete that can eject its ascospores, or a piece of cap or gill tissue from a basidiomycete. In basidiomycetes, a piece of cap or gill tissue is cut from the fruiting body and is placed on the inside of the top of a Petri dish using Vaseline (Fig. 13). It is then sealed with parafilm and left horizontally overnight at 25 C. If the basidia are mature, they will fall on agar surface. In the case of ascomycetes, a small piece

of substrate containing a few fresh ascomata, is cut away from the source. Care is taken to avoid the inclusion of other fungi in order to reduce the chances of contamination. The substrate is then inserted perpendicularly into the agar so that the necks of the ascomata are aimed across the surface of the plate (Fig. 14). The plate is then left on its side overnight at 25 C. If the ascomata are mature enough, ascospores will be ejected and fall on agar surface below the substrate.

The next day, the agar plate surface is checked to see whether spores have been ejected or dropped. If spores are present on the agar surface, their identity can be established by preparing a slide of agar containing spores. If the spore is the correct one, a sterilized glass needle can be used to transfer a piece of agar containing each spore onto the PDA plate as before.

The PDA plate is incubated in 25 C until their colony diam. are about 1 to 2 cm. A small piece of mycelium with agar is then cut and transferred to another PDA plate and the culture is checked after few days, if there is no contamination, a pure culture has been obtained.

If there is any contamination in a culture, subcultures may be obtained by transferring small threads of uncontaminated hyphae with the glass needle. Although difficult, it is possible to rescue cultures by this method.

Storage of cultures

Once a pure culture has been obtained, it can be maintained on an agar slant at 4 C or stored as a few pieces of agar with mycelia in cryo vials with 10% glycerol in liquid nitrogen. Alternatively, it can be stored as a few pieces of agar with mycelia in small bottle with sterilized parafin oil or sterilized water at 4 C. There are several other methods to maintain fungal cultures and these can be reviewed in the "Preservation and Maintenance of Living Fungi" (Smith and Onions, 1994). It is recommended that a mixture of methods are used so that preservation of a culture is more likely (in case one method fails).

Mites

As fungi are an excellent source of food for mites and mites are found everywhere, fungal cultures in storage may soon become contaminated with mites. If there are any mite infestations in fungal cultures, the mites spread spores such as *Aspergillus* sp., bacteria and yeasts from one plate to another and if not dealt with quickly can contaminate all of the cultures. There are no proven methods to eliminate mites, but some precautions can be used to reduce the risk of mite infestations.

1. The bench and the stage of the microscope where work with cultures is carried out must be cleaned with 70% ethanol.

2. All stored cultures should be sealed with parafilm, although parafilm will not keep out mites indefinitely.
3. The equipment use to remove fungi from the substrate must be sterilized by flame or in 70% ethanol.
4. The area for storage of fungal cultures, such as incubator, must be mite-free. This can be achieved by sterilizing with 70% ethanol every month.
5. Naphthalene balls can be stored with fungi and probably slow down the activity of mites, however, Naphthalene balls may also effect the growth of fungi.
6. If a plastic or perspex container is used for storage, the "feet" of the container can be immersed in jars with paraffin oil or water, and therefore, mites cannot climb into container. Once there is mite-contaminated plate in the collection, it must be removed and sterilised immediately.
7. Try to avoid leaving plates in the incubator for long periods and transfer them to storage vessels as soon as possible. This will reduce the risk of build up of mites.
8. Mite proof Petri dishes can also be used (e.g. 60 mm air-tight Petri dishes, Falcon), but they are expensive.

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