Karstenia 20: 19—22. 1980

Simple isolation and inoculation methods for fungal cultures

KARI KORHONEN and VEIKKO HINTIKKA

KORHONEN, K. & HINTIKKA, V. 1980: Simple isolation and inoculation methods for fungal cultures. — Karstenia 20: 19—22.

Methods are described for isolation of single spores, spore tetrads, and hyphal cells, and rapid inoculation of fungal cultures in mating experiments. The instruments used are simple and are made in the laboratory.

Kari Korhonen, The Finnish Forest Research Institute, Unioninkatu 40 A, SF — 00170 Helsinki 17, Finland.
Veikko Hintikka, Institute of General Botany, University of Helsinki, Viikki, SF — 00710 Helsinki 71, Finland.

Interfertility experiments have proved to be a useful aid in the taxonomy of many fungal groups. A large number of single-spore isolates and extensive subculturing may be necessary for such experiments. Several good methods have been described for the isolation of single spores, either by hand from a spore dilution streak or with the aid of a special device fitted on a microscope (e.g. Raper 1963, Seaby 1977, Messner 1980). Methods have also been described for the isolation of basidiospore tetrads, with a micromanipulator (Papazian 1950, Moore 1966) or by hand (Epp 1977).

In our studies on the cytology and interfertility of the *Armillariella mellea* complex and of *Heterobasidion annosum* (Hintikka 1973, Korhonen & Hintikka 1974, Korhonen 1978a, 1978b, 1980), some simple methods were found to be of practical value in the isolation of monosporous mycelia, spore tetrads, and single hyphal cells, and in the inoculation of pure cultures for pairing experiments. No special equipment was needed and the necessary glass instruments were made in the laboratory. On the other hand, some practice in the use of the instruments proved necessary for rapid manipulation.

The equipment. The self-made instruments are:
— Two modified Pasteur pipettes: one for picking up spores and hyphal fragments and the other for inoculation of agar media.
— A piece of hair glued onto the drawn-out tip of a glass rod for the isolation of spore tetrads and isolation of fungal spores from contaminants.
— A microknife made by glueing a tiny piece of glass onto the drawn-out tip of a glass rod, and used for cutting and transfer of hyphal fragments.

The isolation operations are performed by hand under an ordinary research microscope (Wild M20) with a ×4 (or ×10) objective. Small beakers containing water, ethyl alcohol and acetone, and a spirit-lamp are used for rinsing and sterilizing the instruments.

The isolates were picked up from agar medium in petri dishes and the pure cultures were stored on agar slopes in test tubes. The nutrient medium contained 0.1, 1, or 1,5 % malt extract and 1.5 % Bacto agar or 1.7 % unpurified agar.

Preparation of the instruments. The shoulder of a 230-mm-long Pasteur pipette was heated and drawn out into a new elongated tip having a diameter of about 1 mm. If the pipette was intended for isolation, small pieces were snapped off from the tip with a finger-nail until an oblique break was obtained at a length of about 17 cm. The tip was then heated in a very small flame and bent as shown in Fig. 1. The pipette used for inoculation was longer, the tip being broken off transversely at about 25 cm. A cotton plug was inserted in the other end of the pipette to prevent contamination (the pipettes are blown out by mouth).

A piece of hair, about 1 cm long, was glued onto the drawn-out tip of a glass rod with a two-component epoxy glue that hardened in five minutes. The free end of the hair was cut obliquely with scissors.

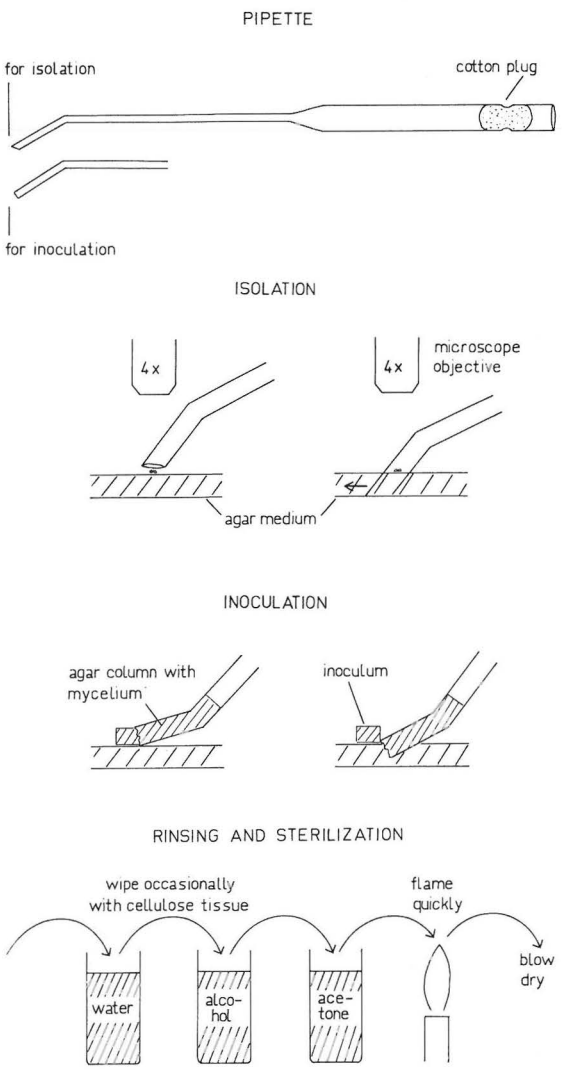

Fig. 1. The modified Pasteur pipettes for isolation and inoculation and their use.

The cutting edges of the microknives were made by smashing glass with a hammer. Tiny pieces of glass that were sharp, narrow and preferably curved were selected under a dissecting microscope. A small drop of glue was placed on the very thin tip of a glass rod and the piece of glass was picked up on the drop and adjusted to the right position just before the glue hardened.

Rinsing and sterilization. A new pipette was sterilized by heating it carefully in a flame. The tip of the pipette was always rinsed after use by sucking up a small amount of water and then blowing it out. The outer surface was occasionally wiped with moist cellulose tissue. The tip was then rinsed with ethyl alcohol and acetone. The acetone was flamed off quickly, care being taken to ensure that the tip did not melt, and the pipette was blown dry (Fig. 1). Rinsing, sterilization, and cooling take only a few seconds. It proved to be unnecessary always to sterilize the whole pipette, sterilization of the first few centimeters of the tip was enough.

The hair and the microknife were rinsed in water after use and sterilized by dipping in alcohol.

Working by hand under the microscope. The instruments were held in the hand and the isolation operations carried out on agar medium in petri dishes under an ordinary research microscope with a $\times 4$ objective and $\times 10$ eyepiece. Occasionally, a $\times 10$ objective was used. At first it proved difficult to work in the inverted sight field of a microscope, but after some practice it was found to be easier than working with a dissecting microscope. An ordinary microscope offers the advantage of easy inspection of the object at larger magnifications and phase contrast illumination.

For isolation operations, the petri dish was fixed to the mechanical stage of the microscope by means of an elastic hook. The dish could be moved with the mechanical stage. The working hand rested on the microscope stage and the elbow on the work table. The tip of the instrument was placed in the light beam of the condenser and its movements were then observed through the microscope.

All the isolation operations were performed in a secluded room without any special disinfection measures. Under normal conditions the danger of contamination proved to be negligible.

Isolation of single spores. A petri dish suitable for the isolation of single spores or germlings can be prepared using any one of the following methods:

1) A sporulating fruit body is held over an open dish until a few spores have dropped onto the nutrient medium. The number of spores on the medium is immediately checked with a microscope.
2) A spore suspension is made in sterile water and streaked over the agar medium. The suspension can be made in the dish by mixing a few spores or a small piece of fruit body with a few drops of water (sterile water can be obtained from, for example, the condensation on the lid of a petri dish). It is preferable to streak the suspension on somewhat dry (1—2 weeks old) medium, since this soon absorbs the fluid, in which contaminating

bacteria might otherwise be swimming around.

3) A piece of gill is taken with a pair of forceps and drawn over the surface of the agar medium.
4) Spores are scraped from a spore print with a scalpel and spread by drawing the scalpel across the surface of the agar.

Method (1) proved to be best when fresh fruit bodies were available. The other methods were used when the spores had to be isolated from spore prints, dried fruit bodies, or from very old contaminated fruit bodies.

A prerequisite for successful isolation is of course that the covering of spores on the agar medium is not too dense. In method (1) a suitable spore density gradient can easily be obtained by covering only a part of the dish with the sporulating fruit body. When the other methods were used it was often necessary to separate some spores from the others or from bacterial contaminants before picking them up. Clean areas were left on the surface of the agar for this purpose when making the streak. Single spores were transferred along the surface to the clean area using a sterilized hair under × 40 magnification. In case the spores were contaminated by bacteria they were moved a sufficient distance to allow the bacteria to drop away. The position of the isolated spores on the clean surface was marked under the microscope by puncturing the surface or by drawing a circle on the surface with the tip of the hair. These marks can be seen by the naked eye in reflected light.

Single isolates are picked up with the isolation pipette under × 40 magnification as shown in Fig. 1. It is possible to see through the microscope what is being taken into the pipette. The tip of the pipette is pushed through the agar layer to the bottom of the dish and moved a little along the bottom to break the agar layer. The pipette is then drawn out and the agar column with the isolate is blown out onto new medium. In order to inspect the isolate at high magnification, a slice having the isolate on the upper surface was cut from the agar column and covered with a cover glass.

It usually proved to be better to let the spores germinate in the dilution dish before isolation, since the germination percentage was often low. In addition, the germlings soon attach themselve to the medium thus making isolation easier on fresh agar media, where the puncturing pipette causes fluid to ooze out from the medium, with the danger that the spore may be displaced. When ungerminated spores or loose hyphal fragments had to be picked up from moist fresh media a small piece of clean agar medium was drawn into the pipette before isolation. This wiped out the pipette when the isolate was blown out, thus preventing the loose isolate from remaining on the wall of the pipette.

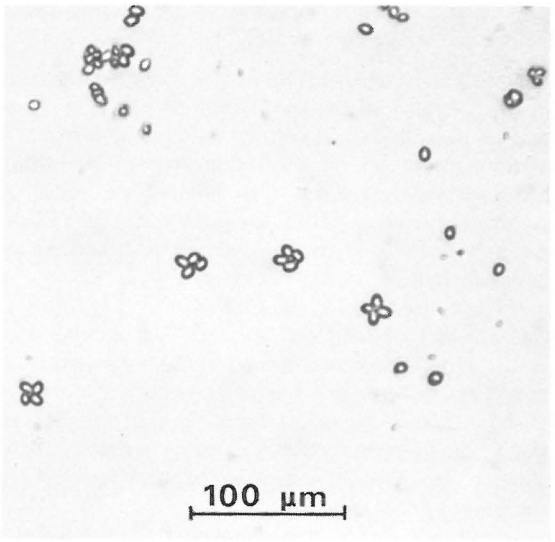

Fig. 2. Basidiospore tetrads of *Armillariella ostoyae* on the surface of agar medium.

Isolation of spore tetrads. Spore tetrads of *Armillariella* were isolated using a method described by Epp (1977). A piece of gill was placed carefully on top of the agar with a pair of forceps and then removed. The area was scanned with a microscope in order to find spores showing the distinct tetrad pattern (Fig. 2). The tetrads were usually found along the border of the spore deposit, where they could quite easily be drawn away from other spores with a hair.

Isolation of hyphal fragments. A single hyphal tip that is growing out from the mycelial colony can often be isolated simply by picking it up with the pipette. It was usually necessary, however, to cut the hypha first with the microknife and then transfer it to a more suitable place for isolation. The transfer was done in the following way: The tip of the knife was pushed beneath the severed end of the hyphal fragment and this end then lifted slightly above the surface. The other end must remain in contact with the agar. The hand holding the knife was kept as steadily in place as possible and the petri dish moved by means of the mechanical stage so that the living part of the hypha glided along the surface to a place suitable for picking it up. In cases where the transfer of a single cell appeared to be too difficult, a larger hyphal fragment was cut and the additional living

cells then killed after transfer by puncturing them with a very sharp microknife.

Inoculation of agar media with a modified Pasteur pipette. The tip of the inoculation pipette (Fig. 1) is pushed into the fungal colony and a column of agar medium about 1.5 cm long containing the mycelium is drawn into the pipette. A small section of the agar column is blown out from the pipette and cut off by pushing the tip of the pipette into the agar medium to be inoculated.

If the consistency of the nutrient media is suitable (not too dry or soft) ten or more inoculations can easily be made from one filling of the pipette. It also minimizes distortion of the stock culture. To ensure easy inoculation, it is important to keep the pipette clean. The pipette is superior to an inoculation needle whenever two or more new subcultures have to be started from the same colony.

Acknowledgements. We thank Mrs. Ritva Pulkkinen for valuable advice and assistance.

References

Epp, B.D. 1977: A rapid method for the isolation of tetrads in higher basidiomycetes. — Mycologia 69: 210—211.
Hintikka, V. 1973: A note on the polarity of Armillariella mellea. — Karstenia 13: 32—39.
Korhonen, K. 1978a: Interfertility and clonal size in the Armillariella mellea complex. — Karstenia 18: 31—42.
-''- 1978b: Intersterility groups of Heterobasidion annosum. — Comm. Inst. For. Fenniae 94(6): 1—25.
-''- 1980: The origin of clamped and clampless basidia in Armillariella ostoyae. — Karstenia 20: 23—27.
Korhonen, K. & Hintikka, V. 1974: Cytological evidence for somatic diploidization in dikaryotic cells of Armillariella mellea. — Arch. Microbiol. 95: 187—192.
Messner, K. 1980: A simple single-spore isolator. — Trans. British Mycol. Soc. 74: 206—207.
Moore, D. 1966: New method of isolating the tetrads of agarics. — Nature 209: 1157—1158.
Papazian, H.P. 1950: A method of isolating the four spores from a single basidium in Schizophyllum commune. — Bot. Gaz. 112: 139—140.
Raper, J.R. 1963: Device for the isolation of spores: — J. Bacteriol. 86: 342—344.
Seaby, D.A. 1977: A single spore isolator. — Bull. British Mycol. Soc. 11: 52—54.

Accepted for publication
on July 14, 1980