Simple methods for crossing and genetic analysis of *Neurospora crassa* strains

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In the asexual cycle of *Neurospora crassa*, the wild-type strains produce only mycelia and conidia when grown at 30o-34oC. In contrast, during the sexual cycle the female reproductive structures - protoperithecia - can be produced and fertilized successfully only if the incubation temperature is around 25oC. Normally, a large number of conidia are also produced at this low temperature. Formation of conidia during the sexual cycle is technically a nuisance and makes handling of the crosses difficult. Currently, several alternative procedures of performing crosses are available and practiced routinely in various labs.

For many purposes such as mating-type tests, the crosses can be performed using mutant strains blocked in macroconidia production as the female parent. Some workers make crosses in liquid synthetic medium containing paper as the sole source of carbon (Davis, R.H. and F.J. de Serres 1970 Meth. Enzymol. 17A:79-143). In this case, enough perithecia are formed at the interface between paper and the liquid medium. Alternatively, non-absorbent cotton can be used successfully to provide the solid support in allowing formation of perithecia in liquid medium. It is also possible to reduce production of conidia if crosses involving wild-type strains are made on synthetic agar medium containing 0.5% sorbose and 0.1% sucrose (Feldman, J.F. and M.N. Hoyle 1971 Neurospora Newslett. 18:12).

Here we describe a crossing procedure which practically eliminates production of conidia and yields a large number of randomly shot ascospores which can easily be harvested. Furthermore, it allows a large number of crosses to be performed simultaneously with little difficulty. The protocol was developed mainly from two observations: (1) that the conidia of the opposite mating type cause massive production of protoperithecia/perithecia when spotted on a mycelial lawn grown on synthetic medium (Degli-Innocenti F., J.A.A. Chambers and V.E.A. Russo 1984 J. Bacteriol. 159:808-810), and (2) that heavy inocula normally result in poor conidiation during the routine preparation of the conidial stocks of several strains (our unpublished observation).

The crossing medium (CM) is the same as the synthetic crossing medium described earlier (Davis, R.H. and F.J. de Serres 1970 Meth. Enzymol. 17A:79-143), except that the concentration of sucrose is 0.1% and contains in addition 0.5% sorbose, 0.1% yeast extract and 0.1% casamino acids. An additional 50 micrograms/ml of the specific amino acids are used if the female parent has amino acid requirements.

Procedure for the crosses:

1. The crosses are performed on pieces (3.5 cm x 4 cm) of Whatman 3 MM paper or Schleicher & Schuell GB 002 paper in disposable plastic Petri dishes. A large number of pieces can be autoclaved in a beaker and stored at room temperature for a long time. As needed, the filters are distributed in Petri dishes (one per dish) and wetted with 1 ml per filter of 1x CM.

2. About 0.2-0.3 ml suspension (in double distilled water) containing 10(5)-10(6) conidia of the female parent are applied dropwise on the paper so that the entire surface of the paper is covered. It is important to inoculate the conidia to cover the entire area of the filter, otherwise a lot of aerial hyphae and conidia are formed on subsequent incubation when the inoculum is placed only as a spot in the center of the paper.

3. The plates are incubated in the dark at 25oC in a loosely covered plastic box. Incubation in the dark is done only because of the technical convenience. We do not know if it is an essential feature of the protocol.

4. Conidia of the male parent are inoculated in the same way as (2) above, after 24-36 h. The mycelium of the female parent cannot always be seen at this stage. The amount of liquid present at this point is just enough to be confined by the surface tension and care must be taken to avoid the liquid spreading away from the paper.

5. The plates are sealed immediately using a piece of parafilm and incubated further at 25oC in the dark. Again, it is not known if incubation in the dark is required.

6. Small perithecia are observed after four or five more days at 25oC.

7. Randomly shot ascospores can be harvested from the lid of the plate after at least 10-12 more days.

8. Ascospores sticking to the lid can be harvested by placing 1 ml of double distilled water on the ascospores and gently scraping them off. About 2 x 10(4) - 2 x 10(5) ascospores are obtained per plate.

Conidial stocks for genetic analysis

Obtaining a large number of conidial stocks of the ascospore isolates is often required for genetic analysis. In our experience, the cultures in reusable glass tubes for this purpose are too expensive, labor intensive, time consuming and occupy a lot of lab space. We have, therefore, developed a "disposable micro-culture" procedure which yields about 10(5) - 10(6) conidia from each culture.

1. The nutrient medium used is essentially Vogel's minimal medium (according to the recipe described by Fincham, J.R.S., P.R. Day and A. Radford. Fungal Genetics, 4th ed. Blackwell Scientific Publications, Oxford, London, Edinburgh, Melbourne 1979) supplemented with 0.1% yeast extract and 0.1% casamino acids, in addition to any other specific requirements of the parents (50 micrograms/ml of the amino acids as needed).

2. A small piece (about 1 sq. mm) of the colony is cut out from the agar medium using a flattened platinum wire and inoculated in 10 microliters medium placed in a 2 ml Eppendorf tube. The lids are closed but not sealed. The tubes are incubated in the dark at room temperature for at least seven days. Several hundred colonies can be handled in a day.

3. The conidial suspensions are obtained by carefully adding 0.5 to 1.0 ml of distilled water in each tube followed by vortexing. The suspensions thus prepared can then be used directly for checking the genetic markers in a spot test.

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