Ethylene glycol treatment of conidia

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Abstract
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Certain morphological and biochemical mutants are female sterile, i.e., fail to produce perithecia and spores, or cross poorly when used as female parents. Reciprocal crosses between two such mutants may either fail completely or may be extremely slow. Our work with female sterile mutants suggests that heterokaryon may be effective in overcoming these difficulties in many instances. Horowitz et al. (1960 J. Mol. Biol. 2: 96) were successful in making a cross between the female sterile tyrosinase mutants $\mu y$-1 and $\gamma$-2 when $\mu y$-1 was in a heterokaryon used as a female parent and $\gamma$-2 was the male parent.

In our laboratory heterokaryon having all pairwise combinations, except one, of seven or eight different female sterile mutants were tested for their ability to produce perithecia and spores when the heterokaryon were forced markers, since they are non-leaky. Our work on one does not develop pigment in crosses made on Cmtotic Sciences, not been established of others, including of OMM: Department of Orthop. En-2 and nit-3 mutants were crossed into the wild type types 74-OR23-1A and 74-ORB-lo used for comparison form a dense block of conidio above a gob of relatively sparse aeral growth.

The female sterile mutants used are all morphologically different from the wild type. Female sterile sterility and abnormal morphology seem to be inherited as a single recessive character. They grow more slowly, ore less pigmented, and form a more continuous culture than the wild type. They were originally detected in making crosses resulted in the production of perithecio sterile mutants of $\mu$-3 (36104) and $\mu$-2 (37401). A typical cross involving one effective combination of mutants failed, probably due to a distorted nuclear ratio in the heterokaryon; attempts in each case produced substantial numbers of perithecia and ascospores.

The female sterile mutants used are all morphologically different from the wild type. One of the $\mu$-3 mutants (33757) in which the female sterility and abnormal morphology seem to be entirely combinatorial does not grow at all at $25^\circ C$ and does not grow at all at $34^\circ C$. The others, including $\mu$-1, are subtly different from the wild type.

S. F. forcing markers, since they ore non-leaky -- -- of pole having on-2 do not develop pigment in crosses made on Cmtotic Sciences, not been established of others, including of OMM: Department of Orthop. En-2 and nit-3 mutants were crossed into the wild type types 74-OR23-1A and 74-ORB-lo used for comparison form a dense block of conidio above a gob of relatively sparse aeral growth.

The mutants pan-2 (B3) and nit-3 (Y3188) were used as heterokaryon forcing markers, since they ore non-leaky and do not seem to affect growth characteristics or fertility. The heterokaryons were grown on a Westergaard and Mitchell crossing medium having 2% sucrose and 1.5% agar. After seven days they were fertilized with a conidial suspension of one female sterile mutant, or, in separate tests, with al-2 (15300). A typical cross $\mu$-3 was: (nit-3 fs-m A + pan-2, fs-n A) $\mu$ x fs-p a (or al-2 a) $\mu$.

All of the dark spores will represent the pan component and half of those from the nit-3 mutant have reduced female sterility. We tested further to see if one of the female sterile mutants could be recovered from a cross where it was present in both parent nuclei. The following was attempted: (pan-2 A + nit-3 fs A) $\mu$ x pan-2 fs a $\mu$.

The success with which the use of heterokaryons in the above crosses resulted in the production of perithecia and ascospores suggests that progeny may be recovered from crosses between various other morphological or biochemical mutants which have reduced female fertility if heterokaryons ore similarly used. In making such crosses, for instance between the mutants mut-1 and mut-2, the following format using pan-2 as an ascospore color marker is convenient: (pan-2 A + nic-3 mut-1 A) $\mu$ x pan-2 mut-2 a $\mu$.

By shaking the tube, the following format using pan-2 as an ascospore color marker is convenient: (pan-2 A + nic-3 mut-1 A) $\mu$ x pan-2 mut-2 a $\mu$.

To enhance the germination of nit-3 ascospores, nicotinamide can be added too cross tube at the time of fertilization. This is done by preparing the conidial suspension of the male parent $\mu$ in solution of 0.04 mg/ml of nicotinamide, adding approximately 1.5 ml to a 15 x 150 mm cross tube containing 5 ml of medium, and spreading the suspension by shaking the tube. If pan-2 must be recovered from such a cross, a similar amount of pantothenic acid can be added. This will cause pan ascospores to darken so they will be indistinguishable from pan, but it is necessary since pole ascospores usually show reduced germination. We are depositing pan-2 and nit-3 mutants crossed into a St. Lawrence (Oak Ridge) genetic background with the Fungal Genetics Stock Center.

Wilson, J. F. and W. K. Bates. Ethylene glycol treatment of Neurospora conidio. Recently we described some effects of treatment of Neurospora conidio with ethylene glycol (Bates and Wilson 1972 Genetics 68: 54). This treatment results in conidio which enlarge, with concomitant weight gain, and which become osmotically sensitive after two or more days. Osmotic disruption of these cells yields large numbers of intact nuclei and mitochondria, while gradual removal of the ethylene glycol results in approximately 75% germination within one hour. We now present some details of the methodology involved.

The conidio routinely used ore from seven-to-forty-one-day-old cultures, grown at $30^\circ C$ on Vogel's minimal agar medium, with supplements as required for mutants. The strain used for most of our studies is a m-isolate of the Oak Ridge wild type. Additional studies with me-3 (36104) FGSC#502, inos (37401) FGSC#406, and [mi-1] (poky, mi-1-1.8) FGSC#1578, with appropriate supplements, have indicated that the effect is not limited to one strain, although variations do occur in the degree of the response.

Wilson, J. F. and W. K. Bates. Ethylene glycol treatment of Neurospora conidio.
Conidia are harvested in sterile water, filtered through four layers of sterile gauze to remove hyphal fragments, and the concentration is determined with a hemacytometer. The conidial suspension is allowed to stand for at least one hour a 25°C before the conidia are transferred to ethylene glycol medium. This pretreatment with water results in faster and more uniform enlargement of the conidia in response to ethylene glycol. Pre-treatment periods longer than one hour produce no additional effect.

The formulation for 100 ml of the ethylene glycol medium is: 2 ml of 50X Vogel's minimal medium; 80 ml of distilled water; 18 ml ethylene glycol, reagent grade (20 grams); 1.5 g sucrose. We routinely double these amounts to obtain 200 ml ethylene glycol medium, and use this volume in 500 ml Erlenmeyer type flasks with stainless steel closures (DeLong culture flasks). All components are autoclaved together in the flask.

We inoculate at 1-3 x 10^9 conidia per ml medium (2-6 x 10^9 per flask) by centrifuging the volume of aqueous suspension of conidia necessary for each flask in a sterile screw-capped tube and decanting the water from the conidial pellet. The conidia are then resuspended in a part of the contents of a flask of ethylene glycol medium and transferred back to the flask. Thus, inoculation is achieved without dilution of the medium. Flasks are then placed on a rotary shaker at 25°C with carriers mounted at a 15° angle and are shaken continuously at 150 rpm. Osmotic sensitivity is demonstrable at 48 hrs, and both size and osmotic sensitivity continue to increase for at least 10 days. We have observed more than 80% viability after 8 days of this treatment.

Osmotic disruption is accomplished by centrifuging a suitable portion of the suspension and re-suspending the pellet in a hypotonic solution to approximately 10% of the original volume. Disruption occurs within a few seconds. For mitochondria, the pellet is resuspended in 2% sucrose-1mM EDTA at 5% of the original volume, followed by an equal volume of 28% sucrose-1mM EDTA at 30 seconds. It should be noted, however, that such mitochondria are not identical to those prepared by sand grinding.

For studies involving germination (including sorbose plating) it is necessary to dilute the ethylene glycol gradually, allowing the conidia to equilibrate at the lower concentrations. We have accomplished this with minimal disruption by non-linear rates of addition of water or minimal medium, according to the following schedule:

10 ml conidial suspension in 20% ethylene glycol in 125 ml Erlenmeyer flask on magnetic stirrer at room temperature.

Add diluent at 1 ml/min for 10 min to yield 10% solution,
Add diluent at 2 ml/min for 10 min to yield 5% solution,
Add diluent at 4 ml/min for 15 min to yield 2% solution.

Diluent is added by a peristaltic pump, aseptically if necessary. If faster dilution is required, rates of addition can be doubled with only a slight increase in disruption.

Ethylene glycol treated conidia are much more susceptible to disruption by sand grinding than are untreated conidia, as judged by comparative extraction yields. This allows preparation of extracts when osmotic shock is not desirable, or in the preparation of mitochondria. The procedure is: dilute as described above; centrifuge to concentrate the conidio; re-suspend in the extraction medium; and grind with sand with a mortar and pestle.

Although various modifications will be necessary to suit specific experimental conditions, the methods outlined above should prove adequate for preliminary studies. A more complete characterization of these conidia and the extracts obtained from them will be presented elsewhere. 

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