**How to make a cross.**
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**Background**

Crosses are essential not only for basic genetic manipulations such as mapping, stock building, and testing for allelism, but also for studying meiosis and ascus development (see Raju 1980), identifying and characterizing mutations that affect sexual-phase traits (Raju 1992), assigning wild isolates to species (Perkins *et al*. 1976), and distinguishing mendelian and nonmendelian inheritance.(Mitchell and Mitchell 1952). Details of chromosome morphology are best seen microscopically during meiotic prophase (McClintock 1945, Singleton 1948, 1953). Chromosome aberrations (Perkins 1974) and meiotic drive elements (Turner and Perkins 1979) can be detected and diagnosed using patterns of ascospore abortion (see Raju 1994).

**Procedure**

The methods described below are based primarily on experience with *N. crassa* but they should apply to other species as well, unless indicated otherwise. Concern here is primarily with crosses from which progeny are to be obtained. If the sole object is to score mating type, see "*How to determine mating type*".

***Basic media.*** For recipes, see: "*How to choose and prepare media*". The basic synthetic crossing medium (SC) of Westergaard and Mitchell (1942) is most widely used. The need to adjust the pH to 6.5 has been avoided by substituting 0.7 g $K_2HPO_4$ and 0.5 g $KH_2HPO_4$ per liter for the monobasic salt in the original formula (Murray and Perkins 1963). The formula in Davis and de Serres (1970, p. 86) incorporates this change. The trace elements used for Vogel's medium N are suitable for SC at 0.1 ml per liter final volume; there is no need for a separate trace element formula. The SC stock solution is made up at 2× concentration and stored at 5°C, with chloroform added to inhibit bacterial growth. Crosses are usually made on media solidified by agar.

Numerous alternatives and variations exist. Cornmeal agar (available from Difco with or without sucrose) was used historically. Russo *et al.* (1985) have modified Vogel's Medium N for use as a crossing medium by reducing ammonium nitrate tenfold. The modified Vogel's can be made up as a 50× stock in contrast to 2× for SC.

Sucrose is commonly used as carbon source, at 1%. Conidiation can be reduced by decreasing sucrose to 0.5% or 0.1%. Paper may be provide an effective substrate for crossing (Singleton 1954, Murray 1960). Some laboratories routinely substitute filter paper for sugar (e.g., Kinsey *et al.* 1980, Catcheside and Austin 1971). Productivity of difficult crosses may be enhanced by using a filter-paper cone with its base set in liquid media (e.g., Murray 1969).

***Supplementation of auxotrophs.*** High ammonium or amino nitrogen inhibits crossing. The level of amino acid, purine. or pyrimidine supplements should be kept at a minimum in crossing medium. If total amino acid concentration does not exceed 0.3 mg/ml there is usually no problem with fertility. Usually the parent with the simplest requirements is preferred as female. With Oak Ridge-compatible strains, problems of inhibition by supplements can be circumvented by combining the potentially difficult parent with an inactive-mating-type helper strain (Perkins 1984, Metzenberg and Sachs 2002) to form a prototrophic heterokaryon that grows and functions as female parent on unsupplemented crossing medium. See "*How to use helper strains for maintaining and crossing handicapped recessive mutants, etc.*".

Rule-of-thumb concentrations for growth (preferably less for crossing): *For vitamins* -- 10 μg/ml (0.01 mg), except inositol, 0.2 mg/ml. This may provide a surplus in some cases, but should not be inhibitory. *For amino acids* -- 0.2 - 0.5 mg/ml. When arginine and lysine requirements are both segregating, use 1 L-arginine : 2 L-lysine. For tryptophan mutants other than *trp-3*, indole at 0.01 -0.02 mg/ml is preferable to tryptophan. *For purines and pyrimidines* -- 0.2-0.5 mg/ml. Some adenine mutants may require 1.0 mg/ml adenosine or adenine.

***Temperature and the effect of light*:** Crosses at Stanford are routinely made in a 25°C incubator illuminated with a 20W fluorescent lamp timed for a 12:12 light:dark cycle. Perithecia do not develop, or do so poorly, above 30°C. (*N. tetrasperma* is an exception.) Because perithecial beaks orient toward light, cross plates are inverted directly above the light when asci are to be collected on an agar surface or when ascospores are to be examined that have been shot to the lid from spotted inocula.

Protoperithecia are induced by blue light in *N. crassa* (Degli-Innocenti *et al.*, 1983). Perithecial production of four homothallic species is drastically reduced either in the dark or in continuous light, but perithecia are abundant under a regime of 12 hr light : 12 hr dark (Raju 1981). The role of light in crossing of heterothallic species has not been investigated critically. .Observatins on heterothallic species are less extensive Perithecial production in *N. crassa* may be reduced in constant darkness (Raju, unpublished). *N. tetrasperma* appears to be fully fertile in constant dark.

***Glassware*.** Where the object of a cross is to obtain progeny, crosses are conveniently made on SC slants in 18 × 150 mm tubes, using foam or cotton plugs. Push-on metal or plastic caps result in more rapid water loss and are more prone to contamination during the extended incubation period. Crosses are made in petri dishes for specific purposes, as when the object is to determine mating type and fertility or to examine or isolate shot asci. A lawn of the female parent is then fertilized by strains being crossed or tested. Plates are more prone to contamination and desiccation than tubes, and less compact for incubation and storage.

***Fertilization*.** Both mating types may be inoculated simultaneously, or one may be used as female (protoperithecial) parent, the other as male (fertilizing) parent. Where there is doubt as to female-fertility of the strains, reciprocal crosses may be set up. Fertilization can be made as soon as protoperithecia are visible under low power (10×). With experience, monitoring protoperithecia is unnecessary. Fertilization will usually employ dry or suspended macroconidia, which are spread over the lawn surface with needle, loop, or pipette tip. When multiple strains are being used to fertilize the lawn in a test plate, fertilizing cells are placed at marked spots. In this situation, fertilizing conidia are picked up in a loop of sterile water to avoid the scatter that would result if dry conidia were used..

***Timing at 25°C.*** When used as the female parent, most strains are ready to fertilize 6 or 7 days after inoculation of tubes or plates. (Depth of agar affects the time of appearnance of protoperithecia and perithecia, which is quicker on shallow plates of high up on slants.) Developing perithecia can sometimes be seen as soon as 2 days after fertilization. The first asci usually mature and are ejected 9 or 10 days after fertilization. Ejected ascospores show poor germination until they have ripened for 7 to 10 days at 25° or 30°C. When both parents are inoculated simultaneously, perithecia may be seen in ~10 days. The first-formed perithecia inhibit development of perithecia from fertilized protoperithecia elsewhere in the same tube or plate
(Howe and Prakash 1969).

***False perithecia.*** In some single-mating-type strains, protoperithecia may enlarge and become pigmented so as to resemble small perithecia. These "false perithecia" are devoid of beaks, asci, and ascospores. Unfertilized strains exhibiting them remain completely sterile. Formation of false perithecia does not

prevent a strain from developing normal perithecia following fertilization with the opposite mating type. False perithecia are encountered sporadically in some *N. crassa* genotypes, most commonly of mating type *a*. Some isolates of *fl a* (*fluffy*) genotype have tended to make false perithecia. False perithecia are characteristic of single-mating-type cultures of *Neurospora tetrasperma* and of the Kirbyville, Texas isolates of *N. discreta*.

False perithecia can be a nuisance for mating-type testing and a cause of alarm if contamination is incorrectly suspected to be responsible. Failure to develop and produce ascospores even after long incubation distinguishes false from true perithecia, and there is usually no serious problem in making the distinction. False perithecia can be of serious concern, however, in crosses where legitimate true perithecia are barren as a result of duplications or of mutations that affect meiosis and ascus development. Mutants in which function of the mating type idiomorphs is impaired may produce barren, rudimentary perithecia in abortive mating reactions with *mat A* or *mat a* testers (Griffiths *et al.* 1978; Griffiths 1982).

***Storage of crosses and and longevity of ascospores.*** Ascospores remain viable for long periods when cross tubes or plates are stored at 5°C, but they do not survive freezing and thawing. Ascospore germination is poor if the cross has become partially or completely dessicated. Hydration can restore good germination (Strickland and Perkins 1973). Ascospores are reported to retain viability when suspended in distilled water (Smith 1973).

When *N. crassa* perithecia become desiccated before all the asci within them have been shot, ascospores in the unextruded asci ripen and remain viable. This occurs when crosses are made in small (10 × 75 mm) culture tubes, which readily dry down before the perithecia have emptied their contents. These can be stored for long periods without losing viability. When the dried perithecia are placed in a drop of water, intact linear asci are extruded singly. (The problem of separating individual asci from attachment in a rosette is thus avoided.) Ascospores from the extruded asci show high germination when they are heatshocked, even after many months (P. St. Lawrence, personal communication). They are a convenient source of asci for ordered tetrad analysis.

***Ascospore germination.*** See "*How to activate ascospores and optimize germination and the recovery of progeny*".

***Reciprocal crosses****:* A distinction between mendelian and nonmendelian inheritance is best made by carrying out reciprocal crosses (e.g., Mitchell et al 1953). If fertilization is delayed until one parent has been grown to the stage where protoperithecia are visible, and if perithecia then appear promptly, the protoperithecial strain can be assumed to have acted as the female. However, the possibility exists that later-formed perithecia have originated from protoperithecia that are formed by the second strain. Convincing proof of maternity can be obtained by using the *per-1* mutation in one of the parents. The perithecial wall is maternal in origin, and perithecia lack melanin pigment when the female parent is *per-1* (Howe and Benson 1974). *per-1* has also been useful in identifying the parentage of perithecia that form on opposite sides of the clear zone (barrage) that is formed when strains of opposite mating type come together on the agar surface of a plate (Fig. 37 in Perkins *et al*. 2001). See "*How to distinguish mitochondrial mutations from nuclear mutations*"**.** See "*How to establish parentage in reciprocal crosses*".

***Highly fertile testers.*** Strains bearing the *fluffy* mutation are highly fertile and they produce no macroconidia. For this reason, *fl* has been incorporated in tester strains that are used for a variety of purposes, including determining mating type and fertility, detecting and diagnosing chromosome rearrangements (Perkins 1974, Perkins *et al.* 1989, Perkins and Pollard 1989), detecting and scoring Spore killers (Turner and Perkins 1979), determining the species of wild strains (Table 1 in Turner and Perkins 2001), and studying the barrage reaction. Testers in all these categories, are listed in the FGSC

catalog under "Special-purpose Stocks". See "*How to use fluffy testers for determining mating type and for other applicationss*".

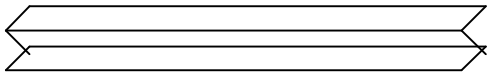
If crosses are to be retained from crosses with *fluffy*, testers of Oak Ridge (OR) heterokaryon-incompatibility genotype (*fl (OR) A*, FGSC 4317 and *fl (OR) a*, FGSC 4347), should be used, thus ensuring that the progeny are OR-compatible. However, inbred OR × OR crosses produce many abortive "bubble" asci (Raju *et al*. 1987). Therefore, when the object of crossing to *fluffy* is to observe asci or ascus development, strains of Rockefeller-Lindegren genetic background (*fl (RL) A*, FGSC 6682 and *fl (RL) a*, FGSC 6683) should be used when testing OR strains, because few bubble asci are present in RL × OR outcrosses. See "*How to minimize ascus abortion in crosses for cytology*".

***Problems with poorly fertile crosses.*** See "*How to enhance perithecial formation and ascospore production of poorly fertile crosses*"..

***Species other than N. crassa.***

(1) *N. tetrasperma*: Perithecia are formed under conditions that would inhibit crossing of other species, Perithecia are produced even on Medium N (which contains ammonium nitrogen) and at termperatures above 30°C (Viswanath-Reddy and Turian 1975). Most self-fertile isolates of this pseudohomothallic species are extremely fertile, promptly producing abundant ascospores..Sussman (1992) has described methods for obtaining ascospores in gram quantities!
(2) *N. intermedia*: Strains of the yellow ecotype are poorly fertile on Synthetic Cross Medium (SC) (Turner 1987) but highly fertile on pieces of corncob (Pandit *et al*. 2000).
(3) *N. sitophila*: Fertility of some strains is greatly increased on SC by substituting filter paper for sucrose (Fairfield and Turner 1993).
(4) *N. africana* (homothallic): As in the homothallic *Aspergillus nidulans*, crosses can be forced between two strains each of which is rendered self-sterile by an auxotrophic or other mutation. (Arnold 1983, Arnold and Howe 1984).

***A rapid method for making large numbers of crosses.*** (Contributed by A. J. F. Griffiths) Often it is necessary to intercross large numbers of strains. A rapid way to do this, a modification of the standard crossing procedure, uses liquid medium in large (~6 inch = 18 × 150 mm) test tubes. Liquid Westergaard and Mitchell medium containing 0.2% sucrose is used. Approximately 5 ml is dispensed into each tube. Into each tube is dropped a 12 × 6 cm piece of filter paper pleated on its long axis in the following way:



If necessary push the paper to the bottom. This piece of paper wicks up medium and acts as a surface on which sexual development will take place. The special pleat prevents sagging and keeps the paper away from the walls of the tube. Rigid plastic or metal caps are ideal because they can be easily removed for inoculations.

When the tubes have been autoclaved and cooled, a drop or two of conidial suspensions of each parent is dripped down the filter paper. If the tubes are arranged in racks in the appropriate grids, many hundreds of crosses can be made by simply inoculating drops along rows and columns of a grid.

Ascospores shot onto the walls can be recovered in the usual way. For ascus analysis, the filter paper and its perithecia can be removed from the tube with ease. Linear asci are dissected out of perithecia, whereas nonlinear asci can be recovered by placing a cut-out section of paper carrying mature perithecia close to a fresh 4% agar collecting surface.

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