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Abstract

Neurospora crassa ergosterol mutants are unable to synthesize ergosterol, the prevalent sterol in most filamentous fungi. These mutants have pleiotropic phenotypes such as reduced mycelial growth, decreased production of conidia, acquired tolerance to the polyene antibiotic nystatin (M. Grindle 1973 Mol. Gen. Genet. 120:283-290) and increased sensitivity to the pea phytoalexin pisatin (K. G. Papavinasasundaram and D. P. Kasbekar 1993 J. Gen. Microbiol. 139:3035-3041). We hoped to use the *erg-1* mutant as a means of isolating pisatin detoxifying genes from other fungi by functional complementation for tolerance to pisatin. However, we observed that standard methods used for *N. crassa* were not suitable for ergosterol mutants because of their low viability on Vogel's minimal medium (H. J. Vogel 1964 American Naturalist 98:435-446).

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Reduced viability of *N. crassa* ergosterol mutants on Vogel's medium.

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Neurospora crassa ergosterol mutants are unable to synthesize ergosterol, the prevalent sterol in most filamentous fungi. These mutants have pleiotropic phenotypes such as reduced mycelial growth, decreased production of conidia, acquired tolerance to the polyene antibiotic nystatin (M. Grindle 1973 Mol. Gen. Genet. 120:283-290) and increased sensitivity to the pea phytoalexin pisatin (K. G. Papavinasasundaram and D. P. Kasbekar 1993 J. Gen. Microbiol. 139:3035-3041). We hoped to use the *erg-1* mutant as a means of isolating pisatin detoxifying genes from other fungi by functional complementation for tolerance to pisatin. However, we observed that standard methods used for *N. crassa* were not suitable for ergosterol mutants because of their low viability on Vogel's minimal medium (H. J. Vogel 1964 American Naturalist 98:435-446).

Vogel's medium (R. H. Davis and F. J. de Serres 1970 Meth. Enzymol. 17A:79-143) and Martin's peptone-glucose agar medium (J. P. Martin 1950 Soil Science 69:215-232; referred to as M2 throughout this manuscript) were prepared as previously described except that glucose was omitted from M2. For both media we used either 1.5% sucrose for unrestricted growth or 2% sorbose, 0.05% glucose and 0.05% fructose for colonial growth. Conidia from the mutant strains *erg-1* (FGSC. stock no. 2721) *erg-3* (FGSC stock no. 2725) and the wild type 74-OR8-1a (74a; FGSC stock no. 988) were suspended in water, and 10, 10(2), 10(3), 10(4) and 10(5) conidia, respectively, were plated on Vogel's and M2 medium and incubated at 28 C for six days before counting colonies. The viability rate of 74a was the same on both media whereas *erg-1* and *erg-3* conidia showed a sharp decrease in plating efficiency when plated on Vogel's minimal medium (Table 1).

The reduced viability of *erg-1* and *erg-3* on Vogel's medium could be overcome by using diluted (0.25x) Vogel's medium. To examine the contribution of each component of Vogel's medium to this effect we reduced the concentration of each ingredient individually to 0.25x, while keeping the concentrations of the other ingredients at normal levels (Table 1). The viability of *erg-1* conidia on Vogel's medium containing reduced amounts of $CaCl_2$, $MgSO_4$, trace elements or biotin did not differ from the viability on 1x Vogel's medium. However, the reduction of the concentrations of KH_2PO_4, NH_4NO_3 or Na_3-citrate to 0.25x restored the viability of *erg* conidia to varying extents (Table 1). The reduction of sodium citrate from 8.6 mM (=1x) to 2.2 mM restored full viability. A decrease in plating efficiency of *erg* mutants could be observed on medium containing 2% sorbose as well as on medium containing 1.5% sucrose. Preliminary results indicate that supplementation of Vogel's medium with 0.5% yeast extract plus 0.5% casamino acids may also restore full viability, perhaps due to the ergosterol found in yeast extract.

Since most *erg* conidia did not grow on Vogel's medium we examined whether the appearance of cfu was due to reversion of the *erg* mutations to wild type. We transferred single colonies of *erg-1* from Vogel's medium to slant tubes for sporulation. Subsequently plated conidia again showed

full viability on M2 and ca. 1% viability on Vogel's medium. Thus, the ability of some erg conidia to grow on Vogel's medium does not seem to be due to reversion to wild type.

Other observations resulting from our experiments include 1) Selection of pMP6-transformed wild type and *erg-1* spheroplasts on M2 plates containing 150 ug/ml hygromycin resulted in up to 16 fold higher transformation efficiencies than did selection on 1x or 0.25x Vogel's medium. 2) Vogel's medium has a limited shelf life: The viability of wild type and *erg* conidia was 98% lower on Vogel's medium prepared from old 50x Vogel's solutions which had been stored for eight months or more in the dark as compared to being plated on medium prepared from fresh 50x Vogel's solution (Table 1). This decline in viability was not prevented by the addition of biotin. 3) Conidia of 74a as well as *erg-1* germinate faster in 1x Vogel's liquid medium than in liquid 0.25x Vogel's or M2 (data not shown); we therefore continue to use 1x Vogel's liquid medium for the preparation of spheroplasts. On solid medium *erg-1* conidia were observed to germinate but they ceased growth shortly thereafter.

The reduced viability of mutant strains *erg-1* and *erg-3* on Vogel's medium could be based on changes in their osmotic sensitivity. We did not measure the osmotic strength of the media used and, thus, cannot support this conjecture with data. However, fungal mutants with resistance to fungicides which target cell membrane or cell wall synthesis, composition, or structure have been observed to show increased sensitivity to osmotically-amended media (R. E. Beever 1983 Trans. Br. Mycol. Soc. 80:327-331, M. Grindle 1983 Pestic. Sci. 14:481-491, A. W. Chabani and M. Grindle 1990 Mycol. Res. 94:523-528, P. Leroux et. al. 1992 Pestic. Sci. 36:255-261). Different growth behaviors on liquid vs. solid media may involve different reactions of these organisms to the different water potentials of those media (D. M. Griffin 1981 in: Advances in Microbial Ecology 5:91-136), and/or that fungi in liquid culture can produce and thus, may depend on different sterols than in surface culture (J. D. Weete 1989 in: Advances in Lipid Research 23:115-167).

Table 1: Viability of conidia from *N. crassa* wild type and *erg-1* on different media.

	standard Vogel's medium			Vogel's medium, reduced in		
Strains	M2(1)	fresh(2)	old(3)	[Na3 Citrate](4)	[KH2PO4](5)	[NH4NO3]6
74-OR8-1a	+++++	+++++	+/-	+++++	+++++	+++++
erg-1	+++++	+/-	-/+	+++++	+	++

All experiments were performed at least twice; viabilities are indicated as follows: +++++: appearance of 7-10 cfu per 10 conidia plated; ++: appearance of 1-3 cfu per 10 conidia plated; +: appearance of 3-5 cfu per 10(2) conidia plated; +/-: appearance of 5-12 cfu per 10(3) conidia plated; -/+: appearance of 3-5 cfu per 10(4) conidia plated. (1,2): M2 and Vogel's medium prepared from new stock solutions. (3): Vogel's medium prepared with 8 month old or older 50x stock solution. (4,5,6): Vogel's medium containing reduced concentrations (0.25x) of the ingredients indicated.