

A rapid method for isolation of stable *niaD* and *crnA* mutants of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*

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Generally *niaD* mutants of fungi are selected by spontaneous mutations on appropriate minimal medium supplemented with various concentrations of KClO₃ and a nitrogen source (Daboussi *et al.* 1989 *Curr. Genet.* **15**:453-456; Johnstone *et al.* 1990 *Gene* **90**:181-192; Malardier *et al.* 1989 *Gene* **78**:147-156; Unkles *et al.* 1989 *Gene* **78**:157-166). But in case of entomopathogenic fungi it has been observed that *niaD* mutants isolated simply by spontaneous mutation on chlorate were not stable, (Table-1). Therefore a method has been developed to isolate stable *niaD* mutants of these fungi by treating protoplasts with ethane methane sulfonate (EMS).

The method reported here is advantageous as it yielded more mutants when compared to the method of spontaneous mutation. Secondly, mutants obtained by EMS treatment were more stable than spontaneous *niaD* mutants. Their reversion frequency was less than one in 10⁷ viable conidia, whereas comparatively high reversion frequency was obtained, 100 and 60 in 10⁷ viable conidia of spontaneous mutants of *Metarhizium anisopliae* and *Beauveria bassiana*, respectively. Protoplasts of *B. bassiana* and *M. anisopliae* obtained by the method of Shimizu, 1986 *J. Seric. Sci. Japan* **5**:510- 517,(approximately 10⁶) were incubated with 15 ul ethane methane sulfonate (d=1.17g, Sigma) in 1 ml of stabilizing medium (0.02M phosphate buffer pH 7.2 containing 0.6M KCl and 2mM MgCl₂) at 28 +/- 1 C for 1 h. These protoplasts were then washed twice with 10 ml of stabilizing medium by centrifuging at 1,000 X g for 10 minutes, diluted and then spread on plates containing a regeneration medium (2% sucrose; 1% peptone; 0.5% NaCl; 3% yeast extract and 2% agar agar, pH 7.0). Conidia from surviving colonies were harvested and suspended in Tween 80 aqueous suspension (50 ul/100 ml distilled water). Dilutions of this suspension were plated again on a minimal medium (1% glucose; 0.1% K₂HPO₄; 0.5% MgSO₄; 0.5% KCl; 0.001% FeSO₄·7H₂O; 0.005% EDTA disodium salt, pH 6.5) containing 10 mM glutamate as sole source of nitrogen and 470 mM Chlorate. These plates were incubated at 28 C +/-1 C for five days.

It was found that mutants arose at a frequency of 10 and 8 in 10⁶ viable conidia of *B. bassiana* and *M. anisopliae* respectively. These mutants were isolated and purified further on a minimal medium containing chlorate and glutamate. The ability of these mutants to grow on nitrate, nitrite, ammonium, hypoxanthin, proline and glutamate as sole source of nitrogen was assessed (Table 2). Of the 18 mutants tested, two each of *B. bassiana* and *M. anisopliae* had phenotypes indicative of *niaD*⁻. Two were found to be *crnA*⁻ of *M. anisopliae* while one *crnA*⁻ of *B. bassiana* was noticed. It was found on testing two *niaD* mutants

(Figure 1) designated *niaD-1* and *niaD-4* of *B. bassiana* and *M. anisopliae* respectively, that reversion to nitrate prototrophy was less than one in 10, sup>7 viable conidia, whereas reversion frequencies of spontaneous mutants was more than 100 and 60 in 10⁷ viable conidia of *B. bassiana* and *M. anisopliae*, respectively. Hence EMS treated *niaD*⁻ mutants should be suitable for future experiments involving protoplast fusion and transformation.

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Table 1. Reversion frequencies of mutants of *B. bassiana* and *M. anisopliae* to wild type growth.^a

Fungus	Number of mutants isolated		Reversion frequencies	
	Spontaneous ^b	EMS	Spontaneous	EMS
<i>B. bassiana</i>	4	10	>100	<1
<i>M. anisopliae</i>	2	8	>60	<1

^aReversion frequencies in 10⁶ viable conidia.

^bMethod of Malairdair *et al.* 1989 (Gene 78:147-156) was followed.

Table 2. Properties of *B. bassiana* and *M. anisopliae* mutants defective in nitrate assimilation.

Gene ^a	Chlorate resistance ^b	Utilization of sole nitrogen source ^c						Summary ^d
		A	B	C	D	E	F	
niaD-	R	-	+	+	+	+	+	Nitrate Reductase structural
gene crnA-nitrate	R	+	+	+	+	+	+	encodes uptake in young cells

^aThe minus superscript denotes loss of functional mutations.

^bR denotes resistance to chlorate.

^cSymbol (+) denotes wild type level of growth and (-)denotes poor growth. A = nitrate; B = nitrite; C = ammonium; D = hypoxanthine; E = proline; F = glutamate.

^d Summary of the role of the genes.



Figure 1. *niaD*-mutants of *Beauveria bassiana* (# 1-3) and *Metarhizium anisopliae* (# 4-6).#1 & 4: *niaD*-mutants not growing on minimal medium with nitrate as sole source of nitrogen; #2 & 5: growth on minimal medium containing chlorate and glutamate; #3 & 6: growth on complete medium.

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