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Molasses-based medium requires no nitrogen supplement for culturing three entomopathogenic fungi

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ABSTRACT: **The suitability and economics of molasses-based media supplemented with different nitrogen sources for mass production of the entomopathogenic fungi *Beauveria brongniartii* (Saccardo) Petch, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschinikoff) were evaluated. Radial growth of all three fungi on molasses agar media differed significantly among different nitrogen supplements. The salts $NaNO_3$ and KNO_3 supported the highest radial growth. Yeast extract, soyflour and defatted soyameal were next best while fertilizer grade urea inhibited growth of *B. brongniartii*. Spore production of *B. brongniartii* or *M. anisopliae* did not differ significantly among molasses broth media fortified with different nitrogen supplements. In *B. bassiana*, however, yeast extract supported significantly highest spore production and fertilizer grade urea the lowest. A comparison of cost of components for the production of the fungi showed that molasses medium without any nitrogen source was least expensive compared to all other media fortified with different nitrogen sources.**

KEY WORDS: *Beauveria bassiana*, *Beauveria brongniartii*, entomopathogenic fungi, mass culture, *Metarhizium anisopliae*, molasses medium, nitrogen supplements, production economics

INTRODUCTION

Large-scale utilization of entomogenous fungi for the control of economically important crop pests calls for standardization of commercially viable mass culture and formulation techniques. Molasses, a major by-product of sugar industry, can serve as an excellent low-cost substrate for mass culturing promising fungi. Molasses-based media have been evaluated to mass produce fungi such as *Beauveria brongniartii* (Saccardo) Petch, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschinikoff) in India (Sharma *et al.*, 1999; Easwaramoorthy *et al.*, 2002; Gupta *et al.*, 2002) and other countries (Calderon *et al.*, 1991 & 1995).

Most molasses-based media developed hitherto included yeast extract as one of the components to provide nitrogen to the fungus, which substantially increases the cost of production of formulation. For example, comparative economics of culturing *B. brongniartii* on standard medium, molasses-yeast medium and sorghum grains indicated decreasing cost of media in the same order (Easwaramoorthy *et al.*, 2002). The higher cost of production of molasses medium was due to yeast extract, the removal or replacement of

which could potentially render it less expensive than sorghum grains. Few studies have been conducted on modification of molasses media with nitrogen supplements for culturing entomopathogenic fungi. Garcia-Gutierrez *et al.* (2002) produced blastospores of *B. bassiana* in a liquid molasses medium with ammonium sulphate as nitrogen supplement. Prasad and Rangeshwaran (2000) attempted such modification for *Trichoderma harzianum* Rifai by substituting yeast extract with soybean flour, sodium nitrate or potassium nitrate in a molasses-based medium.

In the present study, different nitrogen supplements as substitutes for the expensive yeast extract for mass production of *B. brongniartii*, *B. bassiana* and *M. anisopliae* were evaluated. Comparative economics were also worked out for one litre of media with these supplements. The implication of the study for mass production of *B. brongniartii* whose formulations are currently being used for the control of white grub *Holotrichia serrata* Fabr. (Coleoptera: Scarabaeidae) (Easwaramoorthy *et al.*, 2004), a serious pest of sugarcane in southern India with a potential to inflict 80-100 per cent yield losses (David and Ananthanarayana, 1986), is discussed.

MATERIALS AND METHODS

Maintenance of fungal cultures

Pure cultures of *B. brongniartii* (white grub isolate), *B. bassiana* (shoot borer isolate) and *M. anisopliae* (shoot borer isolate) were maintained on Sabouraud's dextrose agar (SDA) or Emerson YPSS media in slants and plates and subcultured periodically on same media to obtain sufficient quantity of inoculum. To eliminate occasional contamination in cultures, Rose Bengal agar medium was used.

Modification of molasses medium with different nitrogen supplements

Molasses medium standardized by Easwaramoorthy *et al.* (2002) was used as the base in the present studies. Broth medium (1litre) comprised molasses (30ml), yeast extract (2.5g), potassium chloride (0.5g) and magnesium sulphate (0.5g); agar-agar (18g) was added for solid medium. This medium was modified by substituting yeast extract with 2.5g each of $NaNO_3$, KNO_3, soybean flour, defatted soyameal, fertilizer grade urea and laboratory grade urea. Media with and without yeast extract were also included for comparison. After adjusting pH to 6.5, media were autoclaved at 120° C and 15 psi for 20 minutes. The cultures were incubated in the laboratory at 28.2 ± 0.7^0C maximum and 25.8 ± 1.6^0 C minimum temperatures. Two parameters, *viz.* radial growth and spore production were assessed from four replications each.

Radial growth

For observations on radial growth, molasses-agar plates were inoculated with 10 mm disc of each fungus previously grown on SDA or YPSS medium for two weeks. Inoculated plates were incubated in the laboratory for 10 days and radial growth (cm) was measured.

Spore production

To study spore production, 100 ml molasses broth taken in 250 ml culture flask was inoculated with 10 mm fungal disc. After 20 days of incubation, fungal mat and broth were blended in a mixer for 2 minutes, filtered through muslin cloth and the volume made up to one litre. Suspensions were diluted serially and spore count was determined using a haemocytometer and expressed as number per 100 ml of broth.

Data analysis

The data on radial growth and spore production were subjected to analysis of variance (ANOVA) after verifying for variance homogeneity and applying suitable transformations. The means were compared using Duncan's Multiple Range Test (DMRT). Comparative economics of components required for one litre media with different nitrogen supplements were estimated.

RESULTS AND DISCUSSION

Sugar industry by-product molasses has been widely used for mass culturing *B. brongniartii*, *B.*

Table 1. Radial growth and spore production of three entomopathogenic fungi in molasses media fortified with different nitrogen supplements

Nitrogen supplement	Radial growth (cm)			Spore production (x 10^{10} / 100 ml broth)		
	Beauveria brongniartii	*Beauveria bassiana*	*Metarhizium anisopliae*	*Beauveria brongniartii*	*Beauveria bassiana*	*Metarhizium anisopliae*
Control	4.25 abc*	3.15 a	4.25 a	2.40(1.53)$^{\$}$ a*	5.02 ab	3.50(1.83) a
Yeast extract	4.38 bc	3.70 b	4.63 bc	3.10(1.68) a	7.10 b	3.85(1.84) a
$NaNO_3$	4.33 abc	4.08 d	4.80 c	1.80(1.33) a	6.90 b	2.90(1.68) a
KNO_3	4.70 c	4.00 cd	4.65 bc	1.80(1.28) a	6.20 ab	8.10(2.73) a
Soybean flour	4.35 bc	3.68 b	4.38 ab	2.30(1.50) a	5.20 ab	4.20 (1.96) a
Defatted soyameal	4.33 abc	3.73 b	4.15 a	2.60(1.56) a	5.40 ab	3.10(1.73) a
Fertilizer grade urea	3.80 a	3.85 bc	4.20 a	1.60(1.25) a	3.80 a	8.56(2.48) a
Laboratory grade urea	4.00 ab	3.83 bc	4.38 ab	2.70(1.83) a	5.70 ab	8.40(2.65) a

*Means followed by the same letter do not differ significantly ($P>0.05$) by DMRT$^{\$}$.
Figures in parentheses are square root transformed values.

bassiana and *M. anisopliae*, the three fungi that have direct relevance to sugarcane pest control both in India and abroad (Calderon *et al.*, 1995; Sharma *et al.*, 1999; Easwaramoorthy *et al.*, 2002; Gupta *et al.*, 2002). Most of these media incorporated the expensive yeast extract as nitrogen supplement. Alternative nitrogen supplements such as soybean flour (Borges *et al.*, 1997) and ammonium sulphate (Garcia-Gutierrez *et al.*, 2002) in molasses media increased sporulation of *B. bassiana*. Similar results with *T. harzianum* (Prasad and Rangeshwaran, 2000) strengthen the idea that low-cost substitutes for yeast extract would enhance sporulation and economize mass production of entomopathogenic fungi like *B. brongniartii* too on molasses-based media.

Radial growth

Addition of different nitrogen supplements to molasses-media produced significant differences in radial growth of the three fungi on agar media in plates (Table 1). Radial growth was slightly lower in *B. bassiana* than in the other two fungi. Such differential radial growth for the three species, observed in earlier studies with molasses-yeast media (Easwaramoorthy *et al.*, 2002), probably indicated species-specific response to the media and supplements.

When individual fungi are considered, radial growth of *B. brongniartii* differed significantly among the media fortified with different nitrogen supplements (Table 1). Significantly highest (4.7cm) growth was recorded on medium supplemented with KNO_3 and lowest (3.8 cm) on medium supplemented with fertilizer grade urea. Yeast extract (4.38 cm) showed significantly higher radial growth than fertilizer grade urea (3.8 cm) but it did not differ from the medium without yeast extract. All other media showed intermediate values. In the case of *B. bassiana*, media with nitrogen supplements showed significantly higher radial growth than that without nitrogen supplement. Media with $NaNO_3$ (4.08 cm) and KNO_3 (4.0 cm) supported far higher radial growth than media with soybean flour, defatted soyameal and yeast extract. Radial growth of *M. anisopliae* was significantly higher in media supplemented with $NaNO_3$ (4.8 cm), KNO_3 (4.65 cm) and yeast extract (4.63 cm) than in media fortified with defatted soyameal, fertilizer grade urea and control. The general lower radial growth shown

by fertilizer grade urea could be due to differential sensitivity of the fungi to impurities present in this form of urea.

The higher biomass obtained for *T. harzianum* in molasses medium supplemented with soyflour was ascribed to the suitability of organic form of nitrogen for enhanced growth although earlier studies indicated the opposite (Prasad and Rangeshwaran, 2000). They used unequal quantities of KNO_3 and $NaNO_3$ (2g), and soyflour (5g) per litre of molasses media. In the present study, all nitrogen supplements were added at 2.5 g per litre and it was observed that $NaNO_3$ and KNO_3 produced higher radial growth. Although this indicated the suitability of inorganic supplements, the differences in these studies could be more due to the species used than the quantities of nitrogen supplements, which were not determined on the basis of net nitrogen content.

Spore production

Spore production on molasses media with different nitrogen supplements was higher for *B. bassiana* and *M. anisopliae* than for *B. brongniartii* (Table 1). Among different media, it was more uniform for *B. bassiana* than for the other two fungi. Like radial growth, these differences are suggestive of differential response of the three fungi to the media.

Spore production of *B. brongniartii* did not differ significantly among media with different nitrogen supplements despite the differences in radial growth (Table 1). Similarly, spore production of *M. anisopliae* did not differ significantly on media fortified with different nitrogen supplements. In spite of the fact that fertilizer grade urea, laboratory grade urea and KNO_3 supported high spore production, the differences among treatments were not significant. Thus, nitrogen supplements affected vegetative growth of *B. brongniartii* and *M. anisopliae* but not sporulation. Similar differential radial growth and sporulation in molasses medium were earlier reported for *M. anisopliae* (Gupta *et al.* 2002).

Spore production by *B. bassiana*, differed significantly on media with different nitrogen supplements. The highest spore production on molasses medium with yeast extract (7.1×10^{10}/100ml broth) was significantly different from the lowest spore production on fertilizer grade urea (3.8×10^{10}/100ml broth). Media with $NaNO_3$ and KNO_3 showed intermediate spore production followed by laboratory grade urea, defatted soyameal, soybean flour and control all of which were on par with one another. These results indicated that nitrogen supplement was not important for sporulation of this fungus too but an impure supplement like fertilizer urea proved detrimental to it. It appears that the small quantities of nitrogen available in molasses in the form of protein or free amino acids (Dwivedi, 1995) were efficiently utilized by these three species of fungi for spore production. In an earlier study, soybean flour added to coffee husk and distillery must resulted in better sporulation of *B. bassiana* than 2 per cent molasses-torula yeast medium (Borges *et al.*, 1997) which suggested the importance of nitrogen supplement for this fungus. These variable results could be attributed to the differences in media composition used. Addition of soybean flour doubled viable propagules and spore production of *T. viride* compared to salts or nitrogen supplement free medium (Prasad and Rangeshwaran, 2000). As stated earlier, such higher sporulation could be a species-specific response. Comparable sporulation and toxicity to target insect obtained for a microencapsulated formulation of *B. bassiana* that used ammonium sulphate as nitrogen supplement, with other formulations of the fungus (Garcia-Gutierrez *et al.*, 2002), could be more related to formulation process than the nitrogen supplement used.

Economics of media evaluated

A comparison of cost of components required for the preparation of one litre media showed that molasses medium without any nitrogen source was least expensive compared to all other media fortified with different nitrogen sources (Table 2). Media with fertilizer urea, soybean flour and defatted soymeal were as economical as medium without nitrogen supplement. Media with yeast extract, laboratory grade urea, KNO_3 and $NaNO_3$ were expensive in the increasing order of magnitude.

Since spore production of *B. brongniartii* and *M. anisopliae* did not differ among different media, and that of *B. bassiana* differed only marginally, use of molasses medium without additional nitrogen supplement would reduce the cost of production of these fungi. Computation of cost of material required for producing 1 x 10^{12} spores of *B. brongniartii*, the dosage recommended per acre against *H. serrata* (Easwaramoorthy *et al.*, 2004), indicated that molasses-yeast medium, found more expensive than sorghum grains (Easwarmoorthy *et al.*, 2002), would become less expensive without yeast extract.

Table 2. Economics of culturing three entomopathogenic fungi on molasses media fortified with different nitrogen supplements

Nitrogen supplement	Cost per litre *(Rs.)
Control	0.31
Yeast extract	5.40
$NaNO_3$	11.18
KNO_3	8.70
Soybean flour	0.41
Defatted soyameal	0.94
Fertilizer urea	0.32
Lab grade urea	3.87

*Cost of material alone considered

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