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J. Environ. Biol.
33, 721-727 (2012)
ISSN: 0254-8704
CODEN: JEBIDP

In vivo interactions of entomopathogenic fungi, *Beauveria* spp. and *Metarhizium anisopliae* with selected opportunistic soil fungi of sugarcane ecosystem

Author Details

N. Geetha (*Corresponding author*)	Sugarcane Breeding Institute, Indian Council of Agricultural Research, Coimbatore - 641007, India e-mail: mvsbi@yahoo.com
M. Preseetha	Sugarcane Breeding Institute, Indian Council of Agricultural Research, Coimbatore - 641007, India
K. Hari	Sugarcane Breeding Institute, Indian Council of Agricultural Research, Coimbatore - 641007, India
G. Santhalakshmi	Sugarcane Breeding Institute, Indian Council of Agricultural Research, Coimbatore - 641007, India
K. Subadra Bai	Sugarcane Breeding Institute, Indian Council of Agricultural Research, Coimbatore - 641007, India

Publication Data

Paper received:
27 November 2010

Revised received:
07 April 2011

Accepted:
23 April 2011

Abstract

In the present study, the interactions of entomopathogenic fungi *viz*., *Beauveria bassiana, Beauveria brongniartii* and *Metarhizium anisopliae* among themselves and three other opportunistic soil fungi from the sugarcane ecosystem namely, *Fusarium saachari*, *Aspergillus* sp. and *Penecillium* sp. were assayed *in vivo* against *Galleria mellonella* larvae. The tested fungi were co-applied on IV instar *G. mellonella* @ 1x 10^7 ml^{-1}, in combinations of two, at the interval of 24 hrs either preceding or succeeding each other to assess their efficacy and sporulation rates. Results showed that often mortality rates did not correspond to the spore harvest of the mortality agent and presence of other fungus may be antagonistic. The efficacy of *B. bassiana* (90%) and *B. brongniartii* (100%) was not enhanced further but was negatively affected in most combinations with other fungi. In case of *M. anisopliae* compatibility was higher, resulting in higher mortality by application of *B. bassiana* before (100%) or after (83.3%) *M. anisopliae* than when it was applied alone (70%). During sporulation, *B. bassiana* faced the most intense competition from *M. anisopliae* (2.75x10^6 $larva^{-1}$) and enhancement due to *F. sacchari* irrespective of sequence of application. In case of *B. brongniartii*, sporulation was lowest in the combination of *B. brongniartii* preceding *M. anisopliae* (1.83 x10^6 $larva^{-1}$) and *B. brongniartii* succeeding *B. bassiana* (1.58x10^6 $larva^{-1}$). Of all fungi tested, except *F. sacchari* (65.33 x 10^6 $larva^{-1}$) all the other species affected sporulation of *M. ansiopliae* with the least in treatment of *B. bassiana* application following *M. anisopliae*. Similar kind of interaction was observed during sporulation of soil fungi when combined with entomopathogenic fungi, though individually they could not cause mortality of larvae.

Key words

Beauveria bassiana, *Beauveria brongniartii*, *Metarhizium anisopliae*, *Galleria mellonella, In-vivo* interaction, Opportunistic fungi

Introduction

In an ecosystem, biocontrol agents of numerous insect pests and fungal pathogens do exist together but not much known about their interaction if used together. It is a general principle that complex associations in nature are more stable and there is a better chance of attaining successful biological control with a mixture of several antagonists than with a single one (Baker and Cook, 1982). Investigations on the natural prevalence of entomopathogenic fungi *Beauveria bassiana* (Bals.) Vuill, *Beauveria brongniartii* (Saccardo) Petch and *Metarhizium anisopliae* (Metschn.) Sorok have indicated that these fungi widely occur in the soil as well as on insects in the aerial environment and soil. These fungi have also been isolated from the rhizosphere of their host plants. This shows that there is a

long lasting evolutionary coexistence with other microorganisms that includes different types of interactions. There are several reports on *in-vitro* interactions of *Beauveria* spp. and *M. anisopliae* with hyperparasitic, antagonistic and especially, phytopathogenic fungi (Bark *et al*., 1996; Krauss *et al*., 2004; Markova 1991; Posada *et al*., 2004; Reisenzein and Tiefenbrunner, 1997; Vega *et al*., 2009; Vanninen *et al*., 2000; Zimmerman, 2007) though *in vivo* interactions are reported rarely (Umamaheswara Rao *et al*., 2006). Interactions of entomopathogenic fungi with the parasitoids on their host have been found to be antagonistic (Pell *et al.,* 2001; Dos Santos *et al*., 2006).

A few studies indicating interaction of two strains of a single species of entomopathogenic fungus on the host (Leal-Bertioli *et al*., 2000; Wang *et al*., 2002), or a combination of strains of two species of entomopathogenic fungi (Umamaheswara Rao *et al*., 2006; Inglis *et al*., 1997, 1999, 2001) or a single entomopathogenic fungus with a fungal antagonist against a host (Zimmerman, 2007) are available. However, the impact of an opportunistic fungus which may occur simultaneously in the same ecosystem either in soil or air or plant and generally does not infect the insect host, on the entomopathogenic fungi of the target host and the resultant effect on the host is not explored yet. The interactions *in-vitro* are not necessarily an indicator of their *in-vivo* interactions (Krauss *et al*., 2004) which could be an important issue in biocontrol of target pest.

Hence, in the present study such a relationship among *B. bassiana, B. brongniartii* and *M. anisopliae* among themselves as well as with three other soil fungi from their ecosystem (the sugarcane wilt pathogen *Fusarium sacchari* Butler and Hafiz Khan, *Aspergillus* sp. and *Penecillium* sp.) was explored *in-vivo*. The effect of their combined presence on the host mortality as well as the ability of the fungi tested to sporulate on the laboratory host *Galleria melonella* L. when applied at 24 hrs interval was examined in the present study.

Materials and Methods

Fungal species tested and their cultures: The bioassays were carried out at Sugarcane Breeding Institute, Coimbatore during 2010. For the purpose of this study, fungal isolates of the entomopathogenic fungi (*B. bassiana, B. brongniartii* and *M. anisopliae*) as well as the soil fungi (*F. sacchari*, *Aspergillus* sp. and *Penecillium* sp.), originated from heterologous hosts from sugarcane ecosystem were cultured and maintained at an ambient environment of 25 ± 2°C on sterilized potato dextrose agar (PDA) slants. Conidia of each fungus were harvested from 15 day old cultures by gently scrapping from the surface of colonies and rinsing with sterile distilled water. Conidial suspensions were filtered through sterile muslin cloth to remove debris and resuspended in sterile Tween 20 (0.01% v/v aqueous solution) in distilled water. The number of conidia were estimated with a haemocytometer and adjusted to 1 x 10^7 ml^{-1} by serial dilution with sterile water and were used for the bioassays.

Maintenance of *Galleria mellonella* L. culture: The culture of *G. mellonella* was maintained for several years on a diet based on glycerol and honey (Kings *et al.,* 1979) at ambient temperature (28.0± 2°C) at Sugarcane Breeding Institute, Coimbatore, India. Fourth instar larvae were separated from the diet and were allowed to get acclimatized to 25 ± 2°C for 12 hrs prior to bioassays.

Sequence of application of fungi tested: Petri plate bioassays were carried out against fourth instar *G. mellonella* to study the effect of sequential applications of fungi on host mortality and the ability of the fungus to sporulate in the presence of other fungus. The treatments included application of the six fungus species on the larvae in combinations of two (Table 1) having at least one of the entomophagous fungi in the combination. Tests with combinations of two species of saprophytic fungi (*F.sacchari*, *Aspergillus* sp., *Penecillium* sp.) were not included for analysis, since testing with them resulted in 100% of atypical death and no sporulation was observed. One of the two fungi used in the combination, was applied on the larva on day 1 and the second fungus was applied the next day (24 hr interval). For comparison, treatments with application of single species of fungus as well as a control without application of fungi were maintained. The larvae were treated with the target fungus at the concentration of 1 x 10^7 ml^{-1} using an atomizer. Ten larvae per petridish were used for each replication and three such replications were maintained for each treatment. The treated larvae were incubated at 25 ± 1ºC and the mortality of larvae was observed daily for ten days after completion of treatments. The cadavers that showed hyphal growth characteristic of the fungi and even those that were moribund with typical symptoms were recorded as infected larvae. They were further incubated in petriplates lined with moist filter paper for seven days. The larvae that did not die due to entomophagous fungi (atypical death) were also further incubated separately to observe growth of opportunistic fungus species inoculated, if any. Larvae showing fungal mycelial growth either internally or externally were homogenized in proportionate measure of sterilized water (@ 1 ml $larva^{-1}$). The suspension was filtered though sterilized muslin cloth to remove debris. The observations on spore harvest were done through a haemocytometer.

Statistical analysis: The larval mortality and sporulation rates of each fungus were compared as six clusters of data. The tested parameters of each fungus were evaluated as in when it was applied singly or in combination of one other fungus in either sequence *i.e*., 24 hr prior or later. Data on mortality were subjected to arcsine transformation and data on sporulation rates were passed through log (x ± 0.5) transformation. One-way analysis of variance (ANOVA) of transformed values of mortality was performed using SPSS version 11.5 and means were separated by the Duncan' multiple range test (DMRT) Duncan (1955).

Results and Discussion

The majority of the infected larvae became moribund within four days of treatment though larval death was observed even on 10th day after treatment. All the entomopathogenic fungal species were pathogenic to fourth instar larvae of *G. mellonella*

Table 1 : Combination and sequence of fungi applied in various treatments for bioassays against *G. mellonella* *

S. No.	Species applied on Day 1	Species applied on Day 2
1	*B. bassiana*	-
2	*B. brongniartii*	-
3	*M. anisopliae*	-
4	*Penecillium* sp.	-
5	*F. sacchari*	-
6	*Aspergillus* sp.	-
7	*B. bassiana*	*B. brongniartii*
8	*B. bassiana*	*M. anisopliae*
9	*B. bassiana*	*Penecillium* sp.
10	*B. bassiana*	*F. sacchari*
11	*B. bassiana*	*Aspergillus sp.*
12	*B. brongniartii*	*B. bassiana*
13	*B. brongniartii*	*M. anisopliae*
14	*B. brongniartii*	*Penecillium* sp.
15	*B. brongniartii*	*F. sacchari*
16	*B. brongniartii*	*Aspergillus sp.*
17	*M. anisopliae*	*B. bassiana*
18	*M. anisopliae*	*B. brongniartii*
19	*M. anisopliae*	*Penecillium* sp.
20	*M. anisopliae*	*F. sacchari*
21	*M. anisopliae*	*Aspergillus sp.*
22	*Penecillium* sp.	*B. bassiana*
23	*Penecillium* sp.	*B. brongniartii*
24	*Penecillium* sp.	*M. anisopliae*
25	*F. sacchari*	*B. bassiana*
26	*F. sacchari*	*B. brongniartii*
27	*F. sacchari*	*M. anisopliae*
28	*Aspergillus sp.*	*B. bassiana*
29	*Aspergillus sp.*	*B. brongniartii*
30	*Aspergillus sp.*	*M. anisopliae*

*The fungi were applied individually as well as in combination against fourth instar larvae of *G. mellonella*. The second species was applied 24 hr after application of first species. All combinations tested included atleast one entomophagous fungus. Combinations of two saprophytic fungi were not included for analysis since any mortality due to those fungi was not observed (atypical death) in such treatments

and spores of the fungi could be harvested from the dead larvae (Table 2-7).

The mortality of the larvae was similarly high in treatments in which *B. bassiana* was applied either alone (90%) or applied prior to *M. anisopliae* (100.00%) or following the application of *B. brongniartii* (96.67%) as well as when *B. bassiana* was applied with *Penecillium* sp. irrespective of sequence of application (Table 2). This shows that efficacy of *B. bassiana* was not enhanced by adding any other fungus be it entomopathogenic or otherwise.

Similar results of no synergistic effect had been observed by Umamaheswara Rao *et al*. (2006) in either simultaneous or sequential combination treatments with *Nomuraea rileyi* and *B. bassiana* against *Spodoptera litura*. But sequence of fungi applied had been found to influence mortality rates of insect host in combination treatments in another study (Zukowski *et al*., 1999). Though the efficiency of combination treatments with *Paecilomyces farinosus* and *B. bassiana* was higher than individual treatments, the mortality rates were significantly more when *P. farinosus* application was followed by *B. bassiana* than when the sequence was reverse. However, in the present study antagonistic reactions of *B. bassiana* with either of *F. sacchari* and *Aspergillus* sp. irrespective of the sequence of application of fungus was observed as the mortality rates were significantly reduced (50-70%). Among the soil fungi, *Penecillium* sp. was non-interactive with *B. bassiana* in causing mortality of larvae but resulted in highest spore harvest of the latter.

In the case of spore harvest too, the sequence of application played an important role. Highest spore recovery of *B. bassiana* was obtained when it was inoculated 24 hr prior to *Penecillium* sp. (101.33×10^6 larva^{-1}) and when the sequence was reversed, it resulted in the lowest spore recovery (3.00×10^6 larva^{-1}). We have reports of *Penecillium urticae* negatively influencing the survival of conidia of *B. bassiana* in soil (Lingg and Donaldson, 1981) and no influence of other soil bacteria and actinomycetes (Shimazu *et al*., 2002) on the density dynamics of *B. bassiana*. Irrespective of sequence of application, *M. anisopliae* reduced the sporulation of *B. bassiana* the most while *Aspergillus* sp. enhanced it the most (Table 2).

B. brongniartii caused maximum larval mortality when applied alone (100%) or 24 hrs before *B. bassiana* (96.67%) or after *F. sacchari* (100.00%). Application of *M. anisopliae* a day after *B. brongniartii* was least compatible as it caused the least mortality rates (43.33%) and sporulation of the latter (1.83×10^6 larva^{-1}). Severe competitive effect was observed among the entomopathogenic fungi with lowest recovery of spores from the treatments of *B. brongniartii* preceding *M. anisopliae* (1.83×10^6 larva^{-1}) and *B. brongniartii* succeeding *B. bassiana* (1.58×10^6 larva^{-1}). The soil fungi though had some influence, were not as competitive (Table 3).

Saprophytes and fungal antagonists being competitive to *Beauveria* spp. and the reverse have been reported. A hyperparasitic fungus attacking *B. bassiana* and *B. brongniartii* had differential tritrophic relationship based on the incubation time and infection on host (Markova, 1991). Contrary to this, inhibition of the mycelial growth of several phytopathogens including *Fusarium* sp. by *B. bassiana* (Reisenzein and Tiefenbrunner, 1997; Bark *et al.,* 1996) has been reported.

M. anisopliae was not adversely affected by the combination of other fungi tested (Table 4) in terms of efficacy but was complemented the most by the application of either *B. bassiana* (100%) or *Aspergillus* sp. (93.33%) prior to *M. anisopliae*. *In-vivo* compatibility of B. *bassiana* and *M. anisopliae* has been reported in which the isolate of *B. bassiana* as well as the sequence used, resulted in significant differences (Dal Bello *et al*., 2001). However, sporulation of *M. anisopliae* was reduced by all species of fungi

Table 2 : Effect of co-applicant fungus on the efficacy and sporulation of *B. bassiana*

Combinations of species tested	% Mortality of *G. mellonella* larvae **	Spore harvest/ Larva (x 10^6)
B. bassiana	90.00 ± 10.00 ^{e}	11.43 ±1.01 ^{d}
B. bassiana (F. sacchari)	63.33 ± 5.77 ^{bc}	34.67 ± 16.17 ^{e}
B. bassiana (*Penecillium* sp.)	86.67 ± 23.09 ^{de}	101.33 ± 6.11 ^{g}
B. bassiana (*Aspergillus* sp.)	70.00 ± 10.00 ^{cd}	52.33 ± 7.51 ^{f}
B. bassiana (B. brongniartii)	70.00 ± 10.00 ^{cd}	10.27 ± 2.82 ^{d}
B. bassiana (*M. anisopliae*)	100.00 ± 0.00 ^{e}	4.58 ± 0.63 ^{bc}
B. brongniartii (B. bassiana)	96.67 ± 5.77 ^{e}	5.83 ± 1.59 ^{c}
M. anisopliae (*B. bassiana*)	83.33 ± 5.77 ^{de}	2.75 ± 1.25 ^{a}
Penecillium sp. (*B. bassiana*)	100.00 ± 0.00 ^{e}	3.00 ± 0.00 ^{ab}
F. sacchari (*B. bassiana*)	50.00 ± 0.00 ^{a}	38.67 ± 8.33 ^{ef}
Aspergillus sp. (*B. bassiana*)	53.33 ± 5.77 ^{bc}	57.67 ± 8.51 ^{f}

*Analysis was done with log (x + 0.5) transformed values; **Analysis was done with arc sine transformed values. The fungus in the parenthesis was applied 24 hrs later than the fungus out of parenthesis. Values are mean of replicates ± SD. Means were separated by ANOVA using DMRT and means followed by different alphabets are not significantly different (P= 0.05).

Table 3 : Effect of co-applicant fungus on the efficacy and sporulation of *B. brongniartii*

Combinations of species tested	% Mortality of *G. mellonella* larvae **	Spore harvest / Larva(x10^6)*
B. brongniartii	100.00 ± 0.00 ^{e}	22.70 ± 5.24 ^{e}
B. brongniartii (Penecillium sp.)	50.00 ± 0.00 ^{ab}	5.77 ± 2.04 ^{c}
B. brongniartii (*Aspergillus* sp.)	53.33 ± 5.78 ^{ab}	32.00 ± 12.00 ^{e}
B. brongniartii (*Fusarium* sp.)	56.67 ± 5.78 ^{abc}	12.00 ± 0.00 ^{d}
B. brongniartii (*B. bassiana*)	96.67 ± 5.78 ^{e}	2.92 ± 0.52 ^{b}
B. brongniartii (*M. anisopliae*)	43.33 ± 5.78 ^{a}	1.83 ± 0.14 ^{a}
B. bassiana (*B. brongniartii*)	70.00 ± 10.00 ^{cd}	1.58 ± 0.14 ^{a}
M. anisopliae (*B. brongniartii*)	56.67 ± 5.78 ^{abc}	5.58 ± 1.38 ^{c}
Penecillium sp. (*B. brongniartii*)	63.33 ± 5.78 ^{bc}	7.67 ± 0.58 ^{c}
F. sacchari (*B. brongniartii*)	100.00 ± 0.00 ^{e}	23.67 ± 2.52 ^{e}
Aspergillus sp. (*B. brongniartii*)	76.67 ± 5.78 ^{d}	7.93 ± 0.12 ^{c}

*Analysis was done with log (x + 0.5) transformed values; **Analysis was done with arc sine transformed values. The fungus in the parenthesis was applied 24 hrs later than the fungus out of parenthesis. Values are mean of replicates ± SD. Means were separated by ANOVA using DMRT and means followed by different alphabets are not significantly different (P= 0.05).

Table 4 : Effect of co-applicant fungus on the efficacy and sporulation of *M. anisopliae*

Combinations of species tested	% Mortality of *G. mellonella* larvae **	Spore harvest /Larva(x 10^6)*
M. anisopliae	70.00 ± 10.00 ^{abc}	62.37 ± 5.75 ^{f}
M. anisopliae (*Fusarium* sp.)	73.33 ± 5.77 ^{bc}	60.67 ± 7.02 ^{f}
M. anisopliae (*Penecillium* sp.)	86.67 ± 11.55 ^{cd}	13.73 ± 2.05 ^{c}
M. anisopliae (*Aspergillus* sp.)	86.67 ± 15.28 ^{cd}	25.73 ± 2.05 ^{d}
M. anisopliae (*B. bassiana*)	83.33 ± 5.77 ^{bcd}	2.75 ± 0.87 ^{a}
M. anisopliae (*B. brongniartii*)	56.67 ± 5.77 ^{ab}	14.25 ± 1.75 ^{c}
B. bassiana (*M. anisopliae)*	100.00 ± 0.00 ^{e}	11.77 ± 0.47 ^{c}
B. brongniartii (*M. anisopliae*)	43.33 ± 5.77 ^{a}	7.52 ± 1.51 ^{b}
Penecillium sp. (*M. anisopliae*)	80.00 ± 10.00 ^{bcd}	14.00 ± 2.00 ^{c}
F. sacchari (*M. anisopliae*)	83.33 ± 5.77 ^{bcd}	65.33 ± 8.50 ^{f}
Aspergillus sp.(*M. anisopliae*)	93.33 ± 11.54 ^{de}	38.00 ± 2.00 ^{e}

Table 5 : Effect of co-applicant fungus on the efficacy and sporulation of *Fusarium sacchari*

Combinations of species tested	% Mortality of *G. mellonella* larvae**	Spore harvest/ Larva (x 10^6)*
F. sacchari	0.00 ± 0.00 [a]	3.60 ± 3.82
F. sacchari. (*B. bassiana*)	50.00 ± 0.00 [b]	1.84 ± 0.21
F. sacchari. (*B. brongniartii*)	100.00 ± 0.00 [d]	2.47 ±.0.31
F. sacchari. (*M. anisopliae*)	83.33 ± 5.77 [d]	2.37 ± 0.30
B. bassiana (*F. sacchari*)	63.33 ± 5.77 [bc]	5.33 ± 9.24
B. brongniartii (*F. Sacchari)*	56.67 ± 5.77 [b]	3.87 ± 0.31
M. anisopliae (*F. Sacchari)*	73.33 ± 5.77 [cd]	7.07 ± 1.01

*Analysis was done with log (x + 0.5) transformed values; **Analysis was done with arc sine transformed values. The fungus in the parenthesis was applied 24 hrs later than the fungus out of parenthesis. Values are mean of replicates ± SD. Means were separated by ANOVA using DMRT and means followed by different alphabets are not significantly different (P= 0.05)

Table 6 : Effect of co-applicant fungus on the efficacy and sporulation of *Aspergillus* sp.

Combinations of species tested	% Mortality of*G. mellonella* larvae**	Spore harvest/Larva (x10^6)*
Aspergillus sp.	0.00 ± 0.00 [a]	145.33 ± 25.72 [e]
Aspergillus sp.(*B. bassiana*)	53.33 ± 5.77 [b]	20.00 ± 4.00 [c]
Aspergillus sp.(*B. brongniartii*)	76.67 ± 5.77 [bc]	140.00 ± 20.00 [e]
Aspergillus sp. (*M. anisopliae*)	93.33 ± 11.55 [d]	3.33 ± 0.61 [a]
B. bassiana (*Aspergillus* sp.)	70.00 ± 10.00 [bc]	13.67 ± 3.21 [b]
B. brongniartii (*Aspergillus* sp.)	53.33 ± 5.77 [b]	97.33 ± 3.06 [d]
M. anisopliae (*Aspergillus* sp.)	86.67 ± 15.28 [cd]	97.33 ± 13.01 [d]

Table 7 : Effect of co-applicant fungus on the efficacy and sporulation of *Penecillium* sp.

Combinations of species tested	% Mortality of*G. mellonella* larvae **	Spore harvest/ Larva (x 10^6)*
Penecillium sp.	0.00 ± 0.00 [a]	132.00 ± 10.58 [d]
Penecillium sp. (*B. bassiana*)	100.00 ± 0.00 [e]	168.00 ± 20.00 [e]
Penecillium sp. (*B. brongniartii*)	63.33 ± 5.77[bc]	11.67 ± 0.58 [b]
Penecillium sp. (*M. anisopliae*)	80.00 ± 10.00 [bcd]	3.67 ± 0.58 [a]
B. bassiana (*Penecillium* sp.)	86.67 ± 23.09 [de]	136.00 ± 32.74 [de]
B. brongniartii (*Penecillium* sp.)	50.00 ± 0.00 [b]	216.00 ± 23.07 [f]
M. anisopliae (*Penecillium* sp.)	86.67 ± 11.55[cde]	98.67 ± 6.11 [c]

*Analysis was done with log (x + 0.5) transformed values; **Analysis was done with arc sine transformed values. The fungus in the parenthesis was applied 24 hrs later than the fungus out of parenthesis. Values are mean of replicates ± SD. Means were separated by ANOVA using DMRT and means followed by different alphabets are not significantly different (P= 0.05)

tested barring *F. sacchari*, regardless of sequence of application. These results show that there is no correspondence between the virulence and sporulation ability of a fungus due to a companion fungus or synergy may not be for both high efficacy as well as sporulation. The lowest harvest of *M. anisopliae* spores was in the treatment where in *B. bassiana* was applied 24 hrs after *M. anisopliae* (2.75 x 10^6 $larva^{-1}$). Contrary to the results presented here, competitive colonization by *M. anisopliae* in combined treatments of *B. bassiana* and *M. anisopliae* has earlier been reported (Inglis *et al.,* 1997).

There was no larval mortality when saprophytic fungi alone were applied (Table 5, 6, 7). Further, when two saprophytic fungi were co- applied the larvae suffered atypical death as well as disintegration (100%) and thus were eliminated from analysis. In the combination treatments involving *Fusarium* sp., the influence based on sequence of application was seen clearly in the case of *B. brongniartii*. While application of *F. sacchari* 24 hr prior to *B. brongniartii* resulted in the highest mortality (100%) and was reduced to 56.67% when it followed *B. brongniartii* application. Sporulation of *F. sacchari* in general was very low (Table 5) and was not significantly different in any of the treatments. Mortality of larvae in the treatments involving *Aspergillus* sp. was the highest when it was co-applied with *M. anisopliae* with either *Aspergillus* sp. preceding it (93.33%) or in the reverse (86.67%). Highest sporulation rate of *Aspergillus* sp. was obtained in treatments with *Aspergillus* sp. when applied alone (145 x 10^6 $larva^{-1}$) and it was unaffected (Table 7) only when *B. brongniartii* application followed it (140 x 10^6 $larva^{-1}$).

In the present study, though the mortality of larvae was caused by entomopathogens and not by saprophytic fungi, the

latter could be recovered in substantial quantum from all the treatments they were involved in, sometimes superceding even the mortality agent. For example, in the combination of *B. brongniartii* and *Aspergillus* sp. applied in either sequence, the spore harvest of *Aspergillus* sp. was higher than *B. brongniartii* (Table 3,6). The theory of saprophytes using the resources from insects dying from the infections of entomopathogenic fungi (Vega *et al*., 2009) is proven true here. The co-application of the *M. anisopliae* with mycoparasites on test insects generally enhanced mycoparasite recovery though the mortality was caused by the entomopathogen (Krauss *et al*., 2004).

Sporulation of *Penecillium* sp. was most favourably influenced when *Penecillium* sp. was applied after *B. brongniartii* (86.67%) as well as in combinations with *B. bassiana* (86.67 and 100%) when it preceded *M. anisopliae* application, spore recovery of *Penecillium* sp. was the lowest (3.67×10^6 larva^{-1}) (Table 7) though in two other combinations it sporulated better than when it was applied alone. In several instances saprophytic fungi gained due to entomopathogenic infection of larvae. Such a sporulation dominance of a single fungus in a combination has been observed by Hughes and Boomsma (2004). In their investigation of the interaction between a virulent *M. anisopliae* and a normally avirulent, opportunistic fungal pathogen, *Aspergillus flavus* it was observed that almost all of the cadavers that sporulated produced only *A. flavus* spores. The dynamics was that of a superinfection except that *A. flavus* was, though less virulent parasite of the host emerged as the superior competitor when combined with other fungus. Furthermore, *A. flavus* sporulated from more hosts during mixed infections than when it was applied at the same dose in isolation.

Saprophytic fungi in this study had a substantial role in letting the biocontrol fungi work better or worse than when the latter were used singly. Further, when combined with the entomopathogenic fungi, though the entomopathogenic fungi were mortality agents, often the cadaver was covered with spores of saprophytic fungi indicating the latter' better efficacy in utilizing the resources in dying larvae, made available by entomopathogen. Within-host competition is an important factor in host-pathogen relationships and a dead or dying insect infected by an entomopathogenic fungus leaves a potential source of energy for other, opportunistic microorganisms (Vega *et al*., 2009). Though it is reported that some species of entomopathogens produce secondary metabolites within their insect hosts that are postulated to help the fungus out compete opportunists during the saprotrophic phase of insect utilization (Strasser *et al*., 2000) co-inoculation of sufficient inoculum of saprophytic fungi was able to overcome this mechanism which happened in the present study. Even between entomopathogenic fungi mortality could be due to an agent while mycosis could be induced by another in combination (Umamaheswara Rao *et al*., 2006).

Several interesting aspects about the relationship within a group (entomopathogens) and two different groups (entomopathogenic and opportunistic) of fungi in using the host were revealed in the present study. Though increased mortality rate by entomopathogenic fungi due to the combination was observed, the number of spores of individual species from the cadavers in a co-application was lesser than the harvest from the treatment with a single entomopathogenic fungus. This is due to possible competitions for the same resources and would manipulate the secondary spread of the pathogen (horizontal dispersal) of the pathogen when applied against target pest. In this case, it was suggested to determine the importance of secondary infections in relation to the damage caused by the pests (Brito *et al*., 2008). These observations are important while combining two agents for biological control of target pest. It has been observed that mixed infection with a largely avirulent pathogen can alter the virulence and reproduction of a second, highly virulent pathogen depending on the order of infection and environmental conditions (Thomas *et al.,* 2003).

In order to formulate new biological control agents the evaluation of interacting dynamics among the entomopathogenic organism, host and environment is necessary (Inglis *et al.,* 2001). This is true even in the case of use of the available biocontrol agents in a better manner. Thus, the current findings become imperative to know role of fungi coexisting in the soil in influencing the entomopathogenic fungi when applied for biocontrol of target pest.

Acknowledgments

The authors thank Dr. N. Vijayan Nair, Director, Sugarcane Breeding Institute, Coimbatore -641007 for the support and facilities provided.

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