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Effect of Biopathogens on Honey Bees

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ABSTRACT

The bio-safety of some entomopathogenic bioagents viz. *Metarhizium anisopliae*, *Beauveria bassiana*, *Bacillus thuringiensis* and *Verticilllium lecanii*, was tested in a honey bee colony by three different methods i.e. the "strip method", the "spray method" and the "feeding method". The bioagents were tested at 10^8 spores/colony in strip and spray methods and 10^7 spores in the feeding method. No significant differences were observed in brood and adult bee mortality when these biopesticides were used, whether in strip or spray form. Similarly, no significant differences were observed in the number of incoming and outgoing bees before and after the application of biopesticides whether using the strip or spray method. However, in the feeding method significant differences were observed in bee mortality under caged conditions by *V. lecanii* and *B. bassiana* according to LT_{50} values, although no significant differences were observed with *M. anisopliae*.

Keywords: *Bacillus*, *Beauvaria*, biopesticides, ectoparasitic mite, entomopathogenic bioagents, *Metarrhizium*, spray method, strip method, *Verticillium*

INTRODUCTION

The extensive use of pesticides in agriculture and public health not only controls insect pests and vector-borne diseases but also causes environmental pollution and upsets the balance of nature due to the loss of pollinators such as *Apis* and other beneficial insects (Matsumura and Benezet 1978). The poisoning of bees by pesticides is a major problem affecting the efficiency of bees not only in the production of honey but in crop pollination too. The immature stages of the honey bee are vulnerable to insecticide poisoning. Such poisoning may result in hidden damage to the honey bee colony. It has been noted that loss of brood and new bees as a result of exposure to insecticides can cause deleterious effects (Davis 1989). Therefore, toxicity of pesticides to beneficial insects, mainly honey bees, has been a matter of great concern for plant protection workers.

To overcome harmful effects of chemical pesticides, ecofriendly methods can be applied. Microbial pesticides are one such method, but their safety to non-target organisms, including the honey bees, needs to be demonstrated. Effects of some biopesticides like fungi, bacteria and nematodes have been tested on honey bees and other pollinators (Cantwell *et al.* 1972; Flexner *et al*. 1986; Krieg and Langenbruch 1981; Vandenberg 1990). However, in India no such systematic work has been carried out on the safety of these bioagents on honey bees, although many of these are being used for pest control. The pathogenicity of these bioagents to honey bees, if any, needs to be worked out under different environmental conditions, since under some conditions these may infect or elicit pathological response in honey bees. Most of the earlier studies of biopesticides has been conducted on bees confined to cages which may respond to the treatments in a different manner than the bees in the colony as physical, chemical and biological stress factors, mainly temperature and high humidity, results in the development of some infection in bees (Glinski and Jaroscz 2001). However, exposure of caged bees to microbial insecticides is a useful method for safety testing as no further testing will be required if the results are not good.

There have been contradictory reports regarding the pathogenicity of different bioagents to honey bees. Butt *et al*. (1994) showed that isolates of *Metarhizium anisopliae* were more pathogenic than *B. bassiana* whereas, there are reports indicating that *M. anisopliae* does not harm bees (Flores 2004). Interestingly, scientists in ARS beneficial insect research unit at Weslaco, Texas have even reported a strain of *M. anisopliae* to be deadly to *Varroa* mites but safer to honey bees. A strain of *M. anisopliae* and *Hirsutella thompsonii* have been reported to be deadly to *Varroa* mite which had no effect on the honey bees (Kanga *et al*. 2002). The abilities of these fungi to adapt to heat tolerance and, therefore, in bee hives have made them strong candidate for successful biological control agents for the *Varroa* mites.

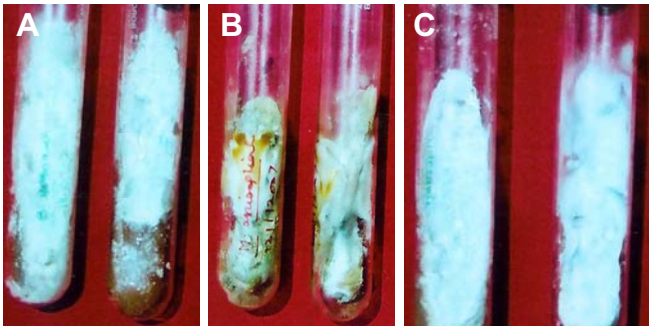

Fig. 1 Culture of *B. bassiana* (A), *M. anisopliae* (B) and *V. lecanii* (C) on SDAY medium.

Keeping in view these developments in the field of bioagents in relation to honey bees, the present studies were undertaken to determine the safety of four most commonly used entomopathogenic bioagents viz. *M. anisopliae*, *B. bassiana*, *B. thuringiensis* and *V. lecanii* to honey bees.

MATERIALS AND METHODS

Raising culture

The culture tubes containing growth medium and inoculated with *B. bassiana*, *V. lecanii* and *M. anisopliae* were kept in an incubator

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Research Note

Fig. 2 Application of biopesticides by the strip method. (**A**) Biopesticide-coated strip depicting its size and position; (**B**) Frames fitted with strips of biopesticides.

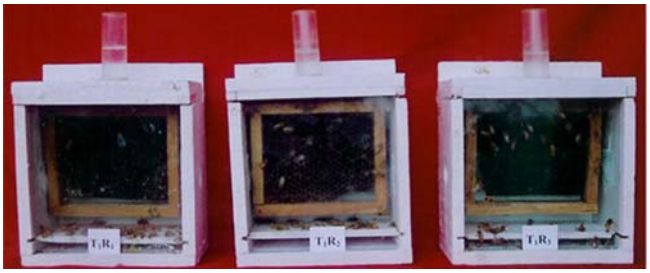

Fig. 3 Feeding of biopesticides to bees in hoarding cages.

at 25-27°C temperature so as to grow the fungus at the given temperature as and when required (**Fig. 1A-C**).

Formulated product of *Bacillus thuringiensis kurstaki* purchased under the trade name Halt (Wockhardt Life Science Ltd.) was used to make the desired concentration of bacterial formulations.

Method of application

Three different methods viz. strip, spray and feeding methods were used to check the pathogenicity of these bioagents to honey bees.

(i) Strip method

Required concentrations of test fungi (in known volume of distilled water) were applied by spreading the suspension evenly on white chart paper strips (15 cm × 10 cm) coated with starch on one side as a base and were allowed to dry for 10-15 min. One such strip was placed in a vertical position between every two frames (**Fig. 2A**). The number of strips varied with the colony strength (**Fig. 2B**).

The bee activity (number of outgoing and incoming bees/min) was also observed before and after the treatments as to observe the bee behavior.

(ii) Spray method

The bioagents (10^8 spores/ ml) were applied to young (0-24 h) and old bee brood (5-days old) by spraying in 6.45 cm^2 marked area at different positions in different frames Observations were recorded on the young brood mortality after 5 days and the mortality of old brood was checked by counting the number of adults emerged from sealed brood.

(iii) Feeding method

Fifty young bees collected from the colony were kept in the hoarding cage at room temperature varying between 16 and 30.5°C. The bioagents (10^7 spores of each) mixed in 20% sucrose solution were fed to bees with the help of feeding tubes fitted on the upper side of the cages (**Fig. 3**). Mortality of bees in the hoarding cages was recorded daily till all the bees died. In control bees were fed with 20% sucrose solution only.

Statistical analysis

The data were statistically analysed using a completely randomized design after appropriate transformation where ever needed (Gomez and Gomez 1986). Per cent cumulative mortality data recorded on the caged bees were used to calculate LT_{50} values by working out probit analysis as outlined by Finney (1971).

RESULTS

Effect of biopesticides on *A. mellifera* colonies

1. Strip method

In the present studies, no significant differences in bee mortality were observed in the colonies treated with *M. anisopliae* (54.67) and *B. bassiana* (53.67) as compared to the control (49.00) (**Table 1**). Flores (2004) and Butt *et al*. (1998) have also reported that honey bees when treated with *M. anisopliae* under field conditions were not affected. However, in the present studies significantly more bee mortality was observed in the colonies treated with *B. thuringiensis* (61.33) and *V. lecanii* (74.67).

2. Spray method

Data on honey bee mortality during 10 days of application of biopesticides using spray method are presented in **Table 2**. The mortality in the colonies treated with *B. bassiana* and

Table 1 Number of dead bees during 10 days of application of biopesticides using the strip method.

Treatment of biopesticides (10^8 spores/colony)	Number of dead bees (10 days post treatment)
Metarhizium anisopliae	54.67
Beauveria bassiana	53.67
Verticillium lecanii	74.67
Bacillus thuringiensis	61.33
Control	49.00
$CD_{0.05}$	11.57

Table 2 Number of dead bees during 10 days of application of four different biopesticides using the spray method.

Treatment of biopesticides (10^8 spores/colony)	Number of dead bees (10 days post treatment)
Metarhizium anisopliae	86.33
Beauveria bassiana	36.67
Verticillium lecanii	79.67
Bacillus thuringiensis	47.00
Control	70.33
$CD_{0.05}$	17.29

Table 3 Mortality of 0-24 h old *Apis mellifera* larvae after 5 days of treatment of biopesticides using the spray method.

Treatment of biopesticides (10^8 spores/6.45 cm^2)	Percent larval mortality (0-24 h old larvae)
Metarhizium anisopliae	18.13
Beauveria bassiana	24.20
Verticillium lecanii	27.20
Bacillus thuringiensis	27.22
Control	17.38
$CD_{0.05}$	NS

Table 4 Mortality of 5-days old *Apis mellifera* larvae after treatment with biopesticides using the spray method.

Treatment of biopesticides (10^8 spores/6.45 cm^2)	Mortality of 5-day old larvae (%)	Mortality in sealed brood (%)
Metarhizium anisopliae	20.38	23.84
Beauveria bassiana	24.95	20.79
Verticillium lecanii	22.68	22.40
Bacillus thuringiensis	21.93	20.34
Control	19.63	23.92
$CD_{0.05}$	NS	NS

Table 5 Mortality of 3-days old *Apis mellifera* larvae at different positions in the treated and control colonies using the spray method.

Position of the brood frame in hive	Control colonies		Treated colonies	
	Per cent larval mortality	Average temperature (°C)	Percent larval mortality	Average temperature (°C)
Centre	4.53 (10.51)*	34.82	22.72 (28.42)*	33.21
Right corner	15.90 (23.23)	29.87	36.37 (37.08)	30.65
Left corner	13.62 (21.52)	31.19	30.67 (33.51)	31.11
Super (centre)	11.35 (19.18)	35.76	36.37 (37.00)	35.07
$CD_{0.05}$	(8.39)	1.34	(5.93)	1.69

* Figures in parentheses are arc sine transformed values

B. thuringiensis were 36.67 and 47.00 bees/colony, respectively the differences being non-significant r. The dead bee count was significantly higher in control (70.33) and colonies treated with *M. anisopliae* (86.33) and *V. lecanii* (79.67), all being on par.

Effect on biopesticides on bee brood using spray method

No significant differences were observed in the brood mortality with theapplication of biopesticides on the larval stage whether young (0-24 h) or old (5-days-old) (**Tables 3, 4**) indicating their safety to bee brood. Thus, it seems that all these biopesticides whether used in the form of strip or spray is almost safe to the bees. More mortality of the adult bees in *V. lecanii* and *B. thuringiensis* treated colonies may be due to some factors other than the biopesticides. Moreover, this mortality is quite low as compared to normal death rate of 0-100 bees/day/colony as given by FAO (1986).

Effect of *M. anisopliae* on 3-days-old bee larvae using spray method at different positions in the colonies

Hive temperature has been reported to influence survival of the bioagents applied inside a colony. Southwick and Heldmaier (1987) have pointed out that the bioagents may not survive in the centre area of brood nest where temperature ranges from 33-36°C. Different bioagents are functional within drone brood areas on the periphery of brood nest where temperature varied between 32.5-33.4°C (Davidson *et al*. 2003). In the present studies the treatment of brood with distilled water only at different positions of the hive revealed that the larval mortality was significantly less in the brood positioned in the centre of the colony where average hive temperature was 34.8°C as compared to brood present on side frames and super (**Table 5**) indicating variations in the brood mortality at different positions in the hive in control treatment also. Further experiment conducted with *M. anisopliae* at different positions in the hive revealed no definite trend in the larval mortality as per position of the brood in the colony. Though there was an indication that the brood mortality was low in the centre as compared to brood in super and on one side of the frame, but on another side in the same colony the mortality was almost same as in the centre. These observations suggest that *M. anisopliae* affects the brood outside the central brood area to some extent which may not be exclusively due to variations in temperature but also to other colony conditions.

Biopathogens and their safety to caged adult honey bees

1. Feeding method

In most of the safety testing of biopesticides, caged bees have invariably been used although they may not respond in the same way as bees in the colony. In the present studies, the data collected on adult honey bee mortality after feeding on sugar syrup containing spores of *M. anisopliae* (107 spores in 20% sugar solution) kept in hoarding cages revealed no significant difference in the daily bee mortality in the treated and the control bees (**Table 6**). There were no significant variations in the LT_{50} values which were 4.7 days for the treated bees as compared to 5.6 days in the control (**Table 10**). This indicates the safety of *M. anisopliae* to the caged bees. However, there are contradictory reports where *M. anisopliae* has been reported to be toxic to bees. Shaw *et al.* (2002) reported that out of six isolates of *Metarhizium*, some caused more mortality (1×10^8 ml^{-1}) of the caged bees as compared to the control. However, they have also pointed out that all the mortality could not be attributed to fungal infection. Similarly, Butt *et al* (1994) have also found LT_{50} values of 4.4 and 8.5 days for two isolates of *M. anisopliae* viz., V_{208} and V_{245}, respectively, at 1×10^{10} conidia/ml. Thus, it seems that even among *M. anisopliae,* the pathogenicity depends on the type of isolate used. This may possibly explain the safety of *M. anisopliae* used in the present study. Kanga *et al.* (2002) have also reported that *M. anisopliae* had no harmful effect on honey bees which further supports the present observation.

Two more fungi tested in the present studies showed more lethal effect on the caged bees. Daily mortality of bees was significantly higher in *V. lecanii* treated bees than the control. The LT_{50} value for the treatment was 3.2 as compared to 5.3 days in the control (**Table 10**). Same trend was observed among the bees treated with *B. bassiana*. These observations suggests that both these fungi can affect adult worker honey bees. Vandenberg (1990) has also found that most bees in the *B. bassiana* treated groups that died after day 4 had mycosis. But he has pointed out that the dose to which bees were exposed (10^8 spores) was probably much higher than they would encounter in the field. On the contrary, Toumanoff (1931) who also conducted similar tests did not observe infection among the treated bees. Thus, there is further need to determine infectivity of these fungi at concentrations that are found under field conditions.

In the present studies, the data collected on adult honey bee mortality after feeding tretament with *B. thuringiensis* revealed that there was no significant difference in the daily bee mortality in the treated and the control (**Table 7**). No

Table 6 Daily mortality (%) of *Apis mellifera* workers in hoarding cages after feeding with *Metarhizium anisopliae* spores.

Treatment / Days after feeding	Feeding with *Metarhizium anisopliae* spores (10^7 spores in 20% sucrose solution)	Control	Mean
1	3.33 (10.40)*	2.67 (9.27)	3.00 (9.83)
2	5.33 (13.17)	4.00 (11.28)	4.67 (12.23)
3	10.67 (19.05)	3.33 (10.40)	7.00 (14.72)
4	9.33 (17.77)	8.00 (16.35)	8.67 (17.06)
5	22.00 (27.96)	9.33 (17.63)	15.67 (22.79)
6	17.33 (24.11)	15.33 (22.98)	16.33 (23.55)
7	16.00 (23.29)	26.00 (30.65)	21.00 (26.97)
8	15.33 (23.04)	30.00 (33.15)	22.67 (28.10)
Mean	12.42 (19.85)	12.33 (18.96)	

$CD_{0.05}$
Treatment: NS
Period: 3.49
Interaction (Treatment x period): 4.99
* Figures in parentheses are arc sine transformed values

Table 7 Daily mortality (%) of *Apis mellifera* workers in hoarding cages after feeding with spores of *Bacillus thuringiensis*.

Treatment / Days after feeding	Feeding with *Bacillus thuringiensis* spores (10^7 spores in 20% sugar solution)	Control	Mean
1	4.67 (12.42)*	1.33 (5.42)	3.00 (8.92)
2	1.33 (5.42)	4.00 (11.28)	2.67 (8.35)
3	7.33 (15.68)	6.67 (14.93)	7.00 (15.30)
4	9.33 (17.63)	8.67 (17.10)	9.00 (17.36)
5	12.67 (20.79)	14.67 (22.48)	13.67 (21.63)
6	15.33 (23.02)	14.67 (22.51)	15.00 (22.76)
7	12.67 (20.76)	16.00 (23.55)	14.33 (22.16)
8	12.67 (20.79)	13.33 (21.37)	13.00 (21.07)
9	12.67 (20.79)	10.67 (19.05)	11.67 (19.92)
10	11.33 (19.66)	10.00 (18.38)	10.67 (19.02)
Mean	10.00 (17.69)	10.00 (17.61)	
$CD_{0.05}$	NS		
Treatment	-		
Period	(2.76)		
Interaction (Treatment x period)	(3.89)		

* Figures in parentheses are arcsine transformed values

significant variations were observed in the LT_{50} values, which were 5.6 days for the treated bees as compared to 5.5 days in the control (**Table 10**). This indicates the safety of *B. thuringiensis* to the caged bees. Cantwell *et al*. (1972) have also observed that with *B. thuringiensis* (Certan) there was no bee mortality in the caged experiments. It is also in total agreement with the work done on various species of *Bacillus* and varieties of *B. thuringiensis* used as microbial control agent (Davidson *et al*. 1977; Lehnert and Cantwell 1978; Krieg *et al*. 1980). Only exotoxin of *B. thuringiensis* poses some threat but most of the commercial preparations being used do not contain exotoxin (Lehnert and Cantwell 1978). However, Vandenberg (1990) reported reduction in honey bee longevity at a very high concentration of *B. thuringiensis* var. *tenebrionis* (10^8 spores/ml) but even at this dose it did not cause any pathology or infection. Thus the present results suggests the biosafety of *B. thuringiensis* to honey bee colonies.

Table 10 Mortality over time (LT_{50} in days) of honey bees exposed to different biopesticides (107 spores in 20% sugar sugar syrup).

Treatment	LT_{50} (95% C.L.)	Slope (95% C.L.)
B. bassiana	4.8 (5.38, 4.28)	0.159 (± 0.311)
Control	6.4 (6.61, 5.81)	0.480 (± 0.941)
B. thuringiensis	5.6 (6.11, 5.22)	0.211 (± 0.413)
Control	5.5 (5.89, 5.22)	0.475 (± 0.9.31)
V. lecanii	3.2 (3.91, 2.58)	1.310 (± 2.571)
Control	5.3 (5.70, 4.89)	0.929 (± 1.822)
M. anisopliae	4.7 (5.71, 3.86)	0.492 (± 0.965)
Control	5.6 (6.05, 5.23)	0.519 (± 1.017)

EFFECT OF BIOPESTICIDES ON BEE ACTIVITY

There are varied reports regarding effect of biopesticides on the behaviour of honey bees. Malone *et al*. (2001) observed no impact on the activity of the bees after treatment with *B. thuringiensis*. However, Israel and Boland (1992) have reported that the various powdered formulations affected the grooming behaviour and time spent outside the hive. In the present study the data recorded on bee activity before and after treatment, revealed that there were no significant differences either in the number of outgoing or incoming bees before and after the application of biopesticides (**Tables 8, 9**). These variations might be related to the mode of application of the biopesticides. In the present study biopesticides were applied only by using strip and spray method and not as powdered formulation.

One or two applications of *B. bassiana* (Balsamo) Vuillemin have been reported to significantly increase the fall of *Varroa destructor* mites in honey bee (*Apis mellifera*) hives without affecting bee health or activity (Meikle *et al*. 2007; 2008a, 2008b). Almazraawi (2007) studied the impact of entomopathogenic fungus *B. bassiana* on honey bees *A. mellifera* and concluded that *B. bassiana* is safe when applied to honey bees under field conditions. Rodriguez *et al.*

Table 8 Effect of biopesticides on bee activity per minute per colony before and after application of biopesticides using the strip method.

Treatment of bio-pesticides	Incoming bees		Outgoing bees	
	Before treatment	After treatment	Before treatment	After treatment
Metarhizium anisopliae	37.00	43.33	51.33	50.00
Beauveria bassiana	40.67	36.67	49.00	54.00
Verticillium lecanii	34.33	41.67	43.67	55.33
Bacillus thuringiensis	37.30	36.33	55.00	56.00
Control	40.00	43.00	53.33	51.00
$CD_{0.05}$	NS	NS	6.19	3.84

Table 9 Effect of biopesticides on bee activity per minute per colony before and after application of biopesticides using the spray method.

Treatment of bio-pesticides	Incoming bees		Outgoing bees	
	Before treatment	After treatment	Before treatment	After treatment
Metarhizium anisopliae	32.00	40.00	44.00	54.00
Beauveria bassiana	34.00	37.67	49.00	53.33
Verticillium lecanii	34.00	39.33	41.00	48.00
Bacillus thuringiensis	31.33	36.00	43.00	48.33
Control	30.33	35.33	42.00	50.33
$CD_{0.05}$	NS	NS	NS	NS

(2009) reported *M. anisopliae* as a promising biological tool to control *V. destructor* as no significant bee mortality was observed between treated and untreated bee hives. Though it has been reported that *M. anisopliae* can infect *A. mellifera* in laboratory trials, at this time, it has not been reported to cause epizootics between bees (Chandler *et al*. 2001). More recently, *Metarhizium* has been considered for controlling other bee pests such as the *Aethina tumida* parasite in South Africa from isolated strains from this insect (Muerrle *et al*. 2006).

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