BIOLOGICAL CONTROL

Compatibility of the Fungus *Beauveria Bassiana* (Bals.) Vuill. (Deuteromycetes) with Extracts of Neem Seeds and Leaves and the Emulsible Oil

Rogério A. Depieri[1,2], Sueli S. Martinez[1] and Ayres O. Menezes Jr.[3]

[1]*Lab. Entomologia, Instituto Agronômico do Paraná, C. postal 481, 86001-970 - Londrina, PR*
rdepieri@sercomtel.com.br, suemart@sercomtel.com.br
[2]*Bolsista CBP & D/Café*
[3]*Depto. Agronomia, Universidade Estadual de Londrina, C.postal 6001, CEP 85051-970 - Londrina, PR*
ayres@uel.br

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Compatibilidade do Fungo *Beauveria bassiana* (Bals.) Vuill. (Deuteromycetes) com Extratos de Sementes e Folhas e o Óleo Emulsionável de Nim

RESUMO – A compatibilidade de uma formulação comercial de óleo emulsionável de nim (*Azadirachta indica* A. Juss.), do extrato aquoso de sementes e do extrato aquoso de folhas de nim com *Beauveria bassiana* (Bals.) Vuill., foi avaliada *in vitro*. Foram conduzidos três experimentos para avaliar o efeito de cada um dos produtos no crescimento vegetativo do fungo, e na produção e viabilidade dos conídios. Os produtos foram incorporados ao meio de cultura (PDA+E) e distribuídos em placas de Petri, nas concentrações, 0,15; 1,5 e 15% (extrato aquoso de folhas), 1; 2 e 4% (extrato aquoso de sementes) e 0,5; 1 e 1,5% (óleo emulsionável). Com base no crescimento vegetativo e na produção de conídios, os extratos aquosos de sementes e de folhas e o óleo emulsionável de nim foram caracterizados segundo o modelo T para classificação de compatibilidade de produtos. Os extratos de sementes e de folhas mostraram-se menos prejudiciais a *B. bassiana* que o óleo emulsionável. Esse produto, nas concentrações testadas, não foi compatível com *B. bassiana*, inibindo significativamente o crescimento vegetativo e reduzindo a produção e a viabilidade dos conídios com efeitos mais acentuados nas concentrações mais altas. Os extratos de sementes e de folhas de nim foram compatíveis com o entomopatógeno em todas as concentrações testadas. O extrato de sementes reduziu o crescimento vegetativo e a produção de conídios, mas não afetou a viabilidade dos esporos produzidos. Embora o extrato de folhas a 15% tenha reduzido um pouco o crescimento vegetativo e a produção e viabilidade dos conídios, ainda se manteve compatível com o fungo *B. bassiana*, segundo o modelo T.

PALAVRAS-CHAVE*: Azadirachta indica*, seletividade, controle biológico, fungo entomopatogênico

ABSTRACT - The compatibility of a commercial formula of emulsible neem oil (*Azadirachta indica* A. Juss.) and of aqueous extracts of neem seeds and leaves with *Beauveria bassiana* (Bals.) Vuill. was evaluated *in vitro*. Three experiments were conducted to evaluate the effect of each product on the fungus vegetative growth and on conidia production and viability. The products were incorporated to a culture medium (BDA+E) and distributed into petri dishes, in the following concentrations: 0.15%; 1.5% and 15% (leaf aqueous extract), 1%; 2% and 4% (seed aqueous extract) and 0.5%; 1% and 1.5% (emulsible oil). Vegetative growth and conidia production were the basis for characterization of the aqueous extracts of seeds and leaves and of the emulsible oil, using the T classification model for compatibility of products. Seed and leaf extracts were less harmful to *B. bassiana* than the emulsible oil. Under the tested concentrations, the oil was not compatible with *B. bassiana*, inhibiting conidia vegetative growth significantly and decreasing production and viability of conidia, particularly at higher concentrations. Neem seed and leaf extracts were compatible with the entomopathogen in all concentrations. The seed extracts reduced conidia vegetative growth and production, but it did not affect the viability of spores. Leaf extract at 15% had a small negative impact on vegetative growth, and on production and viability of conidia, but it was still compatible with the fungus *B. bassiana*, according to the T model.

KEY WORDS: *Azadirachta indica*, selectivity, biological control, entomopathogenic fungus

Biological control, particularly by entomopathogenic fungi, is important for reducing the population density of pests in Integrated Pest Management (IPM) programs. Therefore, preservation of entomopathogens that occur naturally, or are introduced for insect control, should be observed (Oliveira *et al*. 2003). In addition, we must understand the compatibility of entomopathogenic fungi with other crop production techniques such as the use of insecticides, which may inhibit to a smaller or larger extent the development and reproduction of pathogen (Malo 1993).

Coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae), is one of the most serious pests in coffee plantations, attacking the fruit in all developmental stages. Endosulfan, an organochlorine, is still the most common treatment against *H. hampei*, in spite of its fungitoxic effects on *Beauveria bassiana* (Mourão *et al. 2003*, Oliveira *et al.* 2003), one of the most important, naturally occurring pathogens of coffee berry borer. Continuous utilization of endosulfan may promote the selection of populations that are resistant to the insecticide and to other products of the same chemical group (Brun *et al.* 1989, 1994), as well as cause environmental problems and farmers intoxication.

Research for alternative means to control coffee berry borer includes the use of extracts and oils from insecticide plants. Neem, *Azadirachta indica* (Meliaceae), has a small impact on beneficial fauna and the environment (Schmutterer 1995, Martinez 2002). Research on the use of neem to control coffee berry borer has promising results (Sponagel 1994, Rodrigues-Lagunes *et al*. 1998, R.A. Depieri & S.S. Martinez unpublished). Neem effects on natural enemies, however, particularly on entomopathogenic fungi, need further research.

Studies on the compatibility of *B. bassiana* with neem show conflicting results. For some authors, emulsible neem oil inhibts micelia growth (Bajan *et al.* 1998, Hirose *et al*. 2001) and the production and germination of spores of *B. bassiana* (Hirose *et al*. 2001). Other authors do not report fungitoxic effects caused by emulsible oil (Rodriguez-Lagunes *et al*. 1997), except for concentrations above 4% (E.D. Quintela & P.V. Pinheiro, unpublished), or by neem seed extract in concentrations above 2,5% (Rodriguez-Lagunes *et al*. 1997).

The objective of this study was to evaluate *in vitro,* the compatibility between the fungus *B. bassiana* with neem emulsible oil, and seed and leaf extracts, at concentrations that showed potential for reduction of coffee berry borer in a previous study (R.A. Depieri & S.S. Martinez unpubl.).

Materials and Methods

The trials were conducted at the Laboratório de Manejo Ecológico de Pragas e Plantas Inseticidas, Instituto Agronômico do Paraná, IAPAR, in growth chamber at 25 ± 1ºC, 12h photophase.

We evaluated the effect of each product in three trials regarding vegetative growth, conidia production, and viability of *B. bassiana* spores. Emulsible Dalneem® neem oil (0.5%, 1% and 1.5%) (main compound azadirachtin 0.1%), seed aqueous extract (1%, 2% and 4%), and neem leaf aqueous extract (0.15%, 1.5% and 15%), were incorporated to PDA+S (potato-dextrose-agar with streptomicine sulfate) media and evaluated separately. Each trial had a corresponding control trial in PDA+S medium.

Isolation and Cultivation of *B. bassiana.* The isolate of *B. bassiana* obtained from conidia was extracted from dead coffee berry borer (*H. hampei)* specimens, collected from organically-grown coffee trees in Londrina, PR. The isolate was kept in petri dishes (100 mm ∅) containing PDA+S medium, in growth chamber, similar to isolates selected for the trials, until sporulation was complete.

Incorporation of Emulsible Neem Oil in Culture Medium. The neem emulsible oil was diluted in non-solidified (45ºC) PDA+S medium, to obtain 0.5%, 1% and 1.5% concentrations. For each trial, 1000 ml of treated growth medium were prepared and put on 50 petri dishes, for inoculation of *B. bassiana*.

Preparation of Seed Aqueous Extract and Incorporation to the Culture Medium. Ripe neem fruits of ten-year-old trees grown at IAPAR experimental fields in Paranavaí, PR, had the flesh removed in running water. The seeds were shadow-dried for seven days and stored at 8–10ºC, for approximately 30 days. To prepare the aqueous seed extract at 40%, the seeds were first ground in a blender, in the proportion of 80 g to 200 ml of sterile distilled water. The extract remained at rest, for approximately 24h at room temperature, in the dark, and then it was filtered through a polyester tissue, to separate the solid components. Next, 100 ml of this extract at 40% were mixed with 900 ml of PDA+S medium at 45ºC (prepared with 100 ml less of sterile distilled water), thus reducing the concentration to 4%. To obtain 100 ml of seed extract at 20% and 100 ml seed extract at 10%, 100 ml aqueous seed extract at 40% were submitted to serial dilutions in water. Each extract was incorporated separately, in 900 ml of PDA+S medium at 45ºC, to obtain the concentrations at 2% and 1%, respectively. The culture media treated after homogenization were placed on the same number of dishes as in the previous trial.

Preparation of the Leaf Aqueous Extract and Incorporation to the Culture Medium. Leaves without apparent symptoms of insect or disease attacks were collected from eight-year-old neem trees grown at IAPAR experimental fields, in Londrina, PR. The leaves were washed in running water and immersed for five min in a solution of NaClO at 1%. The folioles were separated and washed in sterile distilled water, and then kept for approximately 4h, in laminar flow hood for surface drying. Next, the folioles were ground in a blender, in the proportion of 300 g to 500 ml of sterile distilled water, to obtain the leaf aqueous extract at 60%. The extract was at rest for approximately 24h, under room temperature and in the dark, and then filtered through a fine polyester tissue. After that, 250 ml of leaf extract at 60% were added to 750 ml of PDA+S melted medium, at

the same temperature as for the previous trial, to reduce the concentration to 15%. To prepare the medium at 1.5%, 25 ml of the extract at 60% were diluted in 225 ml of water and incorporated to the same amount of melted PDA+S medium, as for the previous treatment. To obtain the 0.15% concentration, 2.5 ml of the extract at 60% were diluted in 247.5 ml of sterile distilled water and were added to 750 ml of the previously melted PDA+S medium. Therefore, 1000 ml of treated PDA+S medium for each concentration of leaf aqueous extract were obtained, which were later placed on 50 petri dishes.

Preparation and Inoculation of the Entomopathogen in Treated PDA+E Medium. Spores of isolates were suspended in test tubes containing 10 ml of sterile distilled water with Tween® 20 (0.02%), and were stirred mechanically to separate the conidia. The standard was set in 2.5 x 10^7 conidia/ml. Quotes of 0.1 ml of the suspension were distributed in each 100 mm petri dish with PDA culture medium. The dishes were kept in growth chamber for two days, until conidia germination. After the incubation period, discs with 4 mm in diameter were cut from the culture medium containing micelia. The side of the dish containing the growing micelia was put in contact with the solidified PDA+S medium with concentrations of neem emulsible oil, and of aqueous seed and leaf extracts.

Vegetative Fungal Growth. Three days after incubation, 35 colonies per trial were randomly selected and measured with a caliper in two transverse directions, subtracting 4 mm, to determine the mean diameter of the colonies. The mean diameter of the colonies was obtained six days after incubation, using the same procedures.

Evaluation of Conidial Production. On the sixth day of incubation, a 4 mm-in-diameter disc was cut from the edge of each colony, to quantify the conidia. Each disc was placed in a test tube containing 10 ml of sterile distilled water with Tween 20 (0.02%). The discs were stirred for 30 seconds in vortex, to extract conidia from the medium surface. A suspension quote of each test tube was pipeted into Neubauer chamber and the number of conidia/ml was counted.

Evaluation of Conidial Viability. Suspensions for counting conidia in the treated media were diluted in a concentration of approximately 10^6 conidia/ml. Suspensions of the conidia produced in each of the evaluated concentrations were sprayed for one second on three microscopic glass slides containing an agar-water layer. There were five replications per trial. The material was incubated for approximately 20h in growth chamber. After this period, the viability of conidia was determined by counting the germinated spores with an optical microscope (400x).

Compatibility Calculations. Compatibility was calculated by using the formula proposed by Alves *et al.* (1998) to classify chemical products according to their toxicity to entomopathogenic fungi *in vitro*. This classification is based on calculations of the T factor, which relate vegetative growth (VG) and sporulation values (conidiogenesis) (SP) to the control (%): T = [20 (VG) + 80 (SP)]/100. T values between 0 and 30 classify products as very toxic; from 31 to 45 as toxic; from 46 to 60, moderately toxic; and above 60, products are considered compatible with the fungus being studied.

Statistical Analysis. The experimental design for all trials was completely randomized. The data were submitted to ANOVA and the mean values were compared by using the Tukey test ($P \leq 0.05$).

Results and Discussion

Emulsible neem oil, in all tested concentrations, reduced vegetative growth of colonies and conidiogenesis of *B. bassiana* significantly, as compared with the control. At 1% and 1.5%, the emulsible oil also reduced significantly the viability of spores produced by the colonies ($P \leq 0.05$). In general, the effect of neem oil on all parameters depended on concentrations, even though the differences were not always significant (Table 1).

Concentrations of emulsible neem oil below 5% do not cause significant fungitoxicity effects, according to Rodriguez-Lagunes *et al*. (1997) and E.D. Quintela & P.V. Pinheiro (unpublished). The difference between the results reported in those studies and the results observed in this work may be due to the variability in the amount de triterpenoids and other compounds in neem seeds, which are used for the formulation of commercial products (Sidhu *et al*. 2004), or to emulsifiers and stabilizers used in manufacturing. However, negative effects caused by emulsible neem oil, to the entomopathogen, are reported by Bajan *et al.* (1998) and Hirose *et al*. (2001).

The action mechanism of neem by-products on vegetative growth and reproduction of fungi, is still unknown (Locke 1995). However, phytoalexines, sulfurade compounds, and triterpenoids in these products have fungitoxic action (Singh *et al.* 1984, Bandopadhyay 2002).

The colonies in culture media containing seed aqueous extract had their vegetative growth and conidiogenesis significantly reduced as compared with the control trial. The concentration of seed aqueous extract at 1% was enough to cause significant inhibition of micelia growth and conidiogenesis of *B. bassiana,* with greater reductions among the highest concentrations. Incorporation of the seed aqueous extract to the culture medium, however, did not affect the viability of spores produced in none of the concentrations (Table 2). Rodriguez-Lagunes *et al*. (1997) observed no significant inhibition in vegetative growth and germination of spores of *B. bassiana* due to aqueous seed extract at 5%. Variation in concentration of components with possible fungitoxic activity in neem seeds (Sidhu *et al*. 2004) might explain the smaller negative effect of the seed aqueous extract used by these authors on the fungus, considering that other studies have shown the difference in concentration of azadirachtin in seeds of different origins (Ermel *et al.* 1984, Devaranavadagi *et al.* 2003).

A significant inhibition of the vegetative growth of *B.*

Table 1. Colony diameter (average ± SE), average amount of conidia and viability percent, of *B. bassiana* on PDA+S amended with different concentrations of neem emulsible oil, at 25 ± 1ºC and photophase of 12h.

Neem emulsible oil (%)	Colony diameter (n = 35)				Conidia amount (n = 35)		Conidia viability (n = 15)	
	3 days		6 days		(x 10^5/ml)	reduction (%)	(%)	reduction (%)
	cm	reduction (%)	cm	reduction (%)				
0	1.33 ± 0.02 a	0.0	2.09 ± 0.07 a	0.0	49.2 ± 4.33 a	0.0	91.5 ± 0.87 a	0.0
0.5	0.75 ± 0.01 b	43.6	1.05 ± 0.01 b	49.8	24.7 ± 1.61 b	49.8	84.8 ± 1.93 ab	7.3
1	0.77 ± 0.01 b	42.1	0.98 ± 0.01 b	53.1	16.6 ± 1.01 bc	66.3	84.3 ± 1.91 b	7.9
1.5	0.74 ± 0.01 b	44.4	0.94 ± 0.02 b	55.0	10.5 ± 0.65 c	78.7	80.2 ± 2.49 b	12.4

Averages followed by the same letter on column are not different from each other by Tukey test (P ≤ 0.05).

Table 2. Colonies diameter (average ± SE), average amount of conidia and viability percent, of *B. bassiana* on PDA+S amended with different concentrations of neem seed aqueous extract, at 25 ± 1ºC and photophase of 12h.

Neem seed aqueous extract (%)	Colony diameter (n = 35)				Conidia amount (n = 35)		Conidia viability (n = 15)	
	3 days		6 days		(x 10^5/ml)	reduction (%)	$(\%)^{n.s.}$	reduction (%)
	cm	reduction (%)	cm	reduction (%)				
0	1.71 ± 0.01 a	0.0	3.25 ± 0.03 a	0.0	48.2 ± 2.65 a	0.0	90.5 ± 1.11	0.0
1	1.45 ± 0.02 b	15.2	2.93 ± 0.02 b	9.8	38.5 ± 2.20 b	20.1	90.4 ± 1.07	0.1
2	1.34 ± 0.03 c	21.6	2.85 ± 0.03 b	12.3	34.3 ± 2.16 bc	28.8	90.3 ± 1.13	0.2
4	1.24 ± 0.02 d	27.5	2.57 ± 0.03 c	20.9	28.2 ± 2.24 c	41.5	90.3 ± 1.61	0.2

Averages followed by the same letter on column are not different from each other by Tukey test (P ≤ 0.05); $^{n.s.}$non-significant.

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Table 3. Colonies diameter (average ± SE), average amount of conidia and viability percent, of *B. bassiana* on PDA+S amended with different concentrations of neem leaf aqueous extract, at 25 ± 1ºC and photophase of 12h.

Neem leaf aquous extract (%)	Colony diameter (n = 35)				Conidia amount (n = 35)		Conidia viability (n = 15)	
	3 days		6 days		(x 10^5/ml)	reduction (%)	(%)	reduction (%)
	cm	reduction (%)	cm	reduction (%)				
0	1.68 ± 0.01 a	0.0	3.24 ± 0.02 a	0.0	52.3 ± 2.20 a	0.0	92.0 ± 0.47 a	0.0
0.15	1.65 ± 0.01 ab	1.8	3.23 ± 0.02 a	0.3	52.0 ± 2.00 a	0.5	92.0 ± 0.37 a	0.0
1.5	1.59 ± 0.01 b	4.8	3.22 ± 0.01 a	0.3	51.3 ± 1.50 a	1.8	90.8 ± 0.47 a	1.3
15	1.15 ± 0.03 c	31.5	2.56 ± 0.03 b	21.0	38.0 ± 2.20 b	27.3	84.9 ± 1.38 b	7.7

Averages followed by the same letter on column are not different from each other by Tukey test (P ≤ 0.05).

bassiana colonies with neem leaf aqueous extract at 1.5% e 15% was observed after three days. However, the vegetative development of colonies on culture media in all concentrations recovered between the third and the sixth day after inoculation (Table 3). Recovery may result from the metabolization of toxic components of the neem leaf extract, by the fungus, as it occurs among some chemical products (Alves *et al.* 1998). Recovery may also result from processes of oxidation and extract decomposition. Recovery of vegetative growth also occurred in colonies in media with seed aqueous extract, between the third and the sixth days after inoculation (Table 2). This process, however, did not occur in colonies in media with emulsible oil (Table 1), possibly due to the greater stability and persistence of the commercial product, as compared with vegetable extracts. The viability of spores was significantly reduced by the leaf aqueous extract at 15%, compared with the control trial. Similarly, conidiogenesis was significantly inhibited by the leaf aqueous extract at 15%, until the sixth day of incubation (Table 3). Castiglioni *et al.* (2003) reported significant inhibiting effects on vegetative growth, conidiogenesis, and germination of *B. bassiana* spores, caused by the commercial formulation of neem leaves, in concentrations that are equal and greater than 5% i.a.

The leaf aqueous extract was, in general, less toxic to *B. bassiana* than the seed aqueous extract or the emulsible oil. The leaf extract at 1.5% did not inhibit vegetative growth six days after fungus inoculation, and did not reduce conidiogenesis or the viability of spores (Table 3). On the other hand, the emulsible oil at the same concentration, inhibited vegetative growth in more than 50% and conidiogenesis in almost 80%, and reduced the viability of spores (Table 1). The seed aqueous extract at 2%, although showing a smaller negative effect on the fungus as compared to the emulsible oil, was more toxic to the fungus than the leaf aqueous extract; also, even though it did not reduce the viability of spores, it reduced in almost 30% the number of spores produced, when compared to the control trial.

According to results presented in the classification table for plant protection products and toxicity for *B. bassiana* (Table 4), the emulsible neem oil in the tested concentrations was not compatible with the isolate, and may be toxic to other isolates of *B. bassiana* used to control *H. hampei*. In colonies submitted to seed and leaf aqueous extracts, however, these products were compatible with the fungus in all concentrations. The emulsible oil was more toxic to *B. bassiana* than the seed and leaf aqueous extracts. At the concentration 0.5%, the emulsible oil caused greater reduction in vegetative growth and production and viability of spores than the botanical extracts, even when these were in their highest concentrations.

The formula developed by Alves *et al.* (1998) represents the toxic effect of plant protection products on entomopathogenic fungi *in vitro*. Laboratory compatibility tests have the advantage of exposing the pathogen to the maximum activity possible of chemical products, a situation that does not occur under field conditions. Therefore, when a treatment is compatible *in vitro*, there is a strong evidence of its selectivity under field conditions. However, a high toxicity *in vitro* does not mean that the product will always be toxic for that pathogen in the field (Alves *et al.* 1998). In this situation, inhibition of vegetative growth might be a less representative indication of fungitoxicity than the viability of spores or the effect on germination (Loria *et al.* 1983). Consequently, because the commercial formula does not take into account the effect of treatment on spore viability, research results suggest precaution when using neem emulsible oil in environments where *B. bassiana* affects *H. hamperi* mortality significantly.

Table 4. Neem emulsible oil and seed and leaf aqueous extracts compatibility classification with *B. bassiana* (T value, calculated from formula proposed by Alves *et al* 1998).

Treatments	T	Classification
Neem emulsible oil		
0.5%	50.02	Moderately toxic
1%	36.34	Toxic
1.5%	26.04	Very toxic
Neem seed aqueous extract		
1%	81.96	Compatible
2%	74.51	Compatible
4%	62.62	Compatible
Neem leaf aqueous extract		
0.15%	99.54	Compatible
1.5%	98.5	Compatible
15%	73.96	Compatible

Under field conditions, compatibility between the plant protection product and germination is necessary because insects become infected by means of spore germination, by ingestion or contact (Malo 1993). Hirose *et al.* (2001) observed 45% reduction in spore germination of *B. bassiana* when mixed with neem oil at 2%. Therefore, the mixture of aqueous oil emulsion and spores of the pest-controlling entomopathogen, must be avoided.

So, when the emulsible neem oil or neem extracts are used to control the coffee berry borer in areas where the occurrence of *B. bassiana* is significant, compatible formulations should be preferred in order to not impair the entomopathogen action. Because under field conditions environmental factors decrease the impact of toxic components on the fungus, formulations considered compatible in tests *in vitro* may be safe for the fungus.

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