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Population density of *Beauveria bassiana* in soil under the action of fungicides and native microbial populations

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ABSTRACT. This study investigated whether populations of naturally-occurring soil bacteria, fungi and actinomycetes influence the effect of fungicides on the survival and growth of *Beauveria bassiana*. The toxicity of methyl thiophanate, pyraclostrobin, mancozeb and copper oxychloride at the recommended doses was analyzed in culture medium and in soil inoculated with fungus at various time points after addition of fungicides. All fungicides completely inhibited the growth and sporulation of *B. bassiana* in the culture medium. The fungicides were less toxic in soil, emphasizing the action of the microbial populations, which interfered with the toxic effects of these products to the fungus. Actinomycetes had the greatest influence on the entomopathogen, inhibiting it or degrading the fungicides to contribute to the survival and growth of *B. bassiana* in soil. Native populations of fungi and bacteria had a smaller influence on the population density of *B. bassiana* and the action of fungicides towards entomopathogen. The toxic effect of the fungicides was greater when added to the soil one hour before or after inoculation than at 48h after inoculation.

Keywords: biological control, entomopathogenic fungus, soil microorganisms, compatibility, toxicity.

Densidade populacional de *Beauveria bassiana* no solo sob a ação de fungicidas e de populações microbianas nativas

RESUMO. Este estudo investigou se as populações nativas de bactérias, fungos e actinomicetos do solo influenciam a ação de fungicidas na sobrevivência e crescimento de *Beauveria bassiana*. A toxicidade de tiofanato-metil, piraclostrobina, mancozeb e oxicloreto de cobre usados nas doses recomendadas pelos fabricantes, foi analisada no meio de cultura e no solo inoculado com o fungo após diferentes períodos de adição dos fungicidas. Todos os fungicidas inibiram completamente o crescimento e esporulação de *B. bassiana* no meio de cultura. No solo, os fungicidas foram menos tóxicos, enfatizando-se a ação das populações microbianas que interferiram na toxicidade destes produtos para o fungo. Os actinomicetos mostraram a maior influência; inibiram *B. bassiana* ou degradaram os fungicidas, contribuindo para a sobrevivência e crescimento do fungo no solo. As populações nativas de fungos e bactérias tiveram menor influência sobre a densidade populacional de *B. bassiana* e na ação dos fungicidas para o entomopatógeno. A toxicidade dos fungicidas foi maior quando adicionados ao solo uma hora antes ou após a inoculação do fungo, do que 48h após.

Palavras-chave: controle biológico, fungo entomopatogênico, micro-organismos do solo, compatibilidade, toxicidade.

Introduction

Chemical pesticides can be a problem for integrated pest management (IPM) because they can get contact with non-target organisms in soil, such as entomopathogenic fungi (Mochi, Monteiro, & Barbosa, 2005). The pesticides affect these organisms in various ways (Jaros-Su, Groden, & Zhang, 1999). Tests of the compatibility of pesticides and beneficial fungi are essential for developing successful IPM strategies (Jaros-Su et al., 1999). Most tests are conducted by cultivating the fungi in synthetic culture medium containing these products; however this method does not reflect the conditions in the soil where contact between the two control agents occurs (Mochi et al., 2005).

The survival of entomopathogenic fungi in natural environments is influenced by biotic and abiotic factors. The main biotic factors are reservoirs of invertebrates and natural and artificial antagonists (McCoy, Quintela, & Faria, 2002). Natural antagonists are the native microbial populations of the soil and are one of the most active factors in determining the persistence of fungi in these environments (Jaronski, 2010).

In agricultural ecosystems, pesticides reach the soil directly or indirectly through rainwater and

interact with native microbial populations to inhibit the survival and growth of microorganism populations exposed to chemical products (Ampofo, Tetteh, & Belo, 2009) or suffering degradation (Arbeli & Fuentes, 2007). During microbial degradation, the chemical compound is used as a source of carbon by the soil microbial populations to fuel considerable population growth (Bhuyan, Sreedharan, Adhya, & Sethunathan, 1993). This, in turn, can influence the survival of soil microorganisms such as fungi through fungistasis (Lanza, Monteiro, & Malheiros, 2004). The microbial degradation of chemical products benefits soil fungal populations by transforming chemicals into less-toxic byproducts (Lopes, Batista, Batista, Mitidieri, Bataus, & Fernandes, 2010).

In addition to action against native microbial populations indirectly affect entomopathogenic soil fungi, fungicides can act directly on these natural enemies of pests by inhibiting their development and reproduction, with negative effects on the IPM (Malo, 1993). Due to its cosmopolitan distribution and frequent presence in nature, *B. bassiana* is one of the most important and abundant entomopathogenic fungi (Rehner, 2005). The effect of fungicides on fungi used for biological control has been poorly explored in soil mainly under the effect of microbial populations. Thus, this study investigated whether the populations of naturally-occurring soil bacteria, fungi and actinomycetes influence the action of fungicides applied in various ways on the survival and growth of *B. bassiana*.

Material and methods

Test fungal culture and fungicides

The *B. bassiana* isolate JAB 06 (GenBank - accession No. KX599544 - ITS1-5.8S-ITS2 region) from the collection of the Microbiology Laboratory of the Department of Plant Production in the Faculty of Agrarian and Veterinary Sciences, São Paulo State University, was used for all tests. The fungus was cultivated in a medium of potato, dextrose, and agar (PDA) and kept in an incubator at 27 ± 1ºC in the dark for 25 days.

Information on active ingredients, chemical groups, recommended dose of commercial product (c. p.), and spraying volume of the fungicides is shown in Table 1. The concentrations of products in spraying liquids were: a) thiophanate-methyl: 2.5 mg of c. p. mL^{-1}; b) pyraclostrobin: 0.00015 mL of c. p. mL^{-1}; c) mancozeb: 2 mg of c. p. mL^{-1}; and d) copper oxychloride: 2.5 mg of c. p. mL^{-1}.

Experiment with culture medium

The amount of fungicide (per milliliter of culture medium) needed to obtain a concentration equivalent to the liquid used for spraying was calculated and added to the PDA medium at temperatures ranging from 45 to 50°C to avoid possible alterations of the properties of the fungicide. After pouring the medium into Petri dishes, each dish was inoculated in the center by pricking with a platinum needle dipped in a suspension containing 10^7 conidia mL^{-1} obtained from 15-days-old colonies of the fungus.

The vegetative growth, sporulation, and germination of conidia were used as parameters to evaluate the performance of the fungus after treatment with a fungicide. The growth of the colonies was quantified by measuring, in millimeters, three equidistant diameters previously marked on the bottom outside of the Petri dish. The measurements were taken every three days from day three to fifteen after inoculation. Each dish corresponded to a repetition, and each treatment was replicated five times.

On day 15, sporulation was assessed by collecting three samples per dish (one each from the center, middle and edge of each colony) with the aid of a sterilized metallic 8-mm diameter ring. Three replicate colonies were used for sample collection. Each sample was individually transferred to a test tube containing 10 mL of a 1:1 sterilized solution of NaCl (0.89% w v^{-1}) and Tween 80® (0.1% v v^{-1}) and vigorously agitated in an electric tube agitator. Conidia were counted with an optical microscope using a Neubauer counting chamber, and the suspension was diluted when necessary.

Table 1. Fungicides[a] used in this study with the entomopathogenic fungus *Beauveria bassiana* isolate JAB 06.

Active ingredient	Chemical group	Recommended dose	Spray volume
Thiophanate-methyl	Benzimidazoles	250 g of c.p. 100 liters^{-1} of water	1000 liters ha^{-1}
Pyraclostrobin	Strobilurins	15 ml of c.p. 100 liters^{-1} of water	2000 liters ha^{-1}
Mancozeb	Dithiocarbamates	200 g of c.p. 100 liters^{-1} of water	400 liters ha^{-1}
Copper oxychloride	Cuprics	250 g of c.p. 100 liters^{-1} of water	1000 liters ha^{-1}

[a]Information source: Agrofit (2010); c.p.: commercial product.

Germination was assessed by direct examination of conidia on a microscope slide. After demarcation of three areas, a sterile microscope slide was covered with a thin layer of PDA medium containing fungicides at the recommended doses. A drop (approximately 0.05 mL) of the fungal suspension with 10^5 conidia mL^{-1} was placed in the region of the culture medium corresponding to each of these areas. After incubation for 15h at 27 ± 1°C in the dark, the germination process was stopped with a drop of dye in each area. A dye stock solution was prepared by adding 1 g of methylene blue in 20 mL of lactic acid. The working dye was composed of 1 mL of the stock solution added to 29 mL of lactic acid. A total of 150 conidia were observed in each slide area to establish the percentage of germinated conidia. Three replicate slides were used for each treatment, and the replicates were inoculated with suspensions obtained from different 15 day-old colonies.

A single trial was organized using a completely randomized design. The treatments consisted of media containing fungicides and fungicide-free PDA medium was used as a control. Variance was assessed using the F-test, and mean values were compared using Tukey's test ($p \leq 0.05$). AgroEstat (Barbosa & Maldonado, 2010) software was used for statistical analysis.

To determine the toxic effect of the fungicides, we used the formula described by Rossi-Zalaf, Alves, Lopes, Siqueira Neto, and Tanzini (2008), including parameters for vegetative growth, sporulation, and germination:

$$BI = 47 (VG) + 43 (SP) + 10 (GER) \div 100$$

where: BI = is the Biological Index; VG = is the percentage of vegetative growth of the colony after 15 days, relative to the control; SP = is the percentage of sporulation after 15 days, relative to the control; and GER = is the percentage of germination of the conidia after 15 hours, relative to the control. No decimal places were used in the calculation of BI.

Using the obtained BI values we classified the fungicides according to their level of toxicity using the following scale: 0 to 41 = toxic, 42 to 66 = moderately toxic, and > 66 = compatible (Rossi-Zalaf et al., 2008).

Soil experiment

The toxic effect of the fungicides on *B. bassiana* was also evaluated in soil. A Typic Eutrustox soil composed of 53% clay, 18% silt, and 29% sand was collected at a depth of 0 to 20 cm from an environmental preservation area belonging to the Faculty of Agrarian and Veterinary Sciences. The soil was dried at room temperature, loosened, and sifted through a 1-mm mesh sieve. For each fungicide test, an assay was carried out, for which a new sample of soil was collected. The water saturation capacity of the soil was determined before each trial was conducted.

We used 100-mm Petri dishes containing 80 g of non-autoclaved soil. To provide a gap for gas exchange, the space between the two halves of the dishes was increased by attaching two wooden sticks inside the upper plate. In an aseptic chamber, sterile distilled water was added to the soil until it reached 65% of its saturation capacity, and samples were left to rest for one hour for stabilization.

Fungicide was added in accordance with the volume and concentration of spraying liquid used in the field. Based on the manufacturer's recommendation, we calculated the amount of liquid used for spraying (in milliliters) to be deposited per cm^2 of soil, assuming a direct and homogeneous distribution of fungicide in the field. We calculated the soil surface area in the Petri dish and a proportional volume of liquid used for spraying to be applied in this area. A fungicidal solution with the same concentration of the liquid used for spraying in the field was prepared. The appropriate volume for the Petri dish area was carefully distributed (droplets of approximately 0.05 mL) on the soil surface with the aid of a micropipette, and effort was taken to cover the soil surface homogeneously. Fungicide was applied at the following time points: a) one hour before inoculation of fungus; b) one hour after inoculation of fungus; and c) 48 hours after of inoculation of fungus. Inoculation with a micropipette was performed by distributing 2 mL of fungal suspension containing 1.0×10^7 conidia mL^{-1} onto the entire surface of the soil in the most homogeneous manner possible.

Dishes with soil remained in the incubator at 27 ± 1°C in the dark for 28 days. A tray with water was kept in the incubator to minimize water loss. Dishes were checked for water loss weekly, and sterile distilled water was added to replace water loss by evaporation. The control treatment consisted of soil inoculated with the fungal suspension without the addition of fungicide.

For evaluation of fungal survival, colony-forming units (CFUs) were counted after 0, 7, 14, 21, and 28 days of incubation. In each dish, a 1-g sample of damp soil was collected from 12 to 15 points on the surface of the soil and suspended in 9 mL of a 0.1% (v v^{-1}) solution of Tween 80®. Serial dilutions were performed from the initial suspension, and these dilutions were used to seed 0.1 mL in Petri dishes that contained culture media to determine the populations of *B. bassiana* (Joussier & Catroux 1976, with the exclusion of vegetable

juice and oxygall), bacteria (Bunt & Rovira, 1955), fungi (Martin, 1950), and actinomycetes (Hsu & Lockwood, 1973).

For each fungicide, a trial was performed using a completely randomized design with four repetitions per treatment. The data were subjected to an analysis of variance using an F-test to examine a) growth of each microorganism over the course of the evaluation period in a single treatment and b) growth of *B. bassiana* among the treatments. The averages were compared using a Tukey's test at the 5% probability level. The experiment was analyzed according to a split-plot-in-time design, considering the effects of treatment (1 hour before inoculation, 1 hour after inoculation, and 48 hours after inoculation) and time period (0, 7, 14, 21, and 28 days of incubation) and the treatment *versus* time period interaction. The Pearson coefficients of correlation were determined at a 5% probability level to verify interactions between populations of bacteria, fungi, and actinomycetes with population of *B. bassiana*. AgroEstat software (Barbosa & Maldonado, 2010) was used for the statistical analyses.

Results and discussion

Experiment with culture medium

No vegetative growth or sporulation of *B. bassiana* on media containing the recommended doses of the tested fungicides was observed. With the exception of mancozeb, germination of conidia was not significantly different among the tested fungicides and the control. In spite of these results, all fungicides were rated as toxic to *B. bassiana* based on the calculated BI (Table 2). A similar result was obtained by Loureiro, Moino, Arnosti, and Souza (2002) who found that fungicides containing thiophanate-methyl and mancozeb as active ingredients were also classified as very toxic to the CB 66 isolate of *B. bassiana*.

Table 2. Percent germination of *Beauveria bassiana* conidia cultivated on media containing fungicides at the recommended doses, Biological Index (BI) and fungal toxicity rating of the fungicides.

Fungicide	Germination	BI	Toxicological classification
Control	97.07±1.48 a		
Copper oxychloride	93.95±0.66 a	9	Toxic
Thiophanate-methyl	93.69±0.82 a	9	Toxic
Pyraclostrobin	74.26±27.41 ab	7	Toxic
Mancozeb	50.10± 6.18 b	3	Toxic
F-test	8.37**		
C.V. (%)	12.77		

Non-transformed data reported in the table, but analyses were performed after arc-sine-transformation. Means (± standard deviation) followed by the same lower-case letter are not significantly different (Tukey test with 5% probability). C.V. = coefficient of variation. **Significant at a 1% probability level according to a Tukey's test.

The germination of conidia requires a source of carbon as an extracellular signal for translation to occur which then triggers germination (Osherov & May, 2000). This process can explain the utilization of endogenous sources of carbon and nitrogen, without the use of exogenous sources present in the culture medium (Lefevbre, 1931). Thus, the germination of *B. bassiana* conidia would have occurred before the fungus contacted the fungicides added to the medium, and thus the fungicides could not have inhibited conidia germination. However, for vegetative growth and sporulation hyphal extension must occur after germination. At this point, the fungus requires exogenous sources of carbon and nitrogen from the culture medium, and must therefore come into contact with fungicide which inhibits further growth.

These results are important for IPM because they show that fungicides, although toxic to *B. bassiana*, do not completely inhibit the germination of conidia, which is the event that starts penetration of a fungus into the host (Alves & Lecuona, 1998). In addition, the toxicity of fungicides in soil environment can be smaller due to the influence of native microbial populations.

Soil experiments

Thiophanate-methyl

In soil added one hour after inoculation with *B. bassiana*, we observed a linear and directly proportional correlation ($r = 0.78532$, $p \leq 0.01$) between the growth of the entomopathogen population and that of other fungi (Table 3), suggesting that the growth of these populations may be linked (Figure 1A). Results obtained by Draganova, Donkova, and Georgieva (2008) showed that lineage 412 of *B. bassiana* stimulated the growth of soil fungi 30-fold above the control value. Because these microorganisms are heterotrophic, there may be a synergistic interaction between these populations (Draganova et al., 2008). This interaction is important for the degradation of organic soil composts because degradation takes place in stages and includes the action of many physiologically different microorganisms (Stamford, Rodrigues, Heck, & Andrade, 2005).

In joint degradation of organic composts, both populations benefit by taking advantage of nutrients and show similar growth patterns. In this treatment (Figure 1A), the correlation between populations of actinomycetes and *B. bassiana* was inversely proportional ($r = -0.57798$, $p \leq 0.05$) indicating that the increase in the population of actinomycetes is related to the reduction in the population of the fungus (Table 3). The growth of actinomycetes, starting on the seventh day of evaluation, may

generate a competitive relationship with *B. bassiana* for nutrients and space, resulting in inhibition of the fungus. Another explanation would be amensalism; actinomycetes may release microbiostatic compounds that cause fungistasis (Stamford et al., 2005). Autoclaved soils that are re-infested with actinomycetes have been shown to contain volatile fungistatic substances inhibit the germination of spores of *Trichoderma viride* Pers., *Zygorhynchus vuileminii* Namyslowski, and *Gonatobotrys simplex* Corda (Hora & Baker, 1972).

A similar pattern of *B. bassiana* inhibition by actinomycetes was also observed in treatments (where) in which the fungicide was added to the soil one hour before ($r = -0.49914$, $p \leq 0.05$) (Figure 1B) or 48h after ($r = -0.51566$, $p \leq 0.05$) (Figure 1C) inoculation with the entomopathogen (Table 3). As described above, inhibition can be attributed to the release of compounds with inibitory effects by the population of actinomycete bacteria. Actinomycetes that release odors often described as earthy smell may be a productive source of inhibitory volatile compounds that are responsible for fungistasis (Hora & Baker, 1970).

Table 3. Correlations between *Beauveria bassiana* population and populations of bacteria, fungi and actinomycetes obtained for treatments with different fungicides.

Treatment	Pearson coefficients of correlation (r)[a]		
	Bacteria	Fungi	Actinomycetes
Thiophanate-methyl	-0.17005^{NS}	0.91740**	-0.49914*
Addition 1 hour BIBb	-0.29284^{NS}	0.78532**	-0.57798**
Addition 1 hour AIBb	-0.32250^{NS}	0.88365**	-0.51566*
Addition 48 hours AIBb	-0.55585*	0.82688**	-0.40840^{NS}
Control			
Pyraclostrobin	0.21512^{NS}	-0.65064**	0.58913**
Addition 1 hour BIBb	0.27867^{NS}	-0.17031^{NS}	0.32915^{NS}
Addition 1 hour AIBb	0.04262^{NS}	-0.58207**	0.61853**
Addition 48 hours AIBb	0.22528^{NS}	-0.75968**	0.35766^{NS}
Control			
Mancozeb	0.05629^{NS}	0.06766^{NS}	-0.48714*
Addition 1 hour BIBb	0.08069^{NS}	0.19538^{NS}	0.28576^{NS}
Addition 1 hour AIBb	0.34106^{NS}	0.19314^{NS}	0.16840^{NS}
Addition 48 hours AIBb	0.18944^{NS}	0.58354**	-0.41728^{NS}
Control			
Copper oxychloride	0.69148**	0.84943**	-0.46717*
Addition 1 hour BIBb	0.01847^{NS}	0.91818**	-0.37650^{NS}
Addition 1 hour AIBb	0.82772**	0.72213**	-0.22961^{NS}
Addition 48 hours AIBb	0.58644**	0.95867**	-0.36780^{NS}

[a]Positive values of correlation indicate variables correlated in the same direction; negative values indicate correlation in the opposite direction. The closer a value is to +1 or -1, the more strongly the variables are correlated. BIBb: before inoculation with *Beauveria bassiana,* AIBb: after inoculation with *Beauveria bassiana.* NS Not significant, **. * Significant at 1% and 5% probability, respectively, according to a Tukey's test.

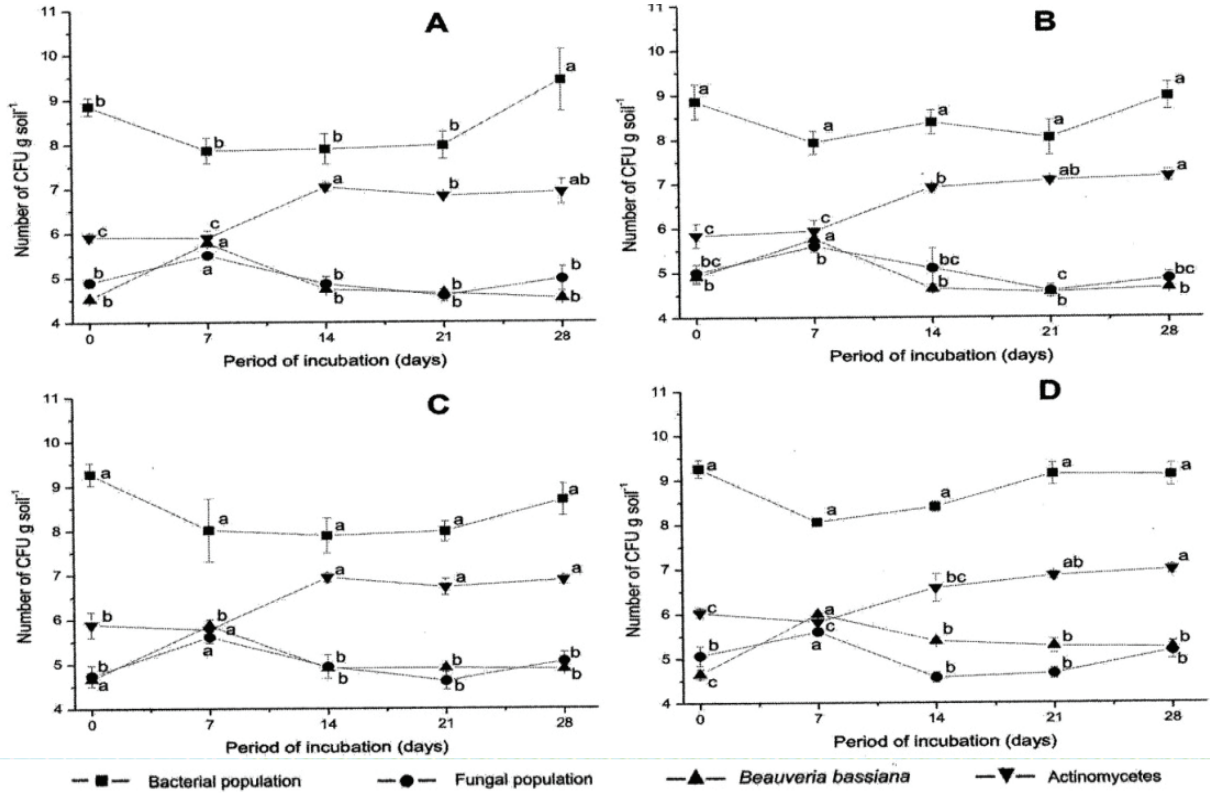


Figure 1. Interactions between *Beauveria bassiana* population and other naturally-occurring microbial populations in soil treated with fungicide containing the active ingredient thiophanate-methyl, applied A) 1 hour before inoculation with fungus, B) 1 hour after inoculation with fungus, C) 48 hours after inoculation with fungus. D is the control, to which no fungicide was added. Means (± standard deviation) with the same lower case letters for the incubation period of the same population were not significantly different (Tukey's test, 5% probability). Statistical analysis performed and graphs plotted with data transformed in log (x+5).

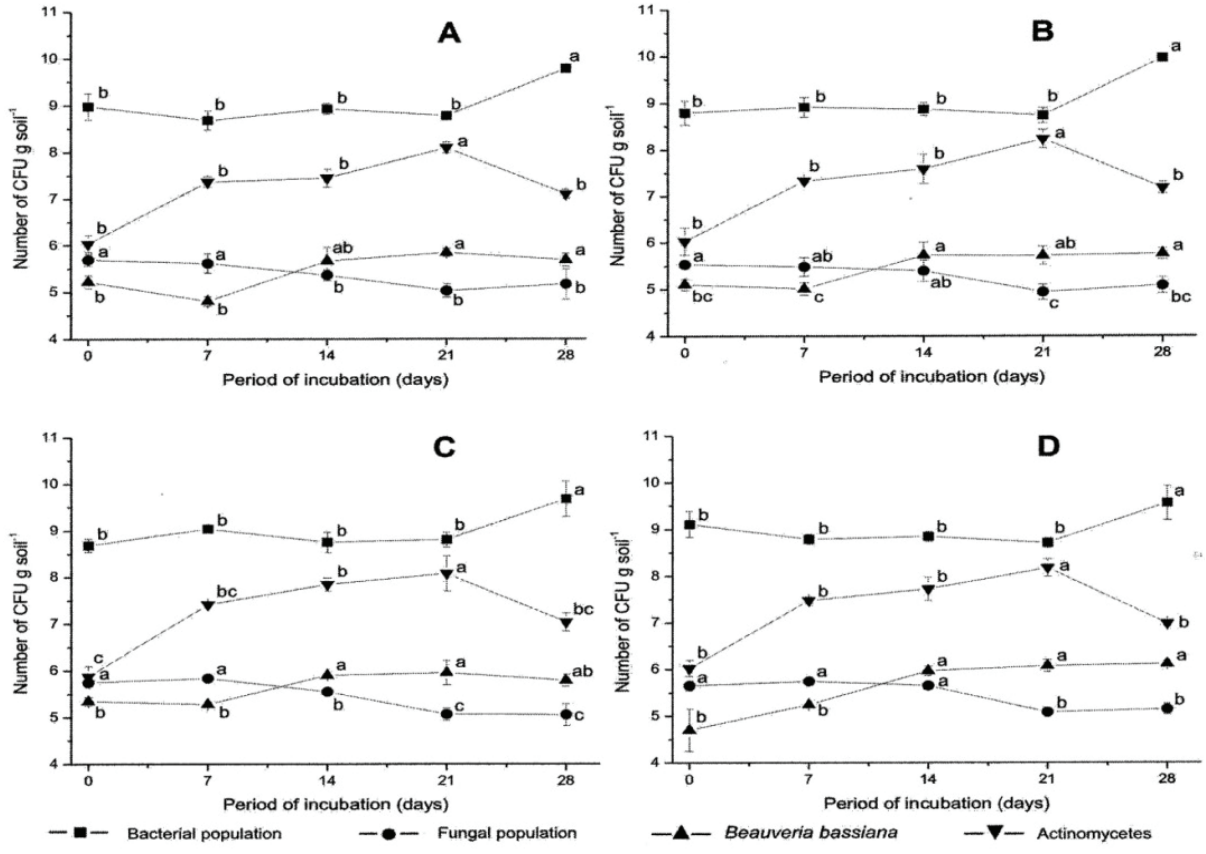
In the control, the negative correlation (r = -0.55585, p ≤ 0.05) (Table 3) between populations of bacteria and *B. bassiana* suggests that the former inhibit the latter (Figure 1D). Bacteria are known to produce substances with fungistatic and even fungicidal effects. Zou, Mo, Gu, Zhou and Zang (2007) isolated 1,018 bacteria from soil and verified that among these, 328 produced volatile compounds with a fungistatic effect that inhibited the germination and mycelial growth of two species of fungi.

Population density averages for *B. bassiana* in different treatments indicated that the fungicide containing thiophanate-methyl had little toxic effect on this population. In the treatment without fungicide, there was greater stability of the fungal population, followed by the treatment where the fungicide was added to the soil 48 h after inoculation with *B. bassiana* (F = 258.55, p ≤ 0.01). The addition of fungicide 48h after inoculation probably provided sufficient time for the fungus to germinate and form hyphae before contacting the fungicide, increasing the chances of pathogen survival.

In treatments in which the product was added to soil one hour before or after inoculation with fungus, the population densities were statistically lower than control densities. The effect of thiophanate-methyl on the population of entomopathogen may be explained by the toxicity of its conversion product methyl-benzimidazol-2-yl carbamate, which inhibits fungi during cellular mitosis (Davidse, 1973). A progressive inhibition of the synthesis of DNA, RNA, and proteins causes flaws in normal mitosis or cytokinesis (Hammerschlag & Sisler, 1972).

Pyraclostrobin

In soils where the inoculation of *B. bassiana* was performed one hour before (Figure 2B) or 48h after (Figure 2C) the addition of fungicide containing pyraclostrobin as an active ingredient, we observed a positive correlation between fungal growth and actinomycete populations (r = 0.58913 and r = 0.61853, respectively, p ≤ 0.01) (Table 3).



]Figure 2. Interactions between *Beauveria bassiana* population and other naturally-occurring microbial populations in soil treated with fungicide containing the active ingredient pyraclostrobin, applied A) 1 hour before inoculation with fungus, B) 1 hour after inoculation with fungus, C) 48 hours after inoculation with fungus. D is the control, to which no fungicide was added. Means (± standard deviation) with the same lower case letters for the incubation period of the same population were not significantly different (Tukey's test, 5% probability). Statistical analysis was performed and graphs plotted with data transformed in log (x+5).

The fungicide may (have been) be used as a source of energy by the actinomycetes, which are the most active microorganisms in the degradation of complex soil composts (Goodfellow, 1983); this would lead to an increase in their population density. The degradation of the product likely led to conversion of the active ingredient into a less-toxic byproduct and favored the growth of the entomopathogen.

The population density of *B. bassiana* was significantly lower ($F = 5.79$, $p \leq 0.05$) in treatments in which pyraclostrobin was added to soil one hour before or one hour after inoculation with fungus. In the control and the treatment in which the fungicide was applied to soil 48 h after fungal inoculation, the population densities were higher. This result suggests the existence of an effect of both the product and of the treatments on the entomopathogen. Inoculation of B. bassiana in the soil one hour before and one hour after the addition of the fungicide did not allow sufficient time for the fungus to germinate, resulting in inhibition. Pyraclostrobin completely controlled the growth of the fungus *Colletotrichum lindemuthianum* (Sacc. and Magn.), showing a fungistatic effect (Sartorato, 2006), in accordance with the results of this study. However, in the treatment in which the fungicide was applied 48 h after inoculation of the fungus, the toxic action was diminished because there was time for germination prior to contact with the chemical, reducing inhibition.

Mancozeb

In the experiment conducted with the fungicide mancozeb, the correlation between the populations of *B. bassiana* and the actinomycetes was inversely proportional ($r = -0.48714$, $p \leq 0.05$) (Table 3) in the treatment where the product was added to soil one hour before the fungus. This indicates that *B. bassiana* may have been slightly inhibited by actinomycetes, although statistical analysis of the growth of both populations (Figure 3B) did not detect this phenomenon. For the control (Figure 3D), however, there was no significant correlation between the two populations (Table 3),

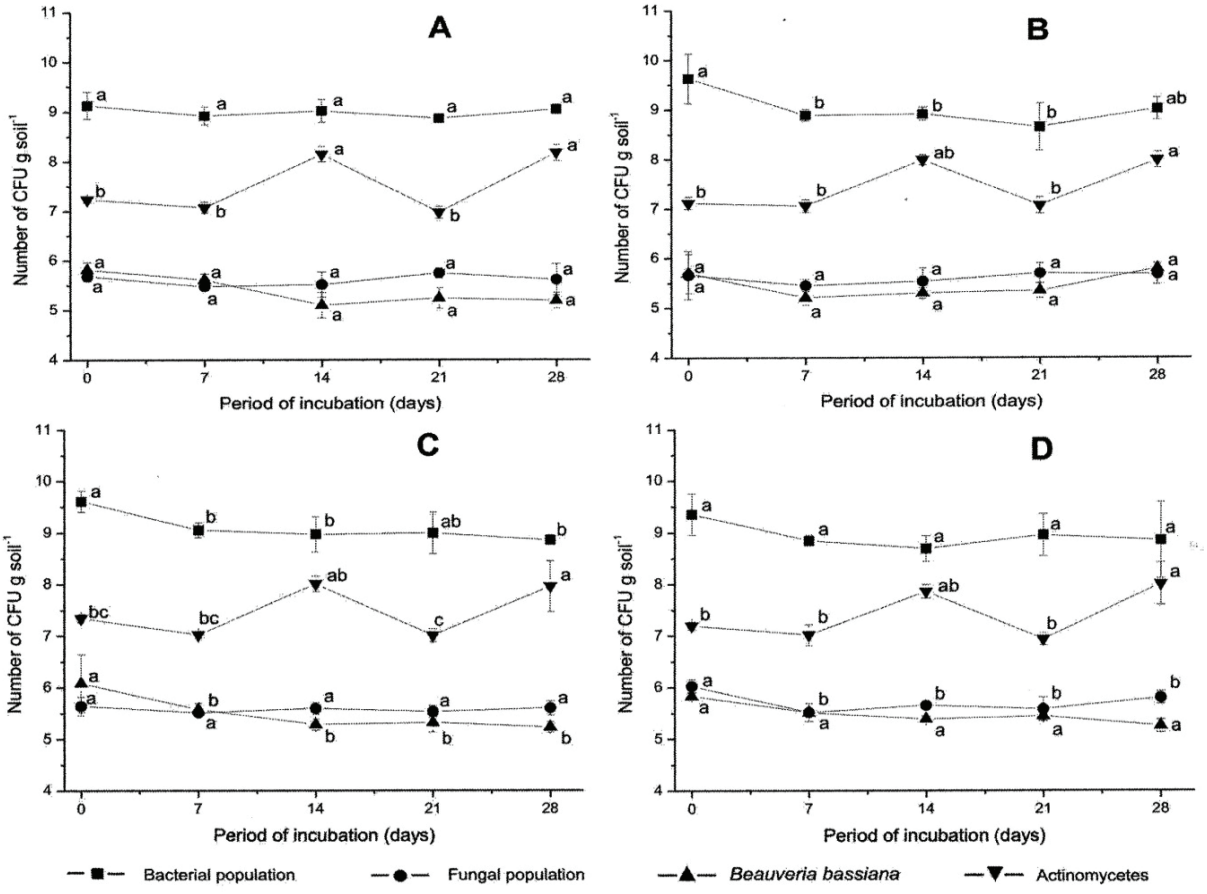


Figure 3. Interactions between *Beauveria bassiana* population and other naturally-occurring microbial populations in soil treated with fungicide containing the active ingredient mancozeb, applied A) 1 hour before inoculation with fungus, B) 1 hour after inoculation with fungus, C) 48 hours after inoculation with fungus. D is the control, to which no fungicide was added. Means (± standard deviation) with the same lower case letters for the incubation period of the same population were not significantly different (Tukey's test, 5% probability). Statistical analysis performed and graphs plotted with data transformed in log (x+5).

suggesting that inhibition of the population of *B. bassiana* may be a consequence of degradation of the fungicide by actinomycetes. In an experiment performed with several species of *Streptomyces*, 80% of the tested species inhibited fungal growth through the production of antibiotics, while the rest of the tested species prevented the fungi from acquiring nutrients (Broadbent, Baker & Waterworth, 1971).

A difference in the toxicity to *B. bassiana* was observed when experiments with mancozeb in culture medium and soil were compared. In the culture medium experiment, the fungicide was classified as toxic to the fungus, while in the soil this toxicity was not observed, as the population of *B. bassiana* in the control did not differ significantly from those in the presence of fungicide ($F = 1.27^{NS}$). This may be due to the influence of soil on the toxicity of chemical products to the microorganisms. The fungicides that had completely toxic effects on *B. bassiana* in the culture medium did not show the same effects when applied in the field (Clark, Casagrande & Wallace, 1982).

The toxic effect of a chemical product on a soil microorganism in a pure culture is different from that produced on the same organism in its natural habitat. In the pure culture, the relationship is only between the product and the organism, while in the field, the relationship is between the chemical product and the organism as protected by the characteristics of the soil (Kreutzer, 1963).

Copper oxychloride

In treatments in which copper oxychloride was added to the soil one hour before (Figure 4B) and 48h after inoculation of fungus (Figure 4C), as well as in the control (Figure 4D), the coefficients of correlation between populations of bacteria and *B. bassiana* were positive ($r = 0.69148$, $r = 0.82772$, and $r = 0.58644$, respectively, $p \leq 0.01$) (Table 3).

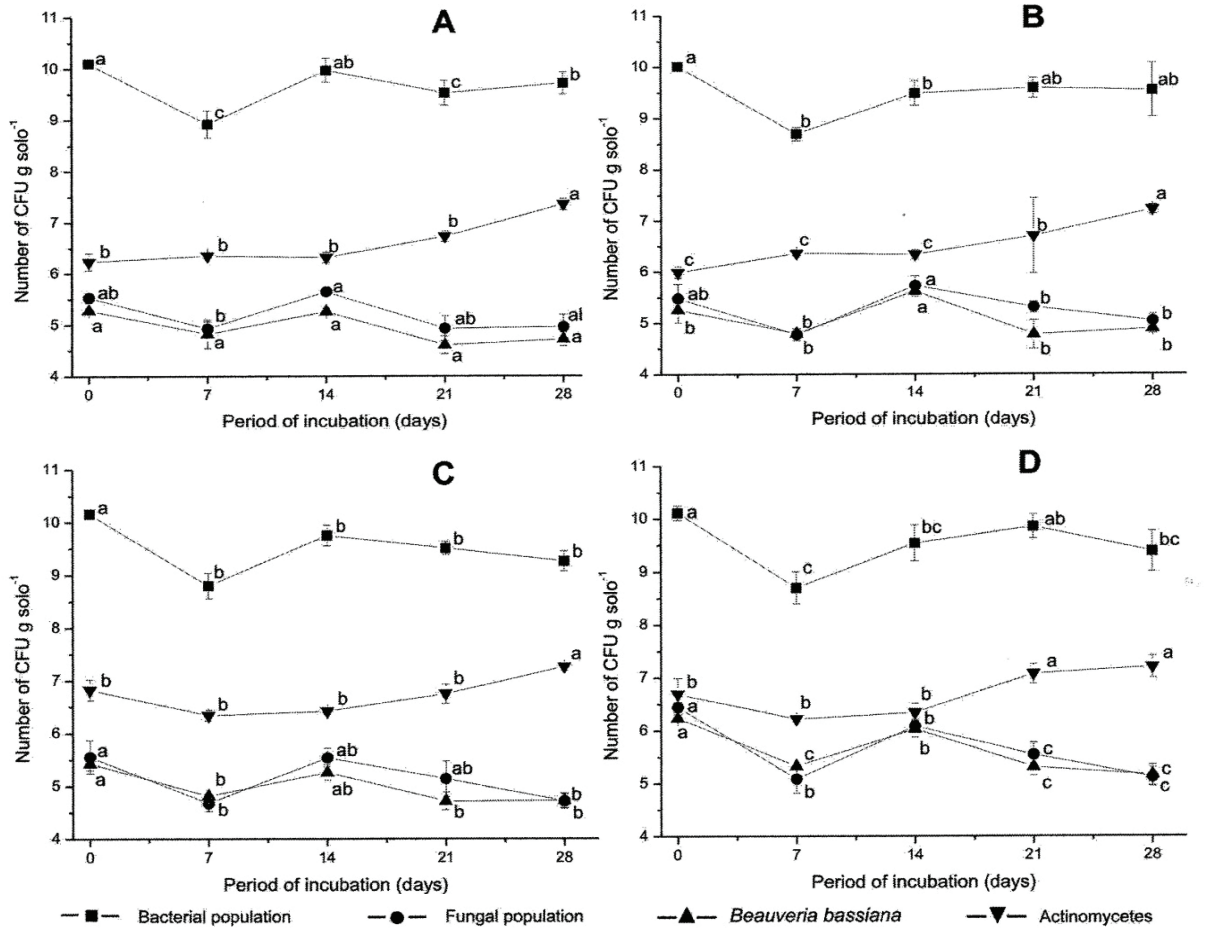


Figure 4. Interactions between *Beauveria bassiana* population and other naturally-occurring microbial populations in soil treated with fungicide containing the active ingredient copper oxychloride, applied A) 1 hour before inoculation with fungus, B) 1 hour after inoculation with fungus, C) 48 hours after inoculation with fungus. D is the control, to which no fungicide was added. Means (± standard deviation) with the same lower case letters for the incubation period of the same population were not significantly different (Tukey's test, 5% probability). Statistical analysis performed and graphs plotted with data transformed in log (x+5).

This result suggests that the development of bacterial populations in soil does not restrict the growth of *B. bassiana* and may even stimulate it, as observed in the control, where the highest population density of the fungus occurred (Figure 4D). Reports indicate that this stimulus can occur due to hormesis, a phenomenon whereby bacteria secrete fungistatic substances that, if released in small quantities, can stimulate the genes of other microorganisms and initiate their growth (Mlot, 2009). Hormesis is a complex process that can have significant implications for organisms that are beneficial for pest management (Duke, 2014).

In the treatment in wich the addition of the fungicide to soil was carried out one hour prior to inoculation of the entomopathogenic fungus (Figure 4B), actinomycetes inhibited the population of *B. bassiana* ($r = -0.46717$, $p \leq 0.05$) (Table 3). Actinomycetes are known to secrete substances that inhibit other microorganisms, such as antibiotics that can inhibit the growth and germination of fungi (Schippers, Bakker, & Bakker, 1987). This secretion may have caused the negative correlation between the two populations.

The populations of fungi and *B. bassiana* had similar growth pattern, with a positive correlation between the growth of both populations in all treatments ($r = 0.84943$, $r = 0.91818$, $r = 0.72213$, and $r = 0.95867$, $p \leq 0.01$) (Table 3). Considering the treated groups, this finding may stem from a lack of selectivity of the fungicide, which inhibited both populations. The actinomycetes grew in all soil, but this did not result in decreased fungicide toxicity for *B. bassiana* in the soil, as shown by the small reduction in entomopathogen population in the majority of the treatments.

Copper oxychloride had a toxic effect on *B. bassiana* in the soil, while in the control, the population density of the fungus was greater than in the other treatments ($F = 52$, $p \leq 0.01$). However, inoculation of the fungus in the soil prior or after fungicide application did not influence the toxic effect.

Conclusion

All fungicides evaluated in this study showed a toxic effect to *B. bassiana* in culture medium, though some variation among compounds was observed. The naturally-occurring soil microbial populations interfered with the effect of the compounds, reducing the toxicity of these fungicides to the fungus. The actinomycetes played an important role by reducing toxicity through degradation of fungicides or inhibition of *B. bassiana* by fungistasis. The population of fungi may inhibit the population of *B. bassiana* through competition for nutrients or through release of toxic metabolites. However, in some cases, the population density of both groups had a similar pattern of increase or decrease. The population of bacteria had little effect. The toxic effect of fungicides was influenced by time point of application: the effect was greater when the addition occurred one hour before or after inoculation of the fungus in the soil than 48h after inoculation.

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