Advanced Journal of Microbiology Research Vol. 2011
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Fungicide toxicity against the growth of lineages of the fungus *Metarhizium anisopliae* var. *anisopliae* (Mestch.) Sorokin

Sideney B. Onofre*, Cristiane R. Kasburg, Danusa de Freitas, Silvana Damin, Andréia Vilani, Jéssica A. Queiroz and Francini Y. Kagimura

Departamento de Ciências Biológicas, Laboratório de Microbiologia, Universidade Paranaense, UNIPAR, Unidade-Campus Francisco Beltrão, Av. Júlio Assis Cavalheiro, 2000 – Bairro Centro - 85601-010,Francisco Beltrão – Paraná – Brasil.

Accepted 24 April, 2011

In this work the fungicidal action of three agrochemicals (Sphere®, Nativo® and Bendazol®) used in soybean for control of fungal diseases on the lineages CG-28 and CG-30 of *Metarhizium anisopliae* var. *anisopliae* was evaluated. It was found that the fungicides inhibited the vegetative growth of the lineages at the concentrations indicated for the field, thereby showing its antifungal effect.

Key words: Biocontrol, crops, pesticides.

INTRODUCTION

Fungi are microorganisms most frequently found attacking insects, responsible for 80% of the epizootic surges occurring in agro-ecosystems. Among the fungi used is *Metarhizium anisopliae*, the first agent used in the microbial control described in the literature and occurring naturally in more than 300 species of insects (Alves and Lopes 2008; Fernandes et al., 2010).

In Brazil, this fungus has been found infecting several species of insects, especially *Mahanarva posticata*, *Diatraea saccharalis, Nezara viridula*, *Piezodorus guildini, Deois* sp., *Zulia* sp., *Bonagota salubricola* and *Anticarsia gemmatalis*, with a large potential for use in biological control. The major example of the successful use of *M. anisopliae* is in the biological control of leafhoppers, mainly in sugar cane and pastures. Its pathogenicity has been demonstrated in ticks of several genera and species (Schrank et al., 2001, Onofre et al., 2002).

An issue that is becoming more serious in the last years is the use of fungicides in the control of fungal diseases in agro-ecosystems, which is causing concern because of their interference with agents of biological control such as the fungi. The impact of the application of phytosanitary chemicals on entomopathogens can vary according to the species and lineage of the pathogen, the chemical nature of the products and the concentrations employed. These products can act by inhibiting vegetative growth, conidial genesis, sporulation and by causing genetic mutations that can lead to reduced virulence against the plagues. Therefore, it is necessary to use the selective products that do not affect the balance between the plagues and their predators, parasites and pathogens (Alves and Lopes, 2008).

Among the commercial products indicated for the control of fungal diseases of soybean we highlight Nativo®, Sphere® and Bendazol®, the first of which having trifloxystrobin and tebuconazole as active agents. Sphere® has trifloxystrobin and cyproconazole as active agents. Both fungicides are from the chemical groups of strobirulins and triazoles. The strobirulins inhibit the respiratory chain by inhibiting complex II and III, interrupting the oxidative phosphorylation and interfering with the action of the ATP-synthase. The triazoles inhibit the biosynthesis of ergosterol (a fungal lipid), an important substance for the maintenance of the cell membrane integrity of the fungal cells (Bayer, 2008). Bendazol® has carbendazim as active agent and belongs to the benzimidazoles, affecting specifically the cell

*Corresponding author. E-mail: sideney@unipar.br Tel: 55-46-35202800.

Table 1. Observed growth of two lineages of *M. anisopliae* var. *anisopliae* in Potato + Dextrose + Agar (PDA) medium, under controlled conditions.

Fungus	96*	144*	192*	240*	288*	336*
CG-30	14.50±0.16[a#]	21.00±0.08[a]	28.00±0.07[a]	33.70±0.06[a]	39.00±0.12[a]	43.80±1.43[a]
CG-28	13.50±0.09[a#]	20.50±0.17[a]	28.00±0.10[a]	32.00±0.10[a]	36.30±0.22[a]	39.30±0.22[a]

[1]Halos of growth in mm. *Time in hours. [#]Data followed by the same lower case letter in the column do not differ by Tukey´s test at the level of 5%.

division by inhibiting the biosynthesis of tubulins (Milenia, 2010).

Moino and Alves (1998) put forward the hypothesis that the microorganism, through a mechanism of physiological resistance, can metabolize the toxic principles of the active chemicals, using the molecules resulting from this process and released in the culture medium as secondary nutrients, promoting its vegetative growth and conidial genesis. Still another possibility is that the fungus, in an activity comparable to that occurring with any living organism, uses its reproductive effort in the presence of a toxic compound that changes its environment and impairs its development, resulting in greater vegetative growth and conidial genesis.

MATERIALS AND METHODS

The fungal lineages assessed were: (a) *M. anisopliae* var. *anisopliae* lieage CG-28(AL), supplied by ESALQ (AL), isolated from *Mahanarva posticata* (Homoptera: Cercopidae) and (b) *M. anisopliae* var*. anisopliae* lineage CG-30(E-6) supplied by ESALQ (E6), isolated from *Deois flavopicta* (Homoptera: Cercopidae). The fungal lineages were supplied by ESALQ/USP (Superior School of Agriculture Luiz de Queiroz – University of São Paulo – Piracicaba – São Paulo – Brazil), lyophilized and conserved at low temperature. The lineages were invigorated using the bovine tick *Boophilus microplus*, later stored for evaluation.

The essays were conducted *in vitro*, in potato-dextrose-agar (PDA) culture medium, adding the phytosanitary products to the fused culture medium, not solidified at 45°C, at the concentration pre-established for the field. Next, the mixture was poured into Petri dishes. The control treatment was prepared inoculating each fungal lineage to the PDA medium in the absence of fungicides. After the solidification of the culture medium containing the fungicide, the inoculation of the fungal lineages to the center of the Petri dishes was proceeded and they were incubated for 336 h at 28°C (Alves and Lopes, 2008).

Three commercial products recommended for the control of fungal diseases of soybean culture in Brazil were tested: Nativo®, whose active agents are trifloxystrobin + tebuconazole, at the concentrations of 2.5 ml/L; 1,250, 650, 350, 150, 70 and 40 µl/L; Sphere®, whose active principles are trifloxystrobin + cyproconazole, at the concentrations of 2.0 ml/L; 1,000, 500, 250, 125, 62.5 and 32 µl/L; and Bendazol®, whose active compound is carbendazim, at the concentrations of 1.43 ml/L; 750; 350; 170, 85, 43 and 22 µl/L.

The determination of the toxic effect was made by evaluating the parameters of vegetative growth, using the model of classification of phytosanitary products for the toxicity against entomopathogenic fungi proposed by Alves and Lopes (2008). The vegetative growth was determined measuring the diameter of the colonies in two orthogonal lines at the surface of the culture medium. The mean diameter of the colonies was considered. All assays were made in triplicate, and the data were submitted to ANOVA. The comparison of the means was made through Tukey´s test at the level of significance of 5%.

RESULTS AND DISCUSSION

The data obtained after 336 h of growth of both lineages of *M. anisopliae* var. *anisopliae* in PDA medium, at 28°C, used as controls, are presented in Table 1 and Figure 1, while the data concerning the behavior of the lineages in the presence of the three fungicides are summarized in Table 2. The analysis of the data of Table 1 reveals that the halos obtained with both lineages are similar, because at all times they did not differ at the level of 5%. From these results, it is verified that both lineages had the same behavior in what concerns their growth under the conditions used.

When the data contained in Table 1 and Figure 1 were analyzed, it was noticed that the halos of growth showed similar kinetics, because the following linear equations did not differ: $y = 16.354\ln(x) + 12.067$ and $y = 14.642\ln(x) + 12.195$, for CG-28(AL) and CG-30(E6), respectively. These results suggest that these two lineages of *M. anisopliae* var. *anisopliae* were very similar. The data contained in Table 2 show that both fungal lineages under study had diverse behaviors in the presence of the three fungicides tested, because after 360 h of growth the fungicide Sphere® inhibited the vegetative growth of the fungus *M. anisopliae* var. *anisopliae* lineage CG-28(AL) at the field concentration of 2.0 ml/L and at the dilutions of 1,000 and 500 µl/L. At the dilutions of 250, 125, 62.5 and 32 µl/L there was mycelial growth of the fungus of 5.30±0.06, 9.20±0.10, 9.80±0.07 and 11.00±0.04 mm, respectively.

With the fungicide Nativo® it was observed that the growth of the lineage CG-28 was inhibited at the field concentration of 2.5 ml/L and at the dilutions of 1,250 and 650 µl/L, while at 350, 150, 70 and 40 µl/L there was development of the fungal mycelia, with halos measuring 1.80±0.02, 3.50±0.04, 4.70±0.06 and 7.50±0.04 mm, respectively. It was also noticed that the fungicide

Bendazol® did not allow the development of the fungus *M. anisopliae* var. *anisopliae* lineage CG-28(AL) at the

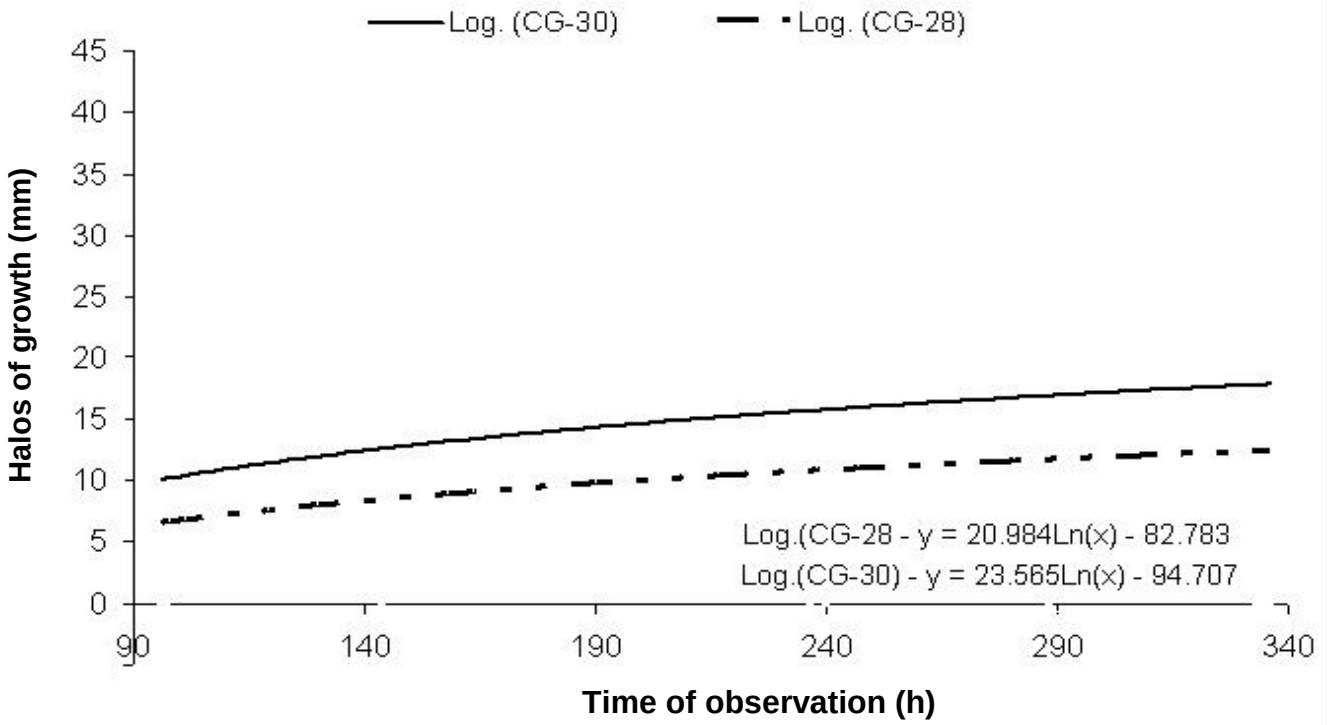

Figure 1. Kinetics of microbial growth of *M. anisopliae* var. *anisopliae*, lineages CG-28(AL) and CG-30(E6), represented by the linear equations y=16.354Ln(x) + 12.067 and y=14.642Ln(x) + 12.195, respectively.

field concentration of 1.4 ml/L, neither at the dilution of 750 µl/L. On the other hand, at the concentrations of 350, 170, 85, 43 and 22 µl/L, there was fungal growth, forming halos of 4.50±0.02, 5.00±0.04, 5.50±0.04, 5.00±0.10 and 5.50±0.06 mm, respectively.

It was observed that the minimum inhibitory concentration (MIC) obtained for the fungicide Sphere® was 500 µl/L; for Nativo® it was 650 µl/L and 750 µl/L for Bendazol®. However, the fungal growth obtained in the presence of the fungicides was lower than that obtained with the control group, which was 41.00±0.22 mm at the same time interval. Therefore, it can be ascertained that the fungicides assayed inhibited the vegetative growth of *M. anisopliae* var. *anisopliae* lineage CG-28(AL). In the presence of the fungicide Sphere® at the concentration of 2.0 ml/L, recommended for field use, the lineage CG-30(E6) of the fungus *M. anisopliae* var. *anisopliae* did not grow. At lower concentrations the fungus did grow, showing halos of 4.80±0.06, 5.30±0.02, 6.50±0.04, 10.10±0.04, 11.00±0.06 and 17.00±0.04 mm for the concentrations of 1,000, 500, 250, 125, 62.5 and 32 µl/L, respectively. Under the action of the fungicide Nativo®, there was no development of the lineage CG-30(E6) at the concentration of 2.5 ml/L, prescribed for field use. At the dilutions of 1,250, 650, 350, 150, 70 and 40 µl/L it was verified that there was fungal growth, with growth halos of 2.00±0.04, 3.00±0.04, 6.60±0.06, 8.50±0.06, 14.50±0.04 and 17.70±0.02 mm, respectively.

As for the fungicide Bendazol®, it was observed that the lineage CG-30(E6) was also inhibited, because it did not develop at the field concentration of 1.4 ml/L and at 750, 350 and 170 µl/L. Only at the concentrations of 85, 43 and 22 µl/L the fungus developed, showing halos of vegetative growth of 2.00±0.04, 3.20±0.08 and 4.50±0.06, respectively. After the evaluation of the data it can be verified that the MIC of the concentrations assayed was 2.0 ml/L for the fungicide Sphere®, 2.5 ml/L for the fungicide Nativo® and 170 µl/L for the fungicide Bendazol®. It must be stressed that, despite the fungal growth in the presence of the fungicides Sphere®, Nativo® and Bendazol®, the values of the halos of growth are lower than that obtained with the control group, which was 46.20±0.14 mm at the same time interval. Therefore, it can be stated that there is a strong inhibition of the fungal growth of the lineage CG-30(E6) of *M. anisopliae* var. *anisopliae* by these fungicides.

The data obtained in this work are compatible with those of Yañez and France (2010), because in the evaluation of the fungicides azoxystrobin, benomyl, captan, chlorothalonil, fenhexamid, fludioxonil, iprodione and metalaxyl on five lineages of the fungus *M. anisopliae* var. *anisopliae*, it was verified that the

Table 2. Growth of *M. anisopliae* var. *anisopliae*, lineages CG 28(AL) and CG 30(E6) in control culture medium and under the action of three commercial fungicides, after 336 h of growth.

Fungicide sphere – CG-30(E6)						
2.0 ml/L	1,000 µl/L	500 µl/L	250 µl/L	125 µl/L	62.5 µl/L	32 µl/L
NG*	4.80±0.06	5.30±0.02	6.50±0.04	10.10±0.04	11.00±0.06	17.00±0.04
Fungicide bendazol – CG-30(E6)						
1.43 ml/L	750 µl/L	350 µl/L	170 µl/L	85 µl/L	43 µl/L	22 µl/L
NG	NG	NG	NG	2.00±0.04	3.20±0.08	4.50±0.06
Fungicide Nativo – CG-30(E6)						
2.5 ml/L	1,250 µl/L	650 µl/L	350 µl/L	150 µl/L	70 µl/L	40 µl/L
NG	2.00±0.04	3.00±0.04	6.60±0.06	8.50±0.06	14.50±0.04	17.70±0.02
Fungicide sphere – CG-28(AL)						
2.0 ml/L	1,000 µl/L	500 µl/L	250 µl/L	125 µl/L	62.5 µl/L	32 µl/L
NG	NG	NG	5.30±0.06	9.20±0.10	9.80±0.07	11.00±0.04
Fungicide bendazol – CG-28(AL)						
1.43 ml/L	750 µl/L	350 µl/L	170 µl/L	85 µl/L	43 µl/L	22 µl/L
NG	NG	4.50±0.02	5.00±0.04	5.50±0.04	5.00±0.10	5.50±0.06
Fungicide nativo – CG-28(AL)						
2.5 ml/L	1,250 µl/L	650 µl/L	350 µl/L	150 µl/L	70 µl/L	40 µl/L
NG	NG	NG	1.80±0.02	3.50±0.04	4.70±0.06	7.50±0.04

NG – No growth.

fungicides benomyl and fenhexamide did not inhibit the fungal growth, while azoxystrobin and fludioxonil inhibited the growth of the five lineages evaluated. The colonies of *M. anisopliae* that grew in the presence of the fungicides Sphere®, Nativo® e Bendazol® showed marked morphologic modifications when compared with the control colonies, such as the presence of irregular edges, vertical growth, change in the color of the conidia and also circular regions with cotton-like mycelium. Although not the focus of this work, it was noticed that the growing colonies showed reduced production of conidia.

According to Oliveira et al., (2002), the vegetative growth of *M. anisopliae* in treatments containing the agrochemicals Vertimec® (abamectin-based), Savey® (hexythioazox-based) and Rufast® (acrinathrin-based) at the concentrations of 1.0 and 0.5 ml/L did not differ statistically compared with the control treatment. However, the formulations of the agrochemicals Parsec® (amitraz) and Sanmite® (pyridaben) at the field concentration of 1.0 and of 0.5 ml/L differed significantly from the control, causing a reduction of the vegetative growth greater than 62%. It must be pointed out that these results coincide with those obtained in this work, although the active agents are different. The active agents abamectin, hexythioazox and acrinathrin did not inhibit the vegetative growth of the fungus *M. anisopliae* var. *anisopliae*, and thus can be recommended for integrated agricultural programs of pest control.

With the data obtained, it is verified that, from the MIC determined, halos of growth are observed which are much smaller that those of the control group at the same time interval. Therefore, it can be inferred that the three fungicides evaluated were capable of inhibiting the fungal growth of the CG-28(AL) and CG-30(E6) lineages of *M. anisopliae* var. *anisopliae*.

When comparing the mechanism of action of each active agent evaluated, it is verified that the strobirulins has translaminar action, specific for the pathogen, and high risk of resistance when compared with the triazoles (cyproconazole and tebuconazole). Strobirulins interfere with mitochondrial respiration by blocking the electron transfer through the cytochrome bc1 complex, at the Qo site, interfering with ATP production and inhibiting the cell respiration of the fungus (Ghini and Kimati, 2002).

The triazoles act by inhibiting the biosynthesis of ergosterol, a fungal lipid important for the maintenance of the cell membrane of the fungi. With the membrane rupture, there is leakage of ionic solutes and the cell death ensues (Ghini and Kimati, 2002).

The benzimidazoles affect specifically the cell division

by inhibiting the biosynthesis of tubulin, which is a microtubular protein (Kendall et al., 1994). Therefore, the formation of microtubules is impaired, and nuclear division and separation do not occur (Ghini and Kimati, 2002).

The *in vitro* studies have the advantage of exposing the microorganism maximally to the action of the chemical, as it does not take place in the field, where several factors prevent this exposure. In this way, once the safety of a product is checked in the laboratory, it is expected to the selective in the field. On the other hand, the high *in vitro* toxicity of a product does not always point to a high toxicity in the field, but the possibility of occurrence of such damages (Moino and Alves, 1998). It has to be stressed as well that the attempt of standardization of the compatibility tests of entomopathogenic fungi with phytosanitary products is recent and amenable to improvement. Factors such as conidial viability and their pathogenicity in the presence of phytosanitary products should also be taken into account in the choice of the most selective products.

Fungicide toxicity is one of the most limiting factors in the use of the group of chemical control; therefore, in a strategy of joint introduction of these with entomo-pathogenic fungi, it is suggested that the use of sub-lethal concentrations, or sub-concentrations of fungicides is equal or lower than the MIC, thus reducing the amount of residues released into the environment and foods, the toxic effect of these phytosanitary chemicals against entomopathogens and farm workers during preparation and application.

Conclusion

It is concluded that the fungicides Nativo®, composed of trifloxystrobin + tebuconazol; Sphere®, composed of trifloxystrobin + cyproconazole, and Bendazol®, with carbendazim as active agent, inhibit the growth of the lineages CG-28(AL) and CG-30(E6) de *M. anisopliae* var. *anisopliae* at their field concentrations. For the lineage CG-28 of *M. anisopliae* var. *anisopliae* the MICs for the fungicides were the following: Sphere®: MIC of 500 µl/L; Nativo®: MIC of 650 µl/L and Bendazol®: MIC of 750 µl/L. For the CG-30 lineage of *M. anisopliae* var. *anisopliae* the MICs were as follows: Sphere®: MIC of 2.0 ml/L (concentration indicated for field use); Nativo®: MIC of 2.5 ml/L (concentration indicated for field use); and Bendazol®: MIC of 170 µl/L. Therefore, it is suggested that studies should be carried out in the field to corroborate the *in vitro* results, once the environmental conditions are variable when compared to laboratory tests. Tests for the determination of conidial genesis are also suggested.

ACKNOWLEDGEMENTS

The authors thank EMBRAPA/CENARGEN – Brazilian Enterprise of Agriculture and Livestock Research/ National Center of Genetic Resources, and ESALQ/USP – Superior School of Agriculture Luiz de Queiroz of the University of São Paulo, for the supply of the fungal lineages, and Bayer of Brazil S.A. and Milênia S.A. for the supply of the fungicides.

REFERENCES

Alves SB, Lopes RB (2008). Controle Microbiano de Pragas na América Latina: Avanços e Desafios. FAELQ: Piracicaba – SP, 414pp.

Bayer CropScience Brasil, (2008) Fungicida Sphere. Disponível em: www.bayercropscinece.com.br/site/nossosprodutos/protecaodecultivosebiotecnologia/detalhedoproduto.fss?produto. Acessado em: 26/02/2010.

Fernandes EKK, Keyser CA, Rangel DEN, Foster RN, Roberts DW (2010) CTC medium: A novel dodine-free selective medium for isolating entomopathogenic fungi, especially *Metarhizium acridum*, from soil. Biol. Control, 54: 197-205.

Ghini R, Kimati H (2002) Resistência de fungos a fungicidas. Jaguariúna: Embrapa Meio Ambiente, p. 78.

Kendall S, Hollomon DW, Ishii H, Heaney SP (1994) Characterization of benzimidazole-resistant strains of *Rhynchosporium secalis*. Pestic. Sci., 40: 175-181.

Milenia - AgroCiência SA (2010) Fungicida Bendazol. Disponível em. www.milenia.com.br. Acessado em: 17 mar 2010.

Moino Jr. A, Alves SB (1998) Efeito de imidacloprid e fipronil sobre *Beauveria bassiana* (Bals.) Vuill. e *Metarhizium anisopliae* (Metsch.) Sorok. e no comportamento de limpeza de *Heterotermes tennuis* (Hagen). An. Soc. Entomol. Brasil, 27: 611-620.

Oliveira RC, Neves PMOJ, Guzzo EC, Alves VS (2002). Compatibilidade de fungos entomopatogênicos com agroquimicos. *Semina: Ciências Agrárias*, 23(2): 211-216.

Onofre SB, Vargas LRB, Rossato M, Barros NM, Boldo JT, Nunes ARF, Azevedo JL (2002). Controle biológico de pragas na agropecuária por meio de fungos entomopatogênicos. In. SERAFINI, L.A., BARROS, N.M., AZEVEDO, J.L. Biotecnologia: avanços na agricultura e na agroindústria. Caxias do Sul: EDUCS, p. 433.

Schrank A, Franceschini M, Guimarães AP, Camassola M, Frazzon AP, Baratto CM, Kogler V, Silva MV, Dutra V, Nakazoto L, Castro L, Santi L, Vainstein MH (2001). Biotecnologia aplicada ao Controle Biológico. O entomopatógeno *Metarhizium anisopliae*. *Biotecnologia Ciência & Desenvolvimento*, 23: 32-35.

Yañez M, France A (2010). Effects of fungicides on the development of the entomopathogenic fungus *Metarhizium anisopliae* var*. anisopliae.* Chilean J. Agric. Res., 70(3): 390-398.