

## Compatibility of conventional agrochemicals used in rice crops with the entomopathogenic fungus *Metarhizium anisopliae*

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**ABSTRACT:** The toxicological impact of chemical pesticides on fungal entomopathogens and their use in tank-mixing can be directly measured through *in vitro* compatibility tests. This study reports the *in vitro* toxicity of eight insecticides, four fungicides and five herbicides in the conidial germination, vegetative growth and conidiation of *Metarhizium anisopliae* (strain CG 168). A conidial suspension containing the pesticide at recommended field dosage was subjected to constant agitation in a rotary shaker for 3h to simulate a tank mixing. Then, aliquots of each suspension were used to determine conidial germination, vegetative growth and conidiation on potato dextrose agar (PDA). The fungicides difenoconazole (69 mL ha<sup>-1</sup>), propiconazole (75 mL ha<sup>-1</sup>), trifloxystrobin (313 g ha<sup>-1</sup>) and azoxystrobin (56 mL ha<sup>-1</sup>) were the most harmful products to all biological stages of *M. anisopliae* and they should not be applied together with this fungus in tank mixing. The insecticides exhibited the least degree of toxicity to this fungal pathogen, whereas the herbicides had the greatest impact on mycelial growth. The agrochemicals compatible with *M. anisopliae* were the insecticides methyl parathion (240 mL ha<sup>-1</sup>), thiamethoxam (31 g ha<sup>-1</sup>), and lambda-cyhalothrin (6.3 mL ha<sup>-1</sup>) and the herbicides glyphosate (1560 mL ha<sup>-1</sup>), bentazon (720 mL ha<sup>-1</sup>), and imazapir+ imazapyr (84 g ha<sup>-1</sup>). The compatible pesticides could be simultaneously used with this bio-control agent for integrated pest management in rice production systems.

**Keywords:** pesticide compatibility, conidiation, entomopathogenic fungi, germination, vegetative growth

### Introduction

The rice stink bug, *Tibraca limbativentris* Stal, 1860 (Heteroptera: Pentatomidae), is one of the most serious pests attacking irrigated rice (*Oryza sativa* L.) crops worldwide, as it can cause yield losses up to 80 % when control measures are neglected (Ferreira et al., 1986; Martins et al., 2004). *Metarhizium* spp. are cosmopolitan entomopathogenic fungi reported to infect more than 300 arthropod species belonging to several insect orders (Alves et al., 1998). These fungi are also adapted to live as saprophytes as well as symbionts in the plant rhizosphere (Hu and St. Leger, 2002), which make the soil their major habitat. *Metarhizium anisopliae* (Metch.) Sorok (Ascomycota: Clavicipitaceae) complex has been observed causing epizootics on populations of *T. limbativentris* under irrigated conditions, and field trials with this fungus have shown promising results for the management of this pest in Brazil (Martins et al., 2004; Rampelotti et al., 2007).

Understanding the effect of chemical pesticides most commonly used by rice farmers in Brazil on different developmental stages of this microbial agent is of paramount importance for establishing an integrated pest management program for this insect. The response of widely used agricultural chemicals on *M. anisopliae* is variable, with fungicides generally being the most toxic compounds followed by insecticides and herbicides (Loureiro et al., 2002; Rachappa et al., 2007; Rampelotti-Ferreira et al., 2010; Tanzini et al., 2002).

Biocontrol agents, such as entomopathogens, cannot totally replace synthetic chemical pesticides in commercial rice production systems. As there is strong evidence indicating the harmful effects of them on the survival of entomopathogenic fungi in the agroecosystem, it is crucial to determine the compatibility of such agrochemicals and to determine which of them are less harmful to *M. anisopliae* in order to guide rice growers. In addition, the combination approach among control measures, such as microbial agents and chemical insecticides, comprises an important component in integrated pest management programs (Quintela and McCoy, 1997). Toxicity of such pesticides to fungal entomopathogens may vary with fungus species and strain, chemical nature of the active ingredient, mode of action, product formulation, and recommended label rate (Alves and Lecuona, 1998). For instance, Tanzini et al. (2002) observed that two different strains of *M. anisopliae* showed different responses when exposed to methamidophos and lambda-cyhalothrin. Therefore, the objective of this study was to determine the compatibility of different agrochemicals registered for rice crops with a selected isolate of *M. anisopliae* under laboratory conditions.

### Materials and Methods

#### Source of fungus inoculum

Bioassays were carried out in Santo Antônio de Goiás (16°28'00" S, 49°17'00" W and 823 m a.s.l.), State

of Goiás, Brazil. *M. anisopliae* was isolated from *T. limbativentris* adult at Santo Antônio de Goiás, GO, in 1985. This strain was identified as being *M. anisopliae* through the sequence analysis of elongation factor 1- $\alpha$  gene, following the protocol described in Bischoff et al. (2009). In 1990, this *M. anisopliae* strain was stored at the culture collection of Embrapa Genetic Resources and Biotechnology (Cenargen), in Brasília, Federal District, Brazil, and is currently identified as *M. anisopliae* strain CG 168.

### Agrochemicals tested

The insecticides, fungicides and herbicides chosen for the bioassays are the most frequently used by rice farmers and described in Table 1. These pesticides are registered for use in rice crops in Brazil. The concentrations of all agrochemicals were tested following the label rates recommended by the manufacturers (MAPA, 2009). When the concentration of a product indicated minimum and maximum doses, the average concentration was used. Methamidophos is a known broad spectrum insecticide not yet registered for rice, though it is widely used by farmers to control *T. limbativentris* (J.A.F. Barrigossi, personal communication, Jul. 31, 2011). The concentration tested for each product was calculated based on the spray volume rate of 200 L ha<sup>-1</sup> of water.

### Conidial germination, mycelial growth and conidial production bioassays

A new procedure is described herein simulating a

tank mixing with the chemical products and the fungus before application. Three most important biological parameters of the fungus were recorded: conidial germination, vegetative growth and conidiation.

In the first experiment, the effect of eight insecticides was assessed on fungal germination rate. Each insecticide concentration was added to 10 mL of fungal suspension containing  $1.9 \times 10^7$  conidia mL<sup>-1</sup> in sterile distilled water plus Tween 80 (polyoxyethylene (80) sorbitan monooleate, Vetec Química Fina Ltda., Rio de Janeiro, RJ, Brazil) at 0.01 % (v/v) using 50-mL centrifuge tubes. Each treatment (i.e., mixture of fungal conidia + pesticide) was replicated four times (i.e., four tubes per treatment). Then, this mixture was vigorously vortexed for 2 min and kept at constant agitation in a rotary shaker at 25 °C with 214 revolutions per minute for 3 h in order to simulate a tank mixing of fungal conidia and pesticide. The suspension was vortexed again and a 500- $\mu$ L aliquot was sprayed onto a thin layer (3 mm) of PDA (20 % potato infusion, 2 % dextrose and 2 % agar) on a glass slide (7.5  $\times$  2.5 cm) using a hand sprayer. Each treatment had three slides (replicates). After conidial inoculation, these slides were placed in a growth chambers at  $26 \pm 1$  °C, relative humidity (RH) of  $70 \pm 8$  % with 12 h photophase. Control consisted of conidia suspended only in 0.01 % aqueous Tween 80 free of any chemical pesticide. Conidial germination was determined after 20 and 48 h of incubation period by examining 200 conidia at random per slide, using a phase-contrast microscope

Table 1 – Description of agrochemicals used in rice production for assessment of *in vitro* compatibility bioassays with *Metarhizium anisopliae* CG 168.

Trade name	Formulation <sup>a</sup>	Chemical group	Technical name	Concentration of active ingredient	Recommended dose
				g L <sup>-1</sup> or g kg <sup>-1</sup> <sup>b</sup>	g or mL ha <sup>-1</sup>
Insecticide					
Standak	SC	Pyrazole	Fipronil	250	240
Bravik	EC	Organophosphate	Methyl Parathion	600	400
Nor-trin	EC	Pyrethroid	Cypermethrin	250	100
Actara	WG	Neonicotinoid	Thiamethoxam	250	125
Engeo Pleno	CS	Neonicotinoid + Pyrethroid	Thiamethoxam + Lambda-cyhalothrin	106 + 141	175
Karate Zeon	CS	Pyrethroid	Lambda-cyhalothrin	50	125
Klap	CS	Pyrazole	Fipronil	200	60
Tamaron	CS	Organophosphate	Methamidophos	600	100
Herbicide					
Roundup Original	CS	Glycine replaced	Glyphosate	480	3250
DMA	SL	Ariloxialconic acid	2,4-Dichlorophenoxyacetic acid (2,4-D)	806	1000
Basagran	SL	Benzothiadiazinone	Bentazon	600	1200
Kifix	WP	Imidazolinone	Imazapic + Imazapyr	175 + 525	120
Herbadox	EC	Dinitroanilines	Pendimethalin	500	3000
Fungicide					
Score	EC	Triazole	Difenoconazole	250	275
Stratego	EC	Triazole	Propiconazole	250	300
Flint	WG	Strobilurin	Trifloxystrobin	500	625
Priori	CS	Strobilurin	Azoxystrobin	250	225

<sup>a</sup>Formulation types : capsule suspension (CS), emulsifiable concentrate (EC), suspension concentrate (SC), soluble concentrate (SL), water dispersible granules (WG), wettable powder (WP). <sup>b</sup>Volume of application = 200 L of water per hectare.

at 400 × magnification. Conidia were considered germinated when the germ tube was longer than the conidial diameter (Luz and Fargues, 1997). This experiment was repeated twice ( $n = 6$  replicates).

In the second bioassay, the effect of the insecticides on mycelial growth and conidial production was assessed. Two microliters of each original conidial suspension mixed with pesticide, whose preparation was described in the previous trial, were inoculated in the center of a 6-cm diameter Petri dish containing PDA (10 mL per plate). Each treatment consisted of four replicates (plates). Control consisted of conidia suspended in 0.01 % aqueous Tween 80. Plates were then placed in a growth chamber at  $26 \pm 1$  °C,  $70 \pm 8$  % RH with 12 h photophase. After nine days of incubation, the fungal radial growth was determined using a digital caliper rule to record length and width of the fungal colony. Afterwards, the entire colony was cut and transferred to a 50-mL centrifuge tube containing 10 mL of distilled water + Tween 80 (0.01 %) and vigorously vortexed for two minutes, so that conidia were dislodged from the PDA medium. Successive dilutions were performed until a desirable suspension was obtained for counting conidia using an improved Neubauer chamber (400 × magnification). The conidial production was recorded and expressed as number of conidia per colony. This experiment was repeated twice ( $n = 8$  replicates).

The compatibility of five herbicides and four fungicides (Table 1) with *M. anisopliae* strain GC 168 was also evaluated by performing the same bioassay procedures as outlined above for the insecticides. The experiments with the herbicides and fungicides were repeated twice.

### Data analysis

The data sets for each experiment in duplicate were joined for statistical analyses. Residuals derived from data sets were submitted to Shapiro-Wilk and Brown-Forsythe tests of significance at 5 % to check the normality assumptions of the parametric statistical model. For those that matched normality assumptions, data were submitted to a one-way analysis of variance (ANOVA, PROC GLM), and treatment means were *post-hoc* compared by Fisher's least significant difference (LSD,  $\alpha = 0.05$ ). When residues did not match parametric assumptions, data were submitted to non-parametric analysis based on Kruskal-Wallis test with rank-transformed data (PROC NPAR1WAY), except data of percentage of conidial germination that were previously subjected to arcsin square root transformation. Afterwards, rank means were compared by Fisher's LSD ( $\alpha = 0.05$ ).

To determine which group of chemical (insecticides, fungicides and herbicides) was more detrimental to each developmental stage of *M. anisopliae*, means within each chemical group were pooled and submitted to the Kruskal-Wallis test, then pooled means were compared by Fisher's LSD test ( $\alpha = 0.05$ ). All statistical analyses were run using SAS software version 9.2 (SAS Institute, 2008).

To determine the selectivity of agrochemicals to entomopathogenic fungi under laboratory conditions, the Biological Index formula (*BI*) proposed by Rossi-Zalaf et al. (2008) was used, calculated as the following:  $BI = [47*VG + 43*SP + 10*GER] / 100$ , where: *VG* is the percentage of vegetative growth of fungal colony in relation to control; *SP* is the percentage of colony sporulation in relation to control; *GER* is the percentage of conidial germination in relation to control. Values of *BI* ( $\alpha = 0.05$ ) were clustered into three categories of toxicological classification of agrochemicals to the fungus: 0 to 41 = Toxic; 42 to 66 = Moderately toxic; > 66 = Compatible. Hence, this formula is considered practical to classify chemical pesticides concerning their toxicity to entomopathogenic fungi.

### Results

All insecticides affected conidial germination at 20 h in contrast to the untreated control ( $F = 261.98$ ;  $df = 8, 45$ ;  $p < 0.0001$ ). The highest detrimental effect on conidial germination was caused by fipronil (25 % w/v) and cypermethrin (reduction of 54 % and 100 %, respectively) (Figure 1). At 48 h, thiamethoxam and methyl parathion had not affected conidial germination ( $p > 0.05$ ), while the other insecticides reduced germination compared to control ( $F = 266.7$ ;  $df = 8, 45$ ;  $p < 0.0001$ ). The lowest germination rates were observed for fipronil (25 % w/v) and cypermethrin which caused a significant inhibition of conidial germination when compared to thiamethoxam, thiamethoxam + lambda-cyhalothrin, methyl parathion, lambda-cyhalothrin, fipronil (20 % w/v) and methamidophos after 20 and 48 h of incubation ( $p < 0.05$ ).

Mycelial growth was reduced in all insecticide treatments when compared to the untreated control ( $\chi^2 = 62.14$ ;  $df = 8$ ;  $p < 0.0001$ ), except methyl parathion which allowed normal fungal growth ( $p > 0.05$ ). No *M. anisopliae* mycelial growth was observed with cypermethrin which was different from the other treatments (Figure 1).

Conidiation of *M. anisopliae* was inhibited by all insecticides ( $\chi^2 = 63.05$ ;  $df = 8$ ;  $p < 0.0001$ ), except methyl parathion which did not differ from the control ( $p > 0.05$ ) (Figure 1). Thiamethoxam + lambda-cyhalothrin, fipronil (20 % w/v), fipronil (25 % w/v) and methamidophos were highly antagonistic (> 73.7 % of reduction) to *M. anisopliae* sporulation ( $p < 0.05$ ). Since cypermethrin inhibited mycelial growth, no conidial production was observed in this treatment (Figure 1).

According to the Biological Index, methyl parathion, thiamethoxam and lambda-cyhalothrin were compatible with *M. anisopliae* CG 168, while fipronil (20 % w/v), fipronil (25 % w/v), thiamethoxam + lambda-cyhalothrin and methamidophos were classified as moderately toxic (Table 2). Among the insecticides tested only cypermethrin was not compatible with *M. anisopliae*.

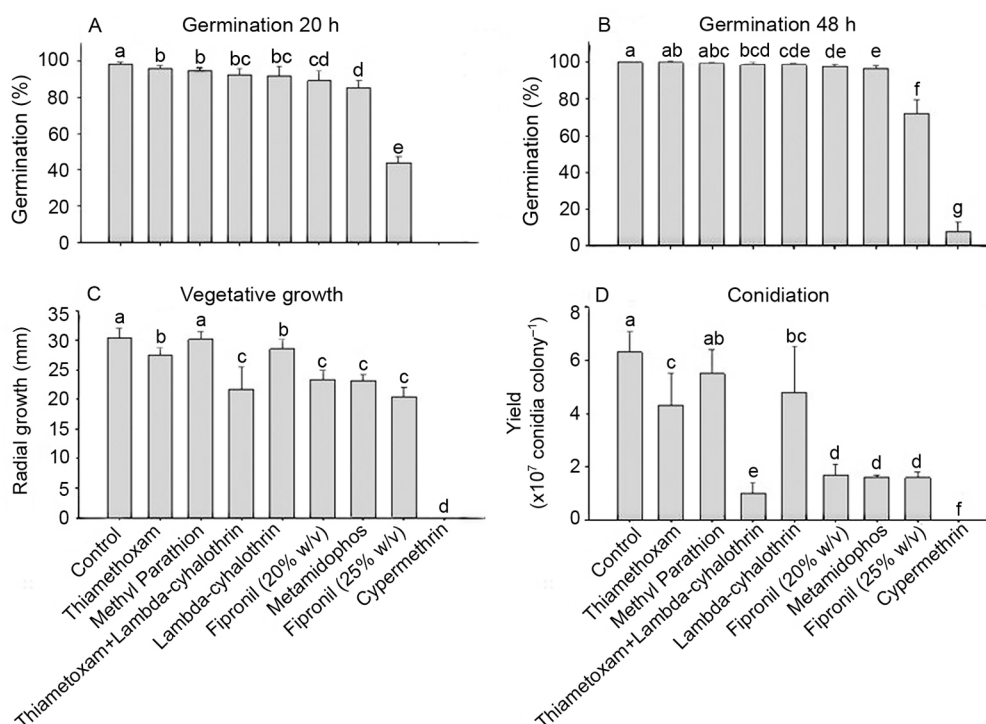


Figure 1 – Effect of insecticides on biological parameters of *Metarhizium anisopliae* CG 168 at 25 °C and 12 h photophase. Means followed by the same letters are not significantly different by Fisher's LSD test ( $p \leq 0.05$ ). Number of replicates per treatment was 6-8. Only distilled water plus Tween 80 (0.01 %) was used in the control group.

Table 2 – Toxicological classification of registered agrochemicals for rice production toward *Metarhizium anisopliae* strain CG 168 based on the Biological Index formula proposed by Rossi-Zalaf et al. (2008).

Agrochemical	Biological Index <sup>a</sup>	Classification
<b>Insecticide</b>		
Standak	47.2	Moderately toxic
Bravik	94.0	Compatible
Actara	81.8	Compatible
Engeo Pleno	49.9	Moderately toxic
Karate Zeon	86.3	Compatible
Klap	56.8	Moderately toxic
Tamaron	55.6	Moderately toxic
Nor-trin	0.0	Toxic
<b>Herbicide</b>		
Roundup	80.5	Compatible
DMA	0.0	Toxic
Basagran	78.4	Compatible
Kifix	83.2	Compatible
Herbadox	43.6	Moderately toxic
<b>Fungicide</b>		
Score	65.0	Moderately toxic
Flint	46.5	Moderately toxic
Stratego	60.9	Moderately toxic
Priori <sup>b</sup>	72.6	Compatible

<sup>a</sup>For computation of the Biological Index, conidial germination at 20 h was used.

<sup>b</sup>Although azoxystrobin was scored as selective to *M. anisopliae* according to the Biological Index formula, we recommend that it not be used in combination with the fungus, as it prevented conidial germination at 20 h.

All herbicides inhibited germination at 20 h and no germination was observed when *M. anisopliae* conidia were exposed to pendimethalin and 2,4-Dichlorophenoxyacetic acid (2,4-D) ( $F = 255.22$ ;  $df = 5, 29$ ;  $p < 0.0001$ ) (Figure 2). After 48 h, the germination rate of the treatments with imazapic + imazapyr and bentazon were similar to the control ( $p > 0.05$ ), whereas the other herbicides considerably reduced the conidial germination ( $F = 715.18$ ;  $df = 5, 29$ ;  $p < 0.0001$ ) (Figure 2). In addition, glyphosate delayed conidial germination with only 8.2 % germination at 20 h but 96.4 % at 48 h.

Most herbicides reduced mycelial growth in comparison with the control ( $\chi^2 = 39.47$ ;  $df = 5$ ;  $p < 0.0001$ ), except for glyphosate ( $p > 0.05$ ). A complete suppression of vegetative growth of *M. anisopliae* was caused by 2,4-D, and consequently no conidia were produced (Figure 2). All herbicides had a deleterious effect on conidial yield compared to the control ( $\chi^2 = 36.53$ ;  $df = 5$ ;  $p < 0.0001$ ), with pendimethalin and 2,4-D causing the highest reduction in fungal conidiation (81.8 % and 100 %, respectively). According to the Biological Index, glyphosate, bentazon and imazapic + imazapyr were compatible with *M. anisopliae* CG 168, while pendimethalin was classified as moderately toxic and 2,4-D toxic (Table 2).

Similar to other chemical groups, all fungicides negatively affected *M. anisopliae* germination at 20 h ( $F = 1246.20$ ;  $df = 4, 20$ ;  $p < 0.0001$ ) and 48 h ( $F = 256.62$ ;  $df$



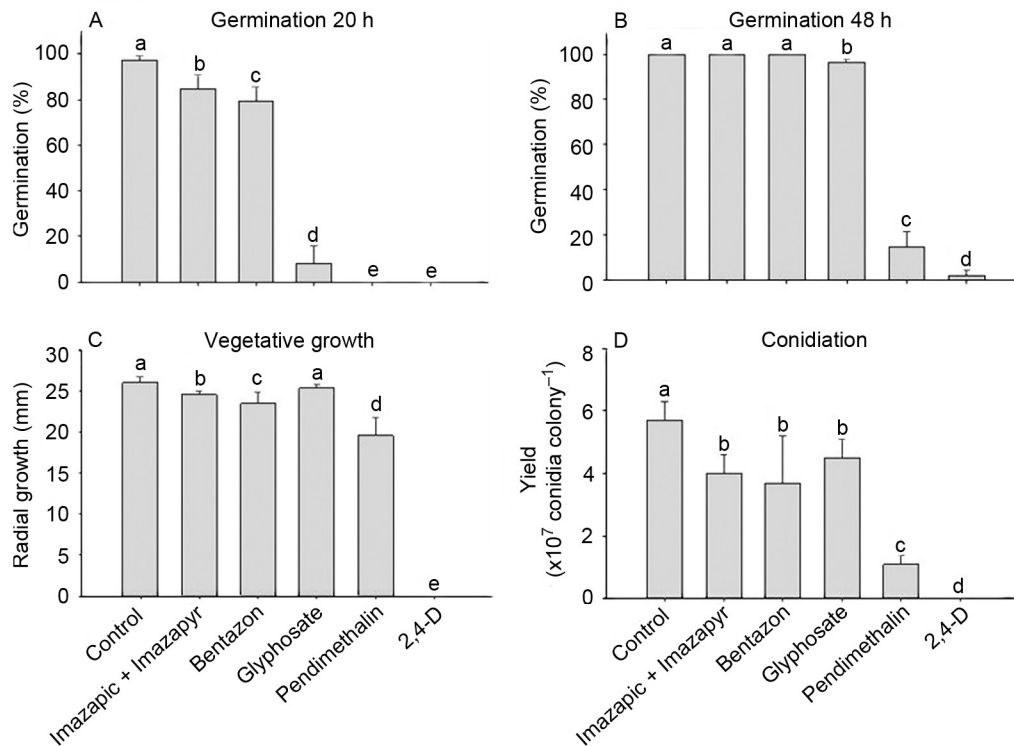


Figure 2 – Effect of herbicides on biological parameters of *Metarhizium anisopliae* CG 168 at 25 °C and 12 h photophase. Means followed by the same letters are not significantly different by Fisher's LSD test ( $p \leq 0.05$ ). Number of replicates per treatment was 6-8. Only distilled water plus Tween 80 (0.01 %) was used in the control group.

= 4, 20;  $p < 0.0001$ ) compared to the controls (Figure 3). Trifloxystrobin and azoxystrobin were the most deleterious fungicides for fungal germination at 20 h, since no germinated conidia were observed. However, some germination occurred after 48 h incubation (48-52 %) indicating that trifloxystrobin and azoxystrobin delayed conidial germination. Vegetative growth and conidial yield were inhibited by all fungicides ( $F = 54.4$ ;  $df = 4, 32$ ;  $p < 0.0001$ ;  $F = 107.51$ ;  $df = 4, 32$ ;  $p < 0.0001$ , respectively). Difenoconazole, propiconazole and trifloxystrobin caused more than 50 % of reduction of *M. anisopliae* conidiation. According to the Biological Index, azoxystrobin was the only compatible fungicide with *M. anisopliae* CG 168, whereas difenoconazole, propiconazole and trifloxystrobin were classified as moderately toxic (Table 2). No fungicide was scored as toxic to this fungus.

For the analysis of the chemical groups through a comparison of pooled means, it was shown that all tested products reduced the biological parameters of *M. anisopliae* CG 168 to some extent. Insecticides comprised the least deleterious products to *M. anisopliae*. Fungicides and herbicides inhibited in a higher degree the mycelial growth of *M. anisopliae* than insecticides ( $\chi^2 = 36.97$ ;  $df = 3$ ;  $p < 0.0001$ ), whereas all pesticides considerably reduced the conidial production compared to the control ( $\chi^2 = 33.06$ ;  $df = 3$ ;  $p < 0.0001$ ) at the same

extent. Fungicides inflicted the most harmful effect on germination at 20 h ( $\chi^2 = 64.28$ ;  $df = 3$ ;  $p < 0.0001$ ) and 48 h incubation ( $\chi^2 = 33.31$ ;  $df = 3$ ;  $p < 0.0001$ ). Moreover, although herbicides reduced the germination at 20 h in comparison to insecticides, there was no difference among chemical groups after 48 h.

## Discussion

None of the tested agrochemicals at label rate recommendations promoted positive effects on developmental stages of *M. anisopliae*. It is possible that those products compatible with *M. anisopliae*, especially the insecticides, might be used at sublethal dosages in combination with the fungus in further studies aimed at improving its virulence against *T. limbativentris* or even to broaden its host range in rice crops.

Although the insecticides methamidophos, methyl parathion, thiamethoxam + lambda-cyhalothrin, Karate™, fipronil (20 % w/v), thiamethoxam and the herbicide imazapic + imazapyr differed from the control, conidial germination in these treatments was high, ranging from 84.6 to 95.8 %. These levels of germination suggest that these insecticides would be suitable for using in combination with *M. anisopliae* for insect control as part of an integrated pest management strategy.

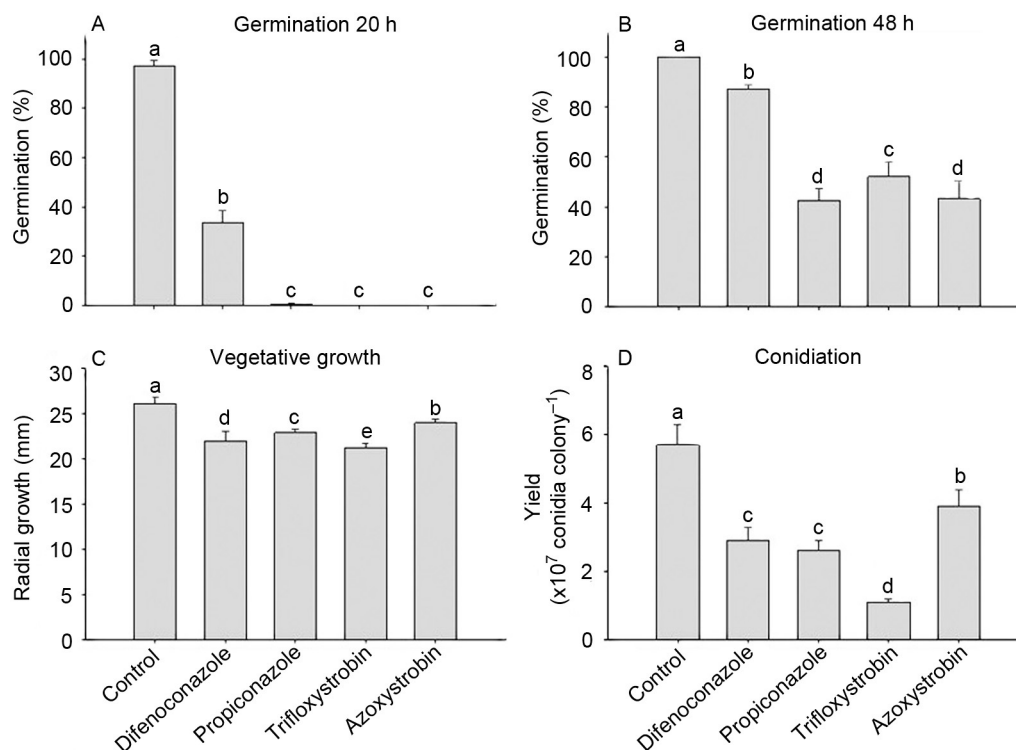


Figure 3 – Effect of fungicides on biological parameters of *Metarhizium anisopliae* CG 168 at 25 °C and 12 h photophase. Means followed by the same letters are not significantly different by Fisher's LSD test ( $p \leq 0.05$ ). Number of replicates per treatment was 6-8. Only distilled water plus Tween 80 (0.01 %) was used in the control group.

Some of the agrochemicals tested delayed conidial germination of *M. anisopliae* as seen in the increased germination from 20 to 48 h incubation. As example, Fipronil (25 % w/v), glyphosate, difenoconazole, propiconazole, trifloxystrobin and azoxystrobin had fungistatic activity on conidial germination at 20 h; however by 48 h germination of the fungus was still occurring after mixing it with these products. The fungicide azoxystrobin inhibited conidial germination at 20 h but allowed larger mycelial growth and greater conidiation compared to the other fungicides, and thus was classified as compatible according to the Biological Index formula. The effect of these products on conidial germination was probably due to the active ingredient and/or to some components of the inert carriers in the formulation. Quintela and McCoy (1998) observed that one component of the inert carrier of the insecticide Admire™ 2 F (Bayer Corporation) affected conidial germination of *M. anisopliae*. The fungicidal activity of the chemical group strobilurins (azoxystrobin and trifloxystrobin) relies on the ability of the active ingredient to disrupt energy production in fungal mitochondria and consequently prevents spore germination. In the triazole fungicides (difenoconazole and propiconazole), ergosterol biosynthesis is inhibited; consequently preventing fungal cell membranes formation (Bartlett et al., 2002). Our results are in agreement

with the mode of action of the strobilurins azoxystrobin and trifloxystrobin as they negatively affected conidial germination at 20 and 48 h, meanwhile the triazoles difenoconazole and propiconazole reduced conidial germination, mycelial growth and conidiation.

Herbicide 2,4-D and the insecticide cypermethrin were the most harmful to *M. anisopliae* CG 168 as they completely inhibited conidial germination, vegetative growth and conidiation. The compound 2,4-D acts by inhibiting the enzyme acetyl-CoA carboxylase, and as a result, prevents the biosynthesis of fatty acids and glucosylceramides, which are components of membrane lipids of animals, plants, and fungi (Leipelt et al., 2001). It remains unclear how the insecticide cypermethrin or other cypermethrin-based insecticides act on fungi. In contrast to the results obtained in the present study, Rachappa et al. (2007) pointed out that pyrethroids were safer to the developmental stages of *M. anisopliae*, but this interaction depends on methodological procedures, pesticide formulations and fungal strains. The compatibility of *M. anisopliae* with thiamethoxam and lambda-cyhalothrin is in agreement with other studies (Batista Filho et al., 2001; Cavalcanti et al., 2002; Loureiro et al., 2002; Neves et al., 2001; Rampelotti-Ferreira et al., 2010). Despite the insecticide thiamethoxam + lambda-cyhalothrin is a combination of two compatible insecticides.

ticides, it was scored as moderately toxic in this study. The combination of two or more active ingredients in an agrochemical may reduce or increase the degree of compatibility via synergism or antagonism (Alves, 1998). In the present study, none of the agrochemicals tested were synergistic to *M. anisopliae*.

At doses of 160 and 240 mL ha<sup>-1</sup> the insecticide fipronil (25 % w/v) was not detrimental to *M. anisopliae* mycelial growth and conidiation; therefore, it was scored as compatible (Rampelotti-Ferreira et al., 2010). However, in our study, this product was classified as moderately toxic to *M. anisopliae* CG 168 when using the same Biological Index. These results cannot be compared because the experimental methodology and fungal isolates were different. These authors as well as Schumacher and Poehling (2012) incorporated the test pesticides into PDA medium. Conversely, in our study the pesticides were added to an aqueous suspension of conidia for three hours of exposure under constant agitation in a rotary shaker. We provided a novel and more reliable compatibility method based on a tank mixing with fungal conidia and agrochemicals for an integrated application approach, enabling conidia to be totally exposed to the chemical product for a certain period of time, and thus can be considered a more realistic method than the other in which the pesticide is incorporated into the solid medium. The precipitation of some chemicals, especially those in granular and wettable powder formulations, cannot be avoided with the previous method (solid medium). Therefore, these pesticides may not have an even dilution in the solid medium due to differences in density, which would compromise the direct contact between the chemical and conidia (Silva et al., 2005).

According to the Biological Index, the fungicide azoxystrobin was classified as compatible with *M. anisopliae*, enabling normal vegetative growth and sporulation, although there were no germinated conidia after 20 h of incubation. This result seems anomalous, because a compatible pesticide should not affect conidial germination. Any chemical pesticide preventing conidial germination up to 20 h should not be used in combination with this fungus, since the conidial germination is the first step to initiate the fungal infection process in the insect. If conidial germination is delayed or inhibited, the potential for the fungus to infect insects in the field will be greatly reduced. In the field, fungal conidia must cope with abiotic and biotic factors detrimental to survival and most of conidial survival on the leaves is lost after 24 h because of environmental constraints such as solar radiation, high temperature, low relative humidity, rainfastness, and plant allelochemicals (Jaronski, 2010). In addition, the high and fast germination rate for fungal entomopathogens has a positive relationship with their virulence toward a host (Altre et al., 1999; Hassan et al., 1989; Rangel et al., 2008). Faster conidial germination indicates non-stressed conidia which play an important role for the success of biological control of insects and should be the major concern in quality control protocols

for conidia-based mycoinsecticides (Faria et al., 2010). Faria et al. (2010) assumed that only vigorous conidia of *Beauveria* and *Metarhizium* which germinated quickly within 24 h incubation should be considered suitable for insect control. On the other hand, conidia germinating after 24 h should be considered debilitated spores (low vigor), as they may cause low insect mortalities and thus should not be used for insect control. As a result, the outcome from conidia that germinated at 20 h instead of at 48 h to calculate the Biological Index was used (Table 2), since those vigorous and non-stressed conidia are able to germinate faster and thus are much more likely to have success in germinating and penetrating through the insect cuticle and escape from environmental stresses.

The fact that insecticides, fungicides and herbicides inhibit mycelial growth of a fungal entomopathogen is not necessarily indicative of reduction in sporulation and conidial germination and vice versa (Zimmermann, 1975). Some agrochemicals can delay mycelial growth, although they can stimulate higher conidiation later, presumably in response to stresses caused by the chemical product, perhaps due to the reduced early mycelial growth. In other cases, the fungus grows well during its vegetative development, but later conidiation does not take place for reasons which are not understood. There is no positive relationship between vegetative growth and conidial yield and the factors that govern this outcome warrant further detailed investigations (Tamai et al., 2002). In the current Biological Index proposed by Rossi-Zalaf et al. (2008), there is a low weight attributed to the germination parameter (i.e., 10 %) in comparison to the other variables (vegetative growth and conidiation). As a result, the Biological Index may in some cases mask the real toxic impact of an agrochemical on entomopathogenic fungi, since 90 % of the formula is attributed to vegetative growth and sporulation.

Conidial germination is more important than vegetative growth and sporulation on cadavers, because the former corresponds to the first step that triggers an epizootic, and the fungus relies on it to infect the host successfully (Alizadeh et al., 2007; Khalil et al., 1985). Thereby, if an agrochemical causes substantial decrease in conidial germination, it may reduce the effectiveness of the entomopathogen toward its target. As mycelial growth develops inside the insect host and the concentration of agrochemicals, especially those with systemic mode of action, are usually found in low titer in the hemolymph, there is little chance of this developmental stage to be negatively affected (Khalil et al., 1985). On the other hand, vegetative growth and conidiation are also important in regards to secondary infections caused by fungi and thus they should be considered for fungal virulence and persistence in the environment (Schumacher and Poehling, 2012). The current Biological Index for toxicological classification of pesticides has merit regarding the ability of a fungus to grow saprophytically in the environment and to produce secondary inoculum sources through sporulation on cadavers. Such factors

are related to the conservational approach for entomopathogens in agroecosystems. Based on all the facts mentioned above, we have identified a situation where this formula is not reliable since it produced a classification of compatible for an agrochemical that reduced conidial germination. Therefore, we strongly contend that the formula of Biological Index proposed by Rossi-Zalaf et al. (2008) must be revised carefully by researchers giving special attention to improving the incorporation of information regarding conidial germination.

For successful control of *T. limbativentris* in rice crops adopting integrated pest management, the awareness of such compatible agrochemicals is very useful to ensure safety fungal application and to facilitate the combination of the fungus with chemical products. On the other hand, for those chemicals not compatible with the fungus, there are two strategies which might be employed to prevent *M. anisopliae* from harmful exposures: i) plan fungus application for two to four days before the chemical spraying (the exact time depends on the residual effect of each product), so that the fungus would have enough time to infect the host (Kouassi et al., 2003); and ii) use of a conidial formulation in oil to reduce the fungistatic effect of pesticides on fungal performance. Recently, Lopes et al. (2011) showed that an oil-based formulation afforded protection to aerial conidia of *M. anisopliae* against products with fungistatic or fungicide activity and this formulation also enhanced fungal virulence on *Diatraea saccharalis* (Lepidoptera: Crambidae) larvae. In addition, there may be some compatible insecticides which can be used at low label rates in combination with the fungus aiming to lower the insect immunity and consequently increase fungal effectiveness.

In summary, our findings from *in vitro* compatibility indicate that the tested fungicides are more detrimental to conidial germination, mycelial growth and sporulation of *M. anisopliae* CG 168 than herbicides and insecticides, and they should not be applied together with this fungus in tank mixing. The agrochemicals compatible with *M. anisopliae* CG 168 are: insecticides methyl parathion (240 mL ha<sup>-1</sup>), thiamethoxam (31 g ha<sup>-1</sup>), and lambda-cyhalothrin (6.3 mL ha<sup>-1</sup>); herbicides glyphosate (1560 mL ha<sup>-1</sup>), bentazon (720 mL ha<sup>-1</sup>), and imazapic + imazapyr (84 g ha<sup>-1</sup>). Although the results of our *in vitro* study did not consider the effect of many variables associated with field use of pesticides, our findings are of paramount importance to guide and advice farmers to use safely this entomopathogen in combination with pesticides registered for rice crops without affecting fungal virulence and germination.

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