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Enhanced susceptibility of *Tibraca limbativentris* (Heteroptera: Pentatomidae) to *Metarhizium anisopliae* with sublethal doses of chemical insecticides

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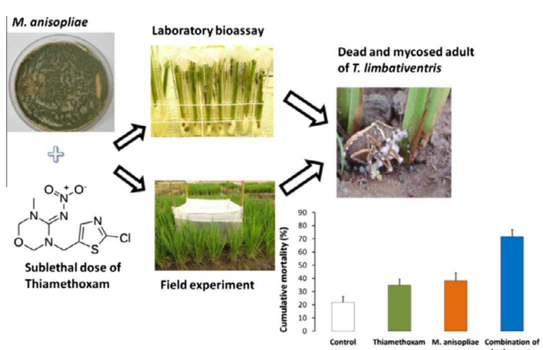
HIGHLIGHTS

- Adults of *Tibraca limbativentris* are more susceptible to thiamethoxam than to lambda-cyhalothrin.
- *T. Limbativentris* exhibited a natural resistance to *Metarhizium anisopliae*.
- Subdoses of chemical insecticides enhanced susceptibility of *T. limbativentris* to *M. anisopliae*.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigated the interaction of the fungus *Metarhizium anisopliae* (Metsch.) Sorok. with sublethal doses of synthetic chemical insecticides thiamethoxam and lambda-cyhalothrin for the control of *Tibraca limbativentris* adults under laboratory and field conditions. Median lethal time (LT_{50}) was reduced significantly when *M. anisopliae* (5×10^6–5×10^8 conidia/mL) was combined with a sublethal dose (0.77 ppm AI) of thiamethoxam compared with fungus only. A similar response on host mortality was observed for *M. anisopliae* at 5×10^7 conidia/mL in combination with sublethal dose of lambda-cyhalothrin at 9.33 ppm (AI). Additionally, the thiamethoxam-fungus combination increased overall mortality and percent mycosed insects in comparison to their counterparts alone. Increasing fungus concentration did not increase insect susceptibility when combined with thiamethoxam either at 0.77 or 0.38 ppm (AI). In a field experiment, the combination of *M. anisopliae* at 1×10^{12} viable conidia/ha with thiamethoxam at 12.5 g (AI)/ha (¼ full dose) synergistically increased mortality and mycosis of adults of *T. limbativentris*. Therefore, enhanced *T. limbativentris* control could potentially be achieved within label rates of fungus (5×10^6 conidia/mL) and sublethal thiamethoxam (0.77 ppm). The strategy of using sublethal doses of chemical insecticides in combination with entomopathogenic fungi is a promising approach to battle the rice stalk stink bug in rice fields.

1. Introduction

The rice stalk stink bug, *Tibraca limbativentris* Stal. (Heteroptera: Pentatomidae), is a major pest of rice-growing areas (*Oryza sativa* L.) in Brazil and other countries in South America (Pantoja, 1997). This insect, particularly the adult stage, can reduce rice yield by 10–80% (Costa and Link, 1992; Ferreira et al., 1997). Nymphs and adults feed on the developing stalks following beginning of vegetative tillering, but the main damage takes place during pre-flowering and panicle formation (Costa and Link, 1992). Direct damages are divided into early attacks, which cause dead hearts,

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and late attacks, which produce white heads where panicles become empty with unfilled grains (Ferreira et al., 1997).

The irrigated environment where nymphs and adults of the rice stalk stink bug are located are conducive for the development of entomopathogenic fungi due to the high humidity conditions (Martins et al., 2004). In the offseason, the rice stalk stink bug hibernates at the base of different plant species along the soil surface where there is high moisture (Link et al., 1996). When the adults migrate to new rice fields, they initially infest the base of plants, among the stalks, where there is generally no standing water (Botton et al., 1996). We have observed epizootics of *Metarhizium anisopliae* (Metsch.) Sorok. (Hypocreales: Clavicipitaceae) complex on populations of *T. limbativentris* under greenhouse conditions and also under irrigated rice field in states of Goiás and Mato Grosso, Brazil. Although environmental conditions are suitable for the use of fungi, many studies have shown that *M. anisopliae* has controlled only 50–60% of this insect (Martins and Lima, 1994; Martins et al., 1997, 2004). Low rate of infection by entomopathogenic hypocrealean fungi on related pentatomid species infesting soybeans has also been observed for *M. anisopliae* and *Beauveria bassiana* (Sosa-Gómez et al., 1993). Subsequently, it was determined that chemical compounds in the *Nezara viridula* (L.) cuticle were able to reduce adhesion, germination and were also fungistatic to conidia of *M. anisopliae* (Borges et al., 1993a; Sosa-Gómez et al., 1997).

One promising approach to overcome host inherent resistance of *T. limbativentris* to entomopathogenic fungi involves exploiting potential synergistic interactions with chemical insecticides, especially the 4A class (neonicotinoids), which can be used to target this pest (Anderson et al., 1989; Boucias et al., 1996; Hiromori and Nishigaki, 2001; Kaakeh et al., 1997; Pachamuthu and Kamble, 2000; Quintela and McCoy, 1998a, 1998b, 1997; Russel et al., 2010). Prior works have demonstrated that sublethal doses of insecticides can increase stress and compromise the immune system or otherwise alter the insect behavior that leads to improved performance of the fungal pathogen (Boucias et al., 1996; Hiromori and Nishigaki, 2001; Quintela and McCoy, 1998a). Integrating sublethal doses of insecticides with fungal entomopathogens can increase pest mortality as well as reduce the time to kill in comparison with either agents alone (Pachamuthu and Kamble, 2000; Paula et al., 2011). However, studies on combinations of entomopathogenic fungi and insecticides for the control of pentatomid stink bugs are lacking.

We hypothesized that the combination of pathogen and chemical insecticides might overcome this natural cuticle-chemical barrier of the insect to the fungus. Our specific objectives were: (1) determine the sublethal concentrations of thiamethoxam and lambda-cyhalothrin to adults; (2) compare the virulence of two strains and an emulsifiable oil formulation of *M. anisopliae* toward adults; (3) evaluate the susceptibility of adults to different rates of the fungus *M. anisopliae* (strain CG168) alone or combined with low rates of thiamethoxam and lambda-cyhalothrin; (4) assess the efficacy of the combined treatment of *M. anisopliae* with subdose of thiamethoxam under flooded rice field conditions.

2. Materials and methods

2.1. Insect colony

Adults of *T. limbativentris* were obtained from a greenhouse colony at the National Rice and Beans Research Center of the Brazilian Agricultural Research Corporation (EMBRAPA Rice and Beans) located at 16°28′00″ S, 49°17′00″ W and 823 m a.s.l. Insects were fed on potted rice plants (*Oryza sativa* L.) cv. BR-IRGA 409. The insect colony was derived from a field population originally collected in Santo Antônio de Goiás, state of Goiás, Brazil. Adults with specific age after emergence were collected from this stock colony and used in all experiments.

2.2. Fungi and insecticides

Strain CG168 (=ARSEF1883, USDA, Ithaca, NY) of *M. anisopliae* was originally isolated from *T. limbativentris* during a greenhouse epizootic at EMBRAPA Rice and Beans in 1985. This strain is preserved at −80 °C in the Laboratory of Invertebrate Mycology, at EMBRAPA Genetic Resources and Biotechnology (Brasília, Brazil). This fungal strain was identified through the multigene sequencing approach described by Bischoff et al. (2009). A commercial strain ESALQ-1037 of *M. anisopliae* sensu lato (Metarril, Itaforte BioProdutos Ltda., Itapetininga, SP, Brazil) isolated in 1992 from *Solenopsis invicta* in Porto Alegre, state of Rio Grande do Sul, Brazil, was also used. The fungi were cultured on PDA (200 g potato infusion +5 g dextrose +15 g agar with 0.2 g tetracycline per liter) and incubated for 10–15 d at 26 ± 1 °C, 70 ± 10% relative humidity (r.h.), 12:12 (L:D) h photoperiod. Conidial viability was determined by counting germ tubes produced on PDA plates after 20 h of incubation using a phase contrast microscope at 400× magnification. Conidia viability was >90% in all cases.

The synthetic chemical insecticides used in the experiments were thiamethoxam (Actara™ 250 WG [dispersible granules], 25% [AI], technical grade 3-(2-cloro-tiazol-5-ilmetil)-5-metil-[1,3,5] × adiazinan-4-ilideno-N-nitroamina) and lambda-cyhalothrin (Karate Zeon™ 50 CS [suspension of microcapsules], 5% [AI], technical grade (S)-a-cyano-3-phenoxybenzyl(Z)-(1R,3R)-3-(2-chloro-3,3,3-rifluoro prop-1-enyl)-2,2-dimethylcyclopropanecarboxylate and (R)-a-cyano-3-phenoxybenzyl(Z)-(1S,3S)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate (Syngenta Crop Protection, 2010). These products are currently registered for the control of *T. limbativentris* in rice crops in Brazil (Agrofit, 2012).

2.3. Determination of sublethal concentration of chemical insecticides to adults of T. limbativentris

In test 1, a topical bioassay with thiamethoxam diluted in sterile distilled water (dH_2O) to concentrations of 6.25, 3.1, 1.6, 0.77, 0.38, and 0.19 ppm (AI) was used to determine the sublethal concentration (LC_{30}) to adults of *T. limbativentris*. Adults 15 days old were anesthetized with CO_2 for 10 s and treated dorsally with 10 µL of each chemical concentration placed on the adult's pronotum using a micropipette. The control group was treated with sterile dH_2O. Treated insects were placed in groups of five into glass tube vial (20 × 2.5 cm) and then fed with three rice stalks (cv. BR-IRGA 409). The rice stalks was previously surface sterilized with bleach solution (containing 2–2.5% of sodium hypochlorite) at 1% v/v and rinsed twice with distilled water. The base of the rice stalk was surrounded by moist cotton. The tubes were sealed with cheesecloth (30-µm mesh size) and fixed by a rubber band. There were six replicates (tubes) containing five insects each (30 insects per treatment). The tubes were incubated at 26 ± 1 °C, 70 ± 10% r.h. and 12:12 (L:D) h photoperiod inside a growth chamber. Adult mortality was monitored daily over 14 days.

In test 2, formulated lambda-cyhalothrin (Karate Zeon™ 50 SC, Syngenta, Greensboro, NC, USA) was tested at concentrations of 2.5, 5.0, 7.5, 10, and 12.5 ppm (AI). The experimental protocol was the same as for test 1. Tests 1 and 2 were repeated twice on different occasions.

2.4. Comparison of virulence between two strains of *M. anisopliae* on *T. limbativentris* adults

The conidial virulence of our experimental strain (CG168) to *T. limbativentris* adults was compared with a commercial oil formulation of *M. anisopliae* strain ESALQ-1037 at 5×10^7 conidia/mL. All conidial suspensions were prepared in 0.01% v/v aqueous polyoxyethylene (80) sorbitan monooleate (Tween 80) (Vetec™ Química Fina Ltda., RJ, Brazil). Tween 80 at 0.01% v/v was included as a control. Adults were topically inoculated as described in 2.3. All treatments were incubated in a growth chamber at 27 ± 2 °C, 70 ± 12% r.h. and 12:12 (L:D) h photoperiod. Each treatment was replicated six times and each replicate contained five insects (30 insects/treatment). Dead adults were recorded daily over 12 days and then transferred to 90-mm Petri dishes containing small pieces of moist cotton to determine sporulation from *M. anisopliae*. This experiment was repeated twice on different dates.

2.5. Interaction between *M. anisopliae* CG168 and two insecticides against *T. limbativentris* adults

Four bioassays were conducted to assess sub-lethal doses of thiamethoxam (0.38–0.77 ppm) and *M. anisopliae* (5×10^7–5×10^8 conidia/ml), tested alone and in combination (Table 2). In the fifth bioassay, lambda-cyhalothrin was tested alone at 9.33 ppm and *M. anisopliae* alone at 5×10^7 conidia/mL and in combination. Conidial suspensions and chemical solutions were prepared in 0.01% v/v aqueous Tween 80. Tween 80 at 0.01% was included as control in all bioassays. Treatments were applied topically onto *T. limbativentris* adultsas described in 2.3. Each treatment was replicated six times with five adults per replicate (30 insects/treatment). The treatments were incubated in a growth chamber at 27 ± 2 °C, 70 ± 12% r.h. and 12:12 (L:D) h photoperiod. Dead and live bugs were recorded daily as well as mycosis on cadavers during 15 days. Each bioassay was repeated twice on different dates.

2.6. Field studies with *M. anisopliae* and thiamethoxam

The experiment was carried out in Palmital Farm at EMBRAPA Rice and Beans, (Brazabrantes, GO, Brazil) located at 16°26′13″ S and 49°23′46.3″ W. The treatments consisted of: (1) Thiamethoxam at 50 g [AI]/ha (recommended full dose); (2) Thiamethoxam at 12.5 g [AI]/ha (sublethal dose), (3) Emulsifiable oil dispersion of *M. anisopliae* ESALQ-1037 (Metarril) at 1 L/ha (1×10^{12} viable conidia/ha), (4) Combination of thiamethoxam (12.5 g/ha) and *M. anisopliae* (1 L/ha), (5) Control (water). The rice cv. Jaçanã was sown (30 cm spacing) in an area of 100 × 15 m in December, 11 2009. The experimental area was immediately flooded and drained after 46 days (Jan/26/2010), when five nylon cages of 1 m^3 per treatment were erected in a randomized complete block design. The distance between cages was 5 × 2 m. Each cage was infested with 20 pairs of 10 day old *T. limbativentris* adults. One day after infestation, treatments were applied with a CO_2 backpack sprayer fitted with a flat nozzle and operating at 25 PSI. The treatments were sprayed at a rate of 30 mL/m^2 inside each cage. Adult mortality inside field cages was checked after seven and 14 days. In addition, both sexes of *T. limbativentris* (10 per cage) were collected one and seven days after spraying and evaluated for mortality over 12 days in the laboratory as described in 2.3.

2.7. Statistical analyses

To estimate the sublethal concentrations for chemical insecticides to adults of *T. limbativentris*, the relationship between cumulative mortality response and insecticide concentrations followed the binomial distribution with response probability given by the equation $p'_i = C + (1 - C)^* p_i$, where p'_i is the probability of mortality, c_i is the insecticide concentration (ppm), C is the mortality in the control group, and p_i is the mortality probability caused by the insecticides (thiamethoxam or lambda-cyhalothrin) (Robertson and Preisler, 1992). In the Probit procedure of SAS (PROC PROBIT) (SAS Institute, 2008), the mortality data was adjusted using Abbott's equation, which considered the mortality in the control group before doing probit analysis. To choose the most appropriate model to fit the mortality data and estimate the sublethal concentrations, the goodness-of-fit of three models (Probit, Gompertz and Logit) were compared on basis of their deviance values (e.g., Pearson χ^2/degrees of freedom) (Robertson and Preisler, 1992). The Probit model (with normal distribution) was chosen to fit the data, as it provided the lower deviance.

Percentage of cumulative mortality and *M. anisopliae* mycosis data from laboratory and field tests were checked for normality assumptions and homogeneity of residual variances prior to analysis. Data did not need to be transformed and were directly subjected to one-way analysis of variance (ANOVA, PROC GLM) with treatment means compared by Fisher's protected least significant difference (LSD) at $\alpha = 0.05$ (SAS Institute, 2008). Data from experiments repeated on different dates were grouped. For all tables and figures, untransformed means are presented.

The median lethal time (LT_{50}) and survival curves were estimated using Kaplan-Meier survival analysis with treatments separated through a Log-Rank (Mantel-Cox) test at $\alpha = 0.05$ (SPSS for Windows, 2008). The interaction between fungus and insecticide was determined by the statistical method reported by Farenhorst et al. (2010), based on the T-Student ($\alpha = 0.05$) test, which compares the observed and expected mortality for the mixture (Mfi). The expected mortality was given as $Me = Mf + Mi(1 - Mf/100)$, where Mf and Mi were % observed mortality caused by the fungus and insecticide alone, respectively. When T-test is significant and $Mfi - Me > 0$ (positive), it is considered synergism. If T-test is significant and $Mfi - Me < 0$ (negative), then the interaction is antagonistic. Finally, if T-test is not significant and the observed mortality for combined treatment is higher than each single agent, thus the effect can be considered additive.

3. Results

3.1. Sublethal concentration of thiamethoxam and lambda-cyhalothrin

Adults of *T. limbativentris* were more susceptible to thiamethoxam than to lambda-cyhalothrin. The estimated LC_{30} values of thiamethoxam for *T. limbativentris* were 12 times lower than lambda-cyhalothrin, at nine days after application (Table 1). The relationship between adult mortality and insecticide concentration (adjusted for control mortality) was best described by the probit model for both thiamethoxam and lambda-cyhalothrin (Table 1).

3.2. Comparison of virulence between two strains of *M. anisopliae*

The virulence of the two strains of *M. anisopliae* toward *T. limbativentris* adults was similar irrespective of formulation, but all fungal treatments killed adults faster than the control ($\chi^2 = 8.14$, $P = 0.0432$) (Fig. 1A). Neither *M. anisopliae* strains reached 50% adult mortality, thus LT_{50} values were not estimated. Adult mortality in all fungal treatments was two-fold higher when compared with the control ($F_{[3,44]} = 6.52$, $P < 0.0001$). The percentage of insects that supported sporulation was similar for CG168 and ESALQ-1037 either applied as aqueous conidial suspension or as oil-based formulation ($F_{[2,33]} = 1.15$; $P < 0.3289$) (Fig. 1B).

Table 1
Determination of sublethal concentrations of thiamethoxam and lambda-cyhalothrin on *Tibraca limbativentris* adults using the Probit model, under laboratory conditions.

Active ingredient	No. insects used	Probit equation[a]	$\chi2$ (*P* value)	LC_{30} (AI ppm) [95% FL][b]
Thiamethoxam	540	$Y = 0.1146 + (1 - 0.1146)[\theta(-0.3357 + 1.6471\ \log(\text{dose})]$	59.92 (0.9976^{ns})	0.77 [0.49 – 1.08]
Lambda-cyhalothrin	360	$Y = 0.1946 + (1 - 0.1946)[\theta(-4.4236 + 4.0214\ \log(\text{dose})]$	33.27 (0.9963)	9.32 [1.52 – 11.11]

[a] *Y* was the proportion of mortality and θ the accumulated normal distribution.
[b] The above lethal concentration (LC) and 95% fiducial limits (FL) refer to effects due to the independent variable (e.g. chemical insecticide) and do not include any effect due to the control mortality. The control mortality was 11.5% and 19.5% in control groups for thiamethoxam and lambda-cyhalotrin bioassays, respectively.

Table 2
Interactions between *M. anisopliae* CG168 and thiamethoxam or lambda-cyhalothrin based on observed mortality from pooled means of two sets of laboratory bioassays.

Combined treatment	Cumulative mortality at day 15 (%)		*T*-test[b]	*P* value
	Observed	Expected[a]		
Thiamethoxam (0.77 ppm) + Ma (5 × 106)	66.7 ± 5.7	57.7 ± 4.3	1.59	0.2212
Thiamethoxam (0.38 ppm) + Ma (5×10^7)	50.0 ± 4.6	51.3 ± 4.3	0.05	0.8336
Thiamethoxam (0.77 ppm) + Ma (5×10^7)	71.7 ± 5.2	59.0 ± 4.9	3.11	0.0915
Thiamethoxam (0.77 ppm) + Ma (5×10^8)	63.3 ± 5.4	71.7 ± 2.3	2.00	0.1714
Lambda-cyhalothrin (9.33 ppm) + Ma (5×10^7)	63.3 ± 5.4	66.3 ± 3.6	0.21	0.6499

[a] Expected mortality Me = Mf + Mi (1 − Mf/100), with Mf and Mi being observed percent mortalities caused by the fungus and the insecticide alone, respectively.
[b] Results show outcomes of paired-samples T-test comparisons of observed and expected cumulative mortality rates at day 15 (mean ± SE), with significant synergy indicated in bold.

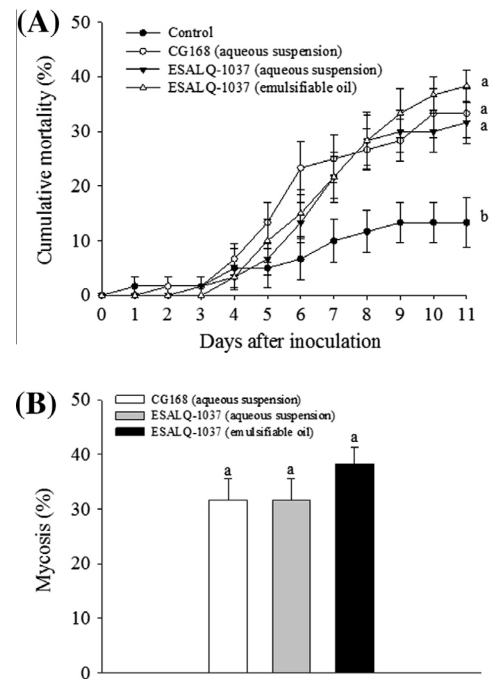

Fig. 1. Cumulative mortality curves (A) and mycosis (B) data for *Tibraca limbativentris* adults inoculated with two strains of *Metarhizium anisopliae*. Mortality curves and mycosed insects (%, mean ± SE) followed by the same letters are not statistically different by the Log-Rank (Mantel-Cox) and LSD test ($P \leqslant 0.05$), respectively.

3.3. Interaction of M. anisopliae with two insecticides

The combination of thiamethoxam at 0.77 ppm with *M. anisopliae* caused 50% adult mortality within 6–7 d (Fig. 2). Adults of rice stalk stink bug exposed to all combined treatments of *M. anisopliae* and thiamethoxam, regardless the label rate, inflicted a significant higher total mortality in relation to each of these agents applied alone, except for the fungus at 5×10^8 conidia/mL. In addition, adults treated with all combinations of fungus with thiamethoxam at 0.77 ppm survived significantly shorter (LT_{50} = 6–7 d) when compared with single applications of *M. anisopliae* and thiamethoxam ($LT_{50} \geqslant 15$ d). The highest LT_{50} (=15 d) was achieved by the mixture of 0.38 ppm thiamethoxam and 5×10^7 conidia/mL *M. anisopliae* resulting in ⩾two-fold slower speed of kill in comparison to the other fungal/insecticide combinations. On the other hand, single treatments of fungus at 5×10^6 or 5×10^7 conidia/mL and thiamethoxam caused <50% mortality of stalk stink bugs, except for the fungus at 5×10^8 conidia/mL. These results suggest that the adults of rice stalk stink bug exhibit a natural resistance to the infection of this mycopathogen.

Survival analysis indicated that *T. limbativentris* adults showed a significant higher susceptibility when exposed to low rates of thiamethoxam (0.38 or 0.77 ppm) combined with different concentrations of *M. anisopliae* (5×10^6–5×10^8 conidia/mL) than the respective single treatments of each agent ($P < 0.05$) (Fig. 2). Thiamethoxam at 0.77 ppm depicted the same fashion of cumulative mortality rate in relation to the single fungal treatments at 5×10^6 ($\chi^2 = 2.985$, $P = 0.084$), 5×10^7 ($\chi^2 = 0.003$, $P = 0.958$) and 5×10^8 conidia/mL ($\chi^2 = 0.909$, $P = 0.34$). The same trend was observed with thiamethoxam at 0.38 ppm with fungus at 5×10^7 conidia/mL ($\chi^2 = 0.993$, $P = 0.319$) (Fig. 2).

Fungal concentrations at 5×10^6 and 5×10^7 conidia/mL with thiamethoxam at 0.77 ppm caused higher mycosis than their single counterparts ($F_{[1,22]} = 55.0$, $P < 0.0001$; $F_{[1,22]} = 16.39$, $P = 0.0005$, respectively) (Fig. 3A, C). While the combination of *M. anisopliae* at 5×10^7 conidia/mL with 0.38 ppm thiamethoxam and *M. anisopliae* at 5×10^8 conidia/mL with 0.77 ppm thiamethoxam did not increase the percentage of mycosis in comparison with the fungus alone ($F_{[1,22]} = 2.17$, $P = 0.1553$; $F_{[1,22]} = 2.0$, $P = 0.1713$, respectively) (Fig. 3B, D).

Adults were equally susceptible to fungus and lambda-cyhalothrin applied alone according to their mortality curves ($\chi^2 = 0.365$; $P = 0.546$) (Fig. 4A). However, when fungus and lambda-cyhalothrin at sublethal dose (9.33 ppm AI) were applied together the mortality rate was higher than their single counterparts ($\chi^2 = 34.64$; $P < 0.0001$) (Fig. 4A) and this mixture rendered a LT_{50} of 8 d (95% FL: 5.9–10.2 d). Lambda-cyhalothrin did not improve the percentage of mycosis by *M. anisopliae* on adult cadavers in relation to the fungus alone ($F_{[1,22]} = 1.11$; $P = 0.3027$) (Fig. 4B).

Finally, T-test showed that combinations of *M. anisopliae* with sublethal doses of thiamethoxam or lambda-cyhalothrin were classified as additive interactions (Table 2).

3.4. Field studies with M. anisopliae and thiamethoxam

Weather conditions during the experiment (monitored from a local weather station) averaged 54.4–82.4% r.h., and 19.5 and 29.4 °C. Ten out of 14 days attained 80% r.h. due to the rain season in January.

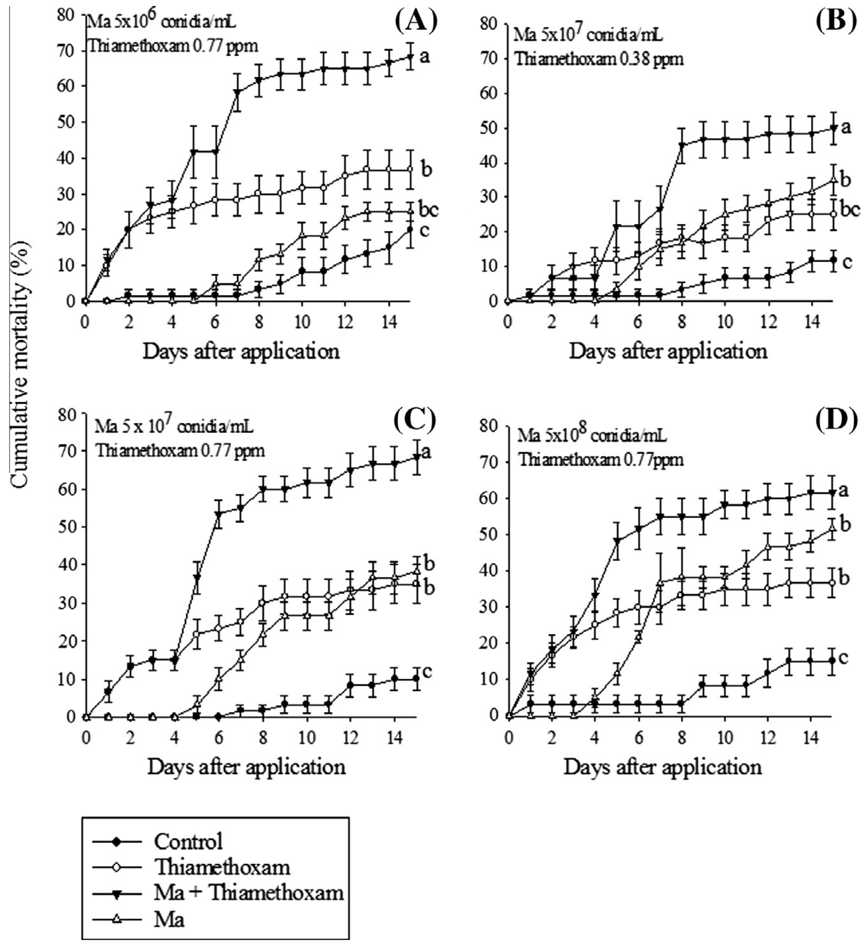

Fig. 2. Cumulative mortality curves (%, mean ± SE) of *Tibraca limbativentris* adults after exposure to single and combined treatments of thiamethoxam and conidial suspensions of *M. anisopliae* (Ma) CG168. Same letters indicate that mortality curves are not significantly different by the Log-Rank (Mantel-Cox) test ($P \leqslant 0.05$).

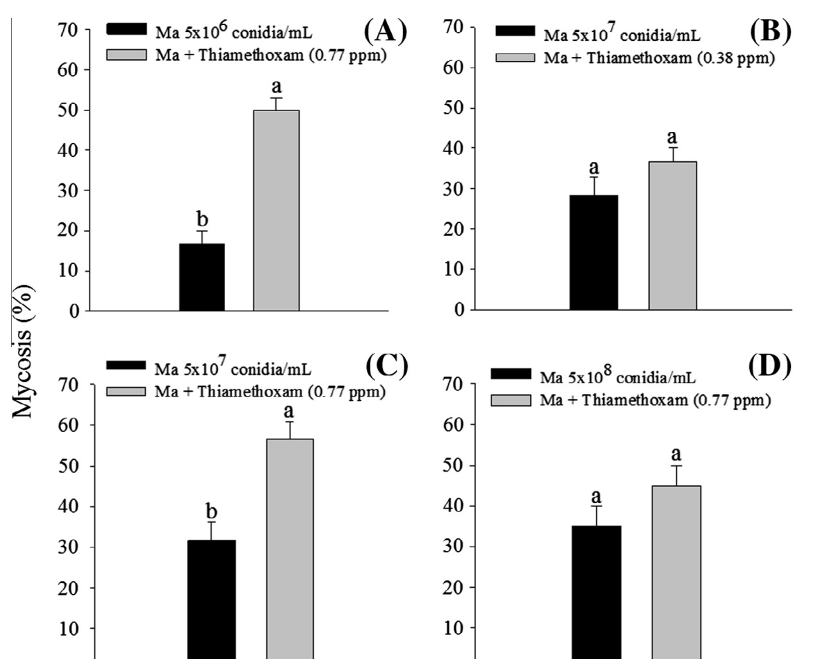

Fig. 3. Percent of mycosis data for *Tibraca limbativentris* after exposure to *M. anisopliae* (strain CG168) applied alone or amended with sublethal doses of thiamethoxam. Bars (%, mean ± SE) followed by the same letters are not significantly different by the LSD test ($P \leqslant 0.05$).

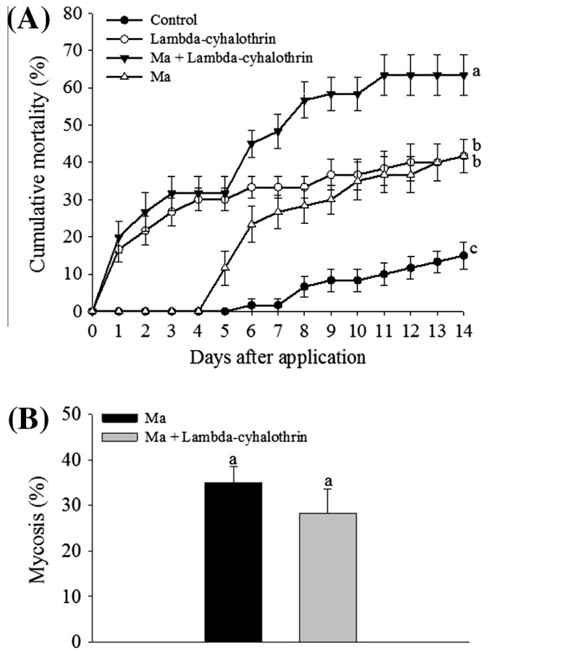

Fig. 4. Cumulative mortality curves (A) and mycosis (B) of *Tibraca limbativentris* adults after exposure to single and combined treatments of lambda-cyhalothrin (9.33 ppm) and conidial suspension of *M. anisopliae* (Ma) CG168 (5×10^7 conidia/mL). Same letters indicate that mortality curves and mycosis (%, mean ± SE) are not significantly different by the Log-Rank (Mantel-Cox) and LSD test ($P \leqslant 0.05$), respectively.

Female and male adults of *T. limbativentris* were equally susceptible to all treatments at 1 day after application (daa) ($\chi^2 = 1.33$, $P = 0.249$) as well as at 7 daa ($\chi^2 = 0.10$, $P = 0.748$) (data not shown).

At 1 daa, adult mortality curve recorded under laboratory conditions after application of thiamethoxam (12.5 g/ha) + *M. anisopliae* (1×10^{12} viable conidia/ha) was not significantly different from the single application of *M. anisopliae* or thiamethoxam at full dose (50 g/ha) ($\chi^2 = 3.05$, $P = 0.061$ and $\chi^2 = 0.35$, $P = 0.552$, respectively) (Fig. 5A). On the other hand, thiamethoxam + *M. anisopliae* mixture caused faster mortality rate in relation to thiamethoxam at sublethal rate (12.5 g/ha) and to the control ($\chi^2 = 7.83$, $P = 0.005$ and $\chi^2 = 18.23$, $P < 0.0001$, respectively). Therefore, the interaction fungus/chemical was considered additive ($t = 0.57$, $P = 0.4731$) (Table 3). The full dose thiamethoxam (50 g/ha) caused rapid adult mortality (18–32%) from day 1 to day 6, while the other treatments did not surpass 10% mortality level during this period (Fig. 5A).

At 7 daa, adult mortality curve by thiamethoxam + fungus application was considerably higher than the other treatments ($\chi^2 = 81.74$, $P < 0.0001$) (Fig. 5B) showing a synergistic effect based on the T-test ($t = 39.67$, $P = 0.0002$) (Table 3). Single application of *M. anisopliae* had higher mortality rate than the control ($\chi^2 = 5.69$, $P = 0.017$), whereas the sublethal rate of thiamethoxam did not promote higher mortality when compared to the fungus alone ($\chi^2 = 2.79$; $P = 0.095$) and to the control ($\chi^2 = 1.03$, $P = 0.31$). However, regarding the total mortality at 12 daa, the mixture of thiamethoxam + fungus resulted in higher mortality than that caused by the other treatments for both field evaluations (1 daa: $F_{[1,16]} = 12.26$, $P < 0.0001$; 7 daa: $F_{[4,16]} = 102.05$, $P < 0.0001$). Moreover, the mycosis level in exposed adults to the interaction was higher than those insects exposed only to the fungus, for the two evaluations (1 daa: $F_{[1,4]} = 10.29$, $P = 0.0327$; 7 daa: $F_{[1,4]} = 45.0$, $P = 0.0026$) (Fig. 5C and D). These results indicate that sublethal rate of thiamethoxam improved the adult susceptibility to mycosis by the fungus. Full dose of thiamethoxam killed only 40% adults at 1 daa and did not cause insect mortality after 7 daa (Fig. 5A and B), which indicates that this insecticide at the recommended dose exhibited a sublethal effect similar to the ¼ of the full dose for field-collected bugs evaluated under laboratory conditions.

When number of dead adults from field and laboratory evaluations were pooled together, the mixture of ¼ dose of thiamethoxam with *M. anisopliae* was the most efficient treatment providing significantly higher number of dead adults/m^2 in comparison to other treatments (Effect of treatment: $F_{[4,16]} = 18.20$, $P < 0.0001$; effect of block: $F_{[4,16]} = 0.78$, $P = 0.553$) (Fig. 6). Meanwhile, the full dose of thiamethoxam resulted in higher dead adults/m^2 than its sublethal dose and the fungus alone.

4. Discussion

Our laboratory studies confirm earlier findings that demonstrate the lower susceptibility of adults of *T. limbativentris* to *M. anisopliae* CG168 (Martins et al., 1997; Martins and Lima, 1994; Rampelotti et al., 2007). Under field conditions, applications of *M. anisopliae* CG168 at 5.0–7.2 $\times$ 10^{13} conidia/ha reduced stalk stink bug in commercial rice farms by 48–62% over three years (Martins et al., 2004). However, the confirmed infection of insects by the fungus was very low ($\leqslant$20% mycosis), suggesting that this insect has low susceptibility to this pathogen. In our study, when the fungus CG168 was tested from 5×10^6 to 5×10^8 conidia/mL, only the highest concentration caused >50% adult mortality. We also showed that an emulsifiable oil-based formulation of *M. anisopliae* (ESALQ-1037) caused <40% adult mortality, similar to CG168. For this reason, the commercial strain ESALQ-1037 was selected to be used in the field experiment, and it is already registered as a mycoinsecticide in Brazil (Agrofit, 2012; Faria and Wraight, 2007).

In order to improve fungus efficacy, sublethal doses of thiamethoxam and lambda-cyhalothrin were combined with the fungus in a series of bioassays. We have previously showed that thiamethoxam and lambda-cyhalothrin were compatible with conidial germination, vegetative growth and sporulation of *M. anisopliae* CG168 (Silva et al., in press). Thiamethoxam is a second-generation neonicotinoid insecticide belonging to the thianicotinyl subclass of chemistry (Jeschke and Nauen, 2008). The pyrethroid product contains the micro-encapsulated lambda-cyhalothrin molecule which has a broad spectrum action against several insect pests (He et al., 2008).

An additive interaction for thiamethoxam/fungus and lambda-cyhalothrin/fungus was observed in all laboratory trials. The median lethal time was reduced with the interactions but diseased insects increased significantly only when the fungus was combined with thiamethoxam. Also, the sublethal concentration of thiamethoxam for *T. limbativentris* adults was lower than lambda-cyhalothrin. Therefore, thiamethoxam was considered to be more efficient to conduct further studies in combination with the fungus. In field studies, when *M. anisopliae* (ESALQ-1037) was combined with ¼ full dose of thiamethoxam, a synergistic effect was observed for this interaction at 7 daa. Thiamethoxam tested at label rate (full dose) had a minimal effect on the insect after 24-hours, providing <40% mortality. We also noticed that this formulation did not persist beyond 7 days after its application (Fig. 5). These results demonstrate the difficulty of controlling *T. limbativentris* population by chemical insecticide, probably because they are located at the base of rice plants, among the stalks. However since thiamethoxam provides excellent control of many other sucking and chewing pests (Jeschke and Nauen, 2008) and has an excellent mammalian and environmental safety profile (Maienfisch et al., 2001), its ability to enhance the susceptibility of the rice stalk stink bug to fungus is significant. Therefore, thiamethoxam is well-suited for use in

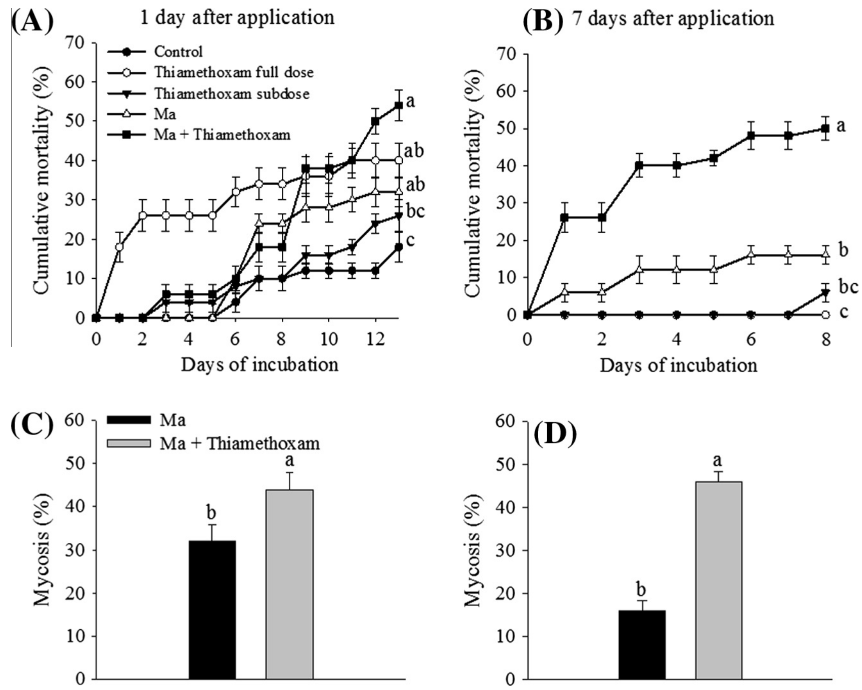

Fig. 5. Cumulative mortality curves (A, B) and mycosed insects (C, D) of field-collected *Tibraca limbativentris* adults at one and seven days after application of single treatments of *M. anisopliae* (Ma) ESALQ-1037 (1×10^{12} viable conidia/ha), thiamethoxam at subdose (12.5 g AI/ha), thiamethoxam at full dose (50 g AI/ha) and one combined treatment of fungus with thiamethoxam (1×10^{12} conidia/ha + 12.5 g AI/ha) under laboratory conditions. Same letters indicate that mortality curves and mycosis (%, mean ± SE) are not statistically different by the Log-Rank (Mantel-Cox) and LSD test ($P \leqslant 0.05$), respectively.

Table 3
Interactions of *Metarhizium anisopliae* at 1×10^{12} viable conidia/ha with thiamethoxam at subdose (12.5 g AI/ha) based on observed mortality assessed in laboratory conditions using insects collected from the field experiment.

Days after application	Cumulative mortality (%)		*T*-test[b]	*P* value
	Observed	Expected[a]		
1	54.0 ± 4.0	49.6 ± 4.3	0.57	0.4731
7	50.0 ± 3.2	21.0 ± 3.3	39.67	0.0002

[a] Expected mortality (Me) = Mf + Mi (1 – Mf/100), with Mf and Mi being observed percent mortalities caused by the fungus and the insecticide alone, respectively.
[b] Paired-samples T-test comparisons of observed and expected cumulative mortality.

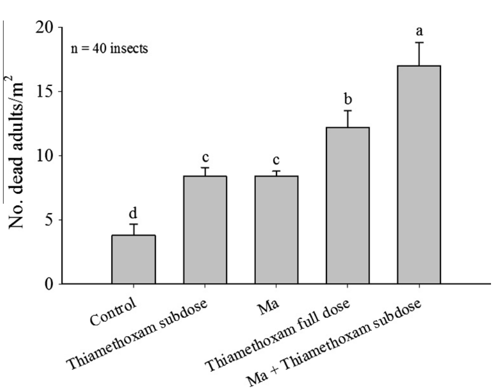

Fig. 6. Effect of thiamethoxam applied alone at sublethal dose (12.5 g AI/ha) and at full dose (50 g AI/ha) and *M. anisopliae* ESALQ-1037 (Ma) at 1 L of emulsifiable oil dispersion/ha (1×10^{12} viable conidia/ha) applied alone or in combination with thiamethoxam at sublethal dose on mortality of *T. limbativentris* adults under field conditions. Means (±SE) followed by the same letter are not significantly different by LSD test ($P \leqslant 0.05$). Bars represent means that were computed by summing the total dead insects recorded in laboratory and at field evaluations.

combination with *M. anisopliae* against *T. limbativentris* in integrated pest management programs.

This fungus is abundant in soil and is found in a large number of ecosystems (Behie et al., 2012). We have observed several epizootics of *M. anisopliae* s.l. on populations of *T. limbativentris,* although fungal epizootics are not generally recorded among the Pentatomidae. For example, field surveys found very low mycosis on stink bugs of soybean (<0.5%) (Moscardi et al., 1988). Prior field research with applications of *M. anisopliae* and *B. bassiana* on caged stink bugs resulted in low mortality levels (<40%) (Sosa-Gómez and Moscardi, 1998). As a result, no reports of natural epizootics of these two major mycopathogens have been mentioned on soybean stink bug populations and other related pentatomid species yet (Sosa-Gómez et al., 1997).

Stink bugs, mainly those members of Pentatomidae, can reduce or even prevent fungal infections by producing antifungal compounds, which can be found impregnated in their epicuticle (Borges et al., 1993b; Moraes et al., 2008). These cuticle extract compounds with fungistatic activity are presented in the alarm pheromone of these stink bugs and they are produced in the scent glands of nymphs and adults (Moraes et al., 2008; Silva et al., unpublished results). As reported earlier by Sosa-Gómez et al. (1997), both the topography and the chemistry of the insect cuticle can affect conidial adhesion of *M. anisopliae*. These authors also pointed out that the aldehyde (*E*)-2-decenal found in the cuticle extract of *N. viridula* inhibited conidial germination of *M. anisopliae*, but did not block the germination of *B. bassiana* and *Isaria fumosorosea*. Our results demonstrated that sublethal concentrations of thiamethoxam enhanced the susceptibility of the rice stalk stink bug adults to *M. anisopliae*, which is considered the most difficult stage to be controlled by the fungus due to the capacity of this insect to produce such aldehydes with fungistatic activity toward *M. anisopliae* CG168 (Silva et al., unpublished results).

There are several studies showing synergistic effects between entomopathogenic fungi and synthetic chemical insecticides toward different insect pests (Anderson et al., 1989; Boucias et al., 1996; Jaramillo et al., 2005; Kaakeh et al., 1997; Quintela and McCoy, 1998a, 1998b, 1997). Some of them identified the factors influencing the synergism. For example, low rates of imidacloprid improved the virulence of entomopathogenic fungi toward the termite *Heterotermes tenuis* (Isoptera: Termitidae) (Moino and Alves, 1998) and *Diaprepes abbreviatus* (Coleoptera: Curculionidae) (Quintela and McCoy, 1998a, 1998b) by affecting the grooming behavior and preventing the larvae from removing conidia from the cuticle, respectively. In another case, Hiromori and Nishigaki (2001) demonstrated that sublethal doses of synthetic insecticides weakened the immune system of *Anomala cuprea* (Coleoptera: Scarabaeidae) facilitating *M. anisopliae* infection. Moreover, Pachamuthu and Kamble (2000) also demonstrated that additive and synergistic effect can vary with the concentration and type of chemical insecticide used in combination with *M. anisopliae* against the cockroach *Blatella germanica* (Dictyoptera: Blattellidae), whereas Jaramillo et al. (2005) observed no significant difference in the susceptibility of *Cyrtomenus bergi* (Hem.: Cydnidae) to different inoculum rates of *M. anisopliae* (strain CIAT) combined with low rates of imidacloprid.

Increased rates of mycosis and sporulation caused by low label rates of thiamethoxam combined with *M. anisopliae* may enhance secondary epizootics. Conversely, Russel et al. (2010) verified that low rates of imidacloprid reduced the fungus mycosis along with the conidial yield per cadaver for *Anoplophora glabripennis* (Coleoptera: Cerambycidae), although they have observed a synergistic effect for the combined treatments of *Metarhizium brunneum* Petch and imidacloprid (at 10 or 100 ppm).

Although there is a great body of literature about the interaction of entomopathogens with sublethal doses of chemical insecticides, our study reports for the first time a synergistic effect of *M. anisopliae* mixed with sublethal dose of thiamethoxam for control of a pentatomid bug. The nymphs and adults of *T. limbativentris* are able to produce a wide variety of chemical compounds by its pheromone scent gland that are antifungal (unpublished data). We speculate that the synergism interaction between *M. anisopliae* and thiamethoxam on *T. limbativentris* might be caused by lowering the production of aldehydes by the alarm pheromone. Additional studies are underway in our laboratory that will help us identify how sublethal concentration of thiamethoxam influence the susceptibility of the rice stalk stink bug to *M. anisopliae* by measuring its effect on the insect immune system and on the production of aldehydes by the alarm pheromone.

In summary, our findings highlight the potential of using sublethal concentrations of the neonicotinoid insecticide thiamethoxam to increase the susceptibility of *T. limbativentris* adults to the fungus *M. anisopliae.* Since rice producing areas are low land often located close to rivers that serve as a source of water for irrigation and also as a drain to receive back the excess of water pumped into the field, it makes rice producing a very fragile environment related to pesticide use. Besides *T. limbativentris*, there is another important stink bug pest of rice, *Oebalus poecilus* (Dallas) that requires treatment with insecticides and is also susceptible to *M. anisopliae*. Therefore, combining biological and chemical control might minimize environmental impacts associated with stinkbug management.

5. Competing interests

The authors declare that they have no competing interests.

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