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Selection of *Beauveria bassiana* sensu lato and *Metarhizium anisopliae* sensu lato isolates as microbial control agents against the boll weevil (*Anthonomus grandis*) in Argentina

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ABSTRACT

The boll weevil (*Anthonomus grandis*) is the main pest of cotton in the Americas. The aim of this work was to evaluate isolates of the entomopathogenic fungi *Beauveria bassiana* sensu lato and *Metarhizium anisopliae* sensu lato virulent against *A. grandis*. Screening was performed to evaluate the pathogenicity of 28 isolates of *M. anisopliae* s.l. and 66 isolates of *B. bassiana* s.l. against boll weevil adults. To select the isolates, LC_{50} values of the most virulent isolates were calculated, and compatibility between the fungi and insecticides was studied. In addition, the effects of these isolates on the feeding behavior of the adults were evaluated. Isolates Ma 50 and Ma 20 were the most virulent against *A. grandis* and their LC_{50} values were 1.13×10^7 and 1.20×10^7 conidia/ml, respectively. In addition, these isolates were compatible with pyrethroid insecticides, but none with endosulfan. On the other hand, infected females reduced the damage caused by feeding on the cotton squares and their weight gain. This shows that entomopathogenic fungi cause mortality in the insects, but also these fungi could influence the feeding behavior of the females. In summary, these results indicate the possibility of the use of *M. anisopliae* s.l. as a microbiological control agent against boll weevils. Also, this species could be included in an Integrated Pest Management program.

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1. Introduction

The boll weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) is the main pest of cotton in North and South America (Burke et al., 1986; Scataglini et al., 2000). Cotton has been cultivated in tropical and subtropical areas, and it is the most important non-food agricultural product in the world. In Argentina, the cotton areas are localized in the northeast region. This crop is important both economically and socially (ICAC, 2011).

Damage to cultivated cotton caused by *A. grandis* was first reported in 1880 in Mexico and has been subject of research since the early 1890s, when it became a cotton pest in the USA. In South America, the boll weevil appeared later and has invaded Brazil, Paraguay, Argentina and Bolivia (Martins et al., 2007; Stadler and Buteler, 2007). This pest causes the most serious damage to cotton crops. Injury to cotton is caused by adults that feed and lay their eggs in the squares (flower buds) and bolls, causing the falling of the squares, fruit abscission, and reduced linter production and quality (Wright and Chandler, 1992).

Chemical insecticides and cultural controls are currently used in the management of the boll weevil population in Argentina (SENASA, 2009). These control tactics have several deficiencies, such as the increase of production costs, reduction of populations of beneficial species, and promotion of the development of insect resistance. Also, insecticides have proved ineffectiveness in the control of this pest (Lange et al., 2009). Problems caused by insecticides used for controlling this pest have led to the application of alternative methods, such as the use of the entomopathogens. Among entomopathogens, fungi have attracted a lot of attention as biologically based pesticides. *Beauveria bassiana* (Bals.-Criv.) Vuillemin and *Metarhizium anisopliae* (Metschn.) Sorokin are two of the most common species of entomopathogenic fungi investigated. Both *Beauveria* spp. and *Metarhizium* spp. are cosmopolitan anamorphic genera of soilborne facultative necrotrophics arthropod-pathogenic fungi (Bischoff et al., 2009; Rehner et al., 2011). These two fungi offer promise in the microbial control of certain economic crop pests (Jaronski, 2010). Among the most important aspects of exposure to entomopathogenic fungi, sublethal effects have been described. There have been studies of such effects, which include modifications of feeding behavior (Ekesi 2001; Fargues et al., 1994; Moore et al., 1992; Seyoum et al., 1994; Tefera and Pringle, 2003) and/or alterations of the survival and reproductive potential of the progeny of populations exposed to entomopathogens (Baverstock et al., 2006; Gindin et al., 2006; Torrado-León et al., 2006). Although insects killed by entomopathogenic fungi often take longer to die than if treated with chemical pesticides, damage to crops is decreased

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during the disease incubation period because infected insects eat less than healthy ones (Roy et al., 2006).

One of the most promising aspects of microbial control of insects is its integration with other pest control measures, particularly the chemical method (Neves et al., 2001). It is important to know the compatibility of pathogens with other agricultural practices to avoid losses of control efficiency. The use of incompatible chemical products may inhibit the development and reproduction of these pathogens, affecting pest control (Jaronski, 2010). The action of chemical insecticides on the entomopathogenic fungi can vary depending on the species or pathogen strain, chemical nature of the product, and dosage used (Purwar and Sachan, 2006). Currently, crop protection is based on Integrated Pest Management (IPM), which includes all the available techniques in a compatible manner, along with reasonable use of different agrochemicals (Shah and Pell, 2003). The knowledge of the interaction between entomopathogenic fungi and pesticides may facilitate the choice of the products used in IPM programs.

The potential of entomopathogenic fungi as biocontrol agents, particularly of *B. bassiana* and *M. anisopliae*, has been evaluated against several weevils species as *Callosobruchus maculatus* (Murad et al., 2006), *Conotrachelus nenuphar* (Alston et al., 2005), *Metamasius spinolae* (Tafoya et al., 2004), *Rhynchophorus ferrugineus* (Dembilio et al., 2010), *Sitophilus zeamais* (Adane et al., 1996). Furthermore, these fungi are able to cause death in the boll weevil, both in the laboratory (Domingues da Silva, 2001; Giometti et al., 2010; Wright and Chandler, 1991) and in the field (Wright, 1993; Wright and Chandler, 1992).

It is desirable to work with properly selected native entomopathogenic isolates to control the boll weevil and to evaluate different aspects of the fungus on the interaction not only with the insect, but also with the chemical insecticides which will be used. For this reason, the main goal of this work was to evaluate different Argentinean isolates of *B. bassiana* s.l. and *M. anisopliae* s.l. for the control of *A. grandis*. Several isolates were researched about their pathogenicity, virulence, effects on feeding behavior and compatibility with different insecticides used in the field. Finally, the most effective isolates were selected for their possible use as mycoinsecticides.

2. Materials and methods

2.1. Insect culture

The boll weevils were collected from rural areas of Santa Fe province, Argentina (28°5′06.11″S; 59°14′37.53″W; Florencia). Insect colonies were reared in the laboratory at 27 ± 1 °C, 50 ± 10% humidity, a 12:12 photoperiod and fed with an artificial diet (Lecuona, 2009). The in vitro assays were carried out using 24-h old red adults and insects were fed using a diet without fungicides. Once the assays were performed, insects were maintained at a controlled room environment with 27 ± 1 °C, 50 ± 10% humidity, and a 12:12 photoperiod. The artificial diet was replaced daily.

2.2. Fungal cultures

2.2.1. Isolates from culture collections

Isolates of *B. bassiana* s.l. and *M. anisopliae* s.l. belonging to the culture collection of the Entomopathogenic Fungi Laboratory (IMYZA, INTA, Castelar, Argentina) were used. All isolates were obtained from different sources, such as insects and soil, and from different regions of this country. All isolates were preservated by freeze-drying (lyophilization). Subcultured isolates of *B. bassiana* s.l. were maintained on Complete Medium Agar (CMA), which is composed of (g/l): KH_2PO_4, 0.4; Na_2HPO_4,1.4; SO_4Mg, 0.6; KCl, 1; NH_4NO_3, 0.7; glucose, 10; agar, 15; and yeast extract, 5; at 4 °C. The isolates of *M. anisopliae* s.l. were maintained on Potato Dextrose Agar (PDA) Oxoid amended with chloramphenicol (0.5 g/l) at the same temperature.

2.2.2. Isolates from soil samples and mummified insects

Also, new isolates were obtained from soil and mummified insects. For isolation from soil, samples were collected from cotton growing fields infected with *A. grandis* from one site located in the province of Santa Fe (28°21′02.44″S; 59°15′37.84″W; Las Toscas) and one site located in the province of Chaco (26°50′09.16″S, 60°26′31.92″W; Roque Saenz Peña). Aliquots of 10 g of soil were resuspended in 90 ml of sterile distilled water, and dilutions were carried out with Tween 80 (0.05%). Then, 100 μl of soil suspension were spread over the surface of plates with selective medium OA-CTAB, which is composed of (g/l): rolled oatmeal, 20; agar, 20, amended with chloramphenicol, 0.5 and Hexadecyltrimethylammonium bromide (CTAB, Alfa – Aesar), 0.6; to retard the growth of bacteria and contaminant fungi (Mini et al., 2008). Cultures were incubated for a week at 26 °C. Colonies were placed on petri dishes with CMA or PDA medium with antibiotic, for purification and identification.

The isolates obtained from cadavers of insects were lifted and placed in petri dishes containing the same culture medium, for purification and identification.

All isolates were identified on a basis of morphology as either *B. bassiana* s.l. or *M. anisopliae* s.l., using a key by Humber (1997) (Fernandes et al., 2011). Then, the isolates were incorporated in the culture collection of the Entomopathogenic Fungi Laboratory.

2.3. Conidial suspensions

Conidial suspensions were obtained from each fungal isolate. For *B. bassiana* s.l., cultures developing on CMA media were used for this purpose. Conidia were collected from one-week old fungal cultures and suspended in Tween 80 (0.05%). For *M. anisopliae* s.l., the conidial suspensions were prepared in Tween 80 (0.05%) from one-week old cultures on sterile polypropylene bags with 90 g of parboiled rice and 45 ml of distilled water (Bahiense et al., 2006).

Concentrations of all suspensions were quantified with an improved Neubauer chamber. Viability of the conidia was assessed by a germination test prior to the experiment and found to be >95%. Also, the colony forming units (cfu) were evaluated and their concentrations were adjusted for each bioassay (Wraight et al., 2007).

2.4. Bioassays

2.4.1. Pathogenicity bioassays

A total of 66 isolates of *B. bassiana* s.l. and 28 of *M. anisopliae* s.l. were screened on *A. grandis* to evaluate their pathogenicity. The assays were performed on 24-h old red unsexed adults of *A. grandis*. For each treatment, all adults were inoculated by the immersion method in a conidial suspension adjusted to 5×10^8 viable conidia/ml for 15 s. Five replicates with 10 adults each were tested for each fungal isolate, and other five replicates with 10 adults each were performed in Tween 80 (0.05%) without the fungus as a control. Due to the large number of isolates, bioassays were performed on several different occasions. Each bioassay consisted of an untreated control and the tested isolates. An isolate standard was run in all bioassays for comparison (Nowierski et al., 1996).

The number of dead insects per day was recorded for 20 d. To confirm fungal infection, dead insects were placed in a humid chamber and incubated for 3 d at 26 °C. The percentage of mortality was calculated for each isolate, and the median survival time (ST_{50}) and 95% confidence intervals for adults receiving each

Table 1
Mean percentage of mortality and median survival time (ST_{50}) (Andersen 95% confidence intervals (CI)) of *A. grandis* inoculated by dipping adults in suspensions with 5×10^8 conidia/ml. Original host or source of *M. anisopliae* s.l. and *B. bassiana* s.l. isolates used in bioassays against *A. grandis*.

Fungal species	Isolate	Percent mortality (SD)	ST_{50} (CI)	Source
B. bassiana s.l.	Bb 2	59 (14)	n.c.	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 5	26 (15)	n.o	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 7	18 (15)	n.o	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 8	26 (18)	n.o	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 9	62 (31)	n.c.	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 10	6 (5)	n.o	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 12	67 (14)	n.c.	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 13	44 (11)	n.o	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 17	42 (12)	n.o	Spilosoma virginica (Lep: Lymantriidae)
	Bb 23	90 (7)	7.00 (6.41–7.59)	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 24	50 (14)	n.o	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 26	12 (11)	n.o	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 36	66 (13)	n.c.	*Tibraca limbativentris* (Hemiptera: Pentatomidae)
	Bb 37	0 (0)	n.o	*Nezara viridula* (Hemiptera: Pentatomidae)
	Bb 38	26 (15)	n.o	*Nezara viridula* (Hemiptera: Pentatomidae)
	Bb 40	44 (19)	n.o	*Euselasia sp* (Lepidoptera: Erycinidae)
	Bb 41	58 (8)	n.c.	Homoptera: Cercopidae
	Bb 45	52 (16)	n.c.	*Diabrotica sp.* (Coleoptera: Chrysomelidae)
	Bb 47	76 (11)	n.c.	*Diatraea saccharalis* (Lep: Pyralidae)
	Bb 65	64 (18)	n.c.	*Nezara viridula* (Hemiptera: Pentatomidae)
	Bb 68	39 (15)	n.o	Coleoptera: Scarabaeidae
	Bb 70	32 (19)	n.o	Coleoptera: Scarabaeidae
	Bb 71	34 (21)	n.o	Soil
	Bb 73	0 (0)	n.o	Soil
	Bb 75	82 (8)	n.c.	Soil
	Bb 79	58 (16)	n.c.	Soil
	Bb 80	16 (15)	n.o	Soil
	Bb 81	47 (10)	n.o	*Phyrdenus muriceus* (Coleoptera: Curculionidae)
	Bb 98	62 (16)	n.c.	*Cyclocephala signaticollis* (Coleoptera: Scarabaeidae)
	Bb 112	84 (11)	n.c.	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 132	20 (12)	n.o	*Deuterocampta quadrijuga* (Coleoptera: Crisomelidae)
	Bb 175	25 (6)	n.o	Rh. (Bo) microplus (Acari: Ixodidae)
	Bb 241	58 (13)	n.c.	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Bb 243	0 (0)	n.o	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Bb 244	52 (24)	n.c.	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Bb 246	22 (13)	n.o	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Bb 247	48 (15)	n.o	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Bb 250	18 (4)	n.o	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Bb 251	34 (13)	n.o	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Bb 257	26 (5	n.o	Soil
	Bb 258	6 (9)	n.o	Soil
	Bb 259	18 (13)	n.o	Soil
	Bb 260	20 (7)	n.o	Soil
	Bb 262	18 (8)	n.o	Soil
	Bb 264	8 (8)	n.o	Soil
	Bb 265	24 (5)	n.o	Soil
	Bb 268	22 (18)	n.o	Soil
	Bb 270	36 (29)	n.o	Soil
	Bb 272	22 (16)	n.o	Soil
	Bb 275	24 (17)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 277	28 (18)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 279	30 (10)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 280	26 (11)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 285	24 (5)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 286	90 (14)	8.00 (7.50–8.50)	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 288	30 (14)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 290	16 (5)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 291	20 (12)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 292	20 (10)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 294	22 (13)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 295	26 (13)	n.o	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 296	70 (7)	n.c.	*Rhammatocerus pictus* (Ortoptoptera: Acrididae)
	Bb 301	92 (8)	9.00 (8.17–9.83)	*Dyscinetus hidrophilides* (Coleoptera: Scarabaeide)
	Bb 302	90 (10)	7.00 (6.54–7.46)	Soil
	Bb 303	72 (20)	n.c.	Soil
	Bb 316	22 (10)	n.o	*Anthonomus grandis* (Coleoptera: Curculionidae)
M. anisopliae s.l.	Ma 1	82 (20)	n.c.	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 2	100 (0)	5.00 (4.87–5.13)	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 4	54 (15)	n.c.	Soil
	Ma 5	92 (13)	5.00 (4.74–5.26)	Soil
	Ma 6	92 (8)	6.00 (5.64–6.36)	*Diloboderus abderus* (Coleoptera: Scarabeidae)
	Ma 7	100 (0)	6.00 (5.75–6.25)	*Diloboderus abderus* (Coleoptera: Scarabeidae)
	Ma 8	42 (25)	n.o	Soil

(*continued on next page*)

Table 1 (*continued*)

Fungal species	Isolate	Percent mortality (SD)	ST_{50} (CI)	Source
	Ma 10	32 (18)	n.o	Soil
	Ma 13	40 (20)	n.o	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 14	98 (4)	6.00 (5.66–6.34)	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 15	66 (24)	n.c.	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 16	92 (8)	5.00 (4.84–5.16)	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 17	92 (8)	5.00 (4.79–5.20)	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 18	86 (5)	n.c.	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 20	100 (0)	6.00 (5.74–6.26)	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 21	44 (15)	n.o	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 25	80 (16)	n.c.	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 27	72 (13)	n.c.	*Acromyrmex lundi* (Hymenoptera: Formicidae)
	Ma 31	90 (7)	6.00 (5.54–6.46)	Soil
	Ma 32	40 (24)	n.o	Coleoptera: Scarabaeidae
	Ma 35	98 (4)	5.00 (4.74–5.26)	*Diloboderus abderus* (Coleoptera: Scarabeidae)
	Ma 36	94 (5)	6.00 (5.48–6.52)	Soil
	Ma 41	96 (5)	6.00 (5.84–6.16)	Coleoptera: Scarabeidae
	Ma 45	90 (10)	6.00 (5.24–6.76)	Soil
	Ma 46	88 (13)	n.c.	Soil
	Ma 47	66 (11)	n.c.	Soil
	Ma 49	98 (4)	6.00 (5.70–6.30)	Soil
	Ma 50	100 (0)	4.00 (3.57–4.43)	Soil

n.o. = 50% mortality was not observed in the bioassays.
n.c. = not calculated.

treatment were calculated based on Kaplan–Meier survival distribution function (Statsdirect, 2008). Based on the bioassays, isolates of *B. bassiana* s.l. and *M. anisopliae* s.l. were preselected. The criteria for selecting isolates included a percentage of mortality upper than 90%, and simplicity to produce massively (data not showed).

2.4.2. Virulence bioassays

The lethal concentrations (LC_{50} and LC_{90}) of the preselected isolates on the cotton boll weevil were assessed. For this purpose, six conidial suspensions adjusted to different concentrations (1×10^6, 5×10^6, 1×10^7, 5×10^7, 1×10^8 and 5×10^8 conidia/ml) were prepared. For each isolate, a different bioassay by the immersion method was done. Three replicates of 40 adults were carried out for each isolate and concentration. A control was performed for each bioassay in Tween 80 (0.05%), and three replicates of 40 adults each were done. The number of dead insects per day was recorded for 15 days. Fungal infection was confirmed as in Section 2.4.1. Data were analyzed using probit software (1989, G.A. Milliken, University of Kansas) and the criterion used to determine differences between isolates was the lack of overlap among 95% confidence intervals (Zhao et al., 1995).

2.4.3. Effect of fungal infection on feeding behavior

To evaluate the effect of infection on the feeding behavior of weevil adults, newly emerged females were inoculated by the immersion method in a conidial suspension adjusted to the LC_{50}. The isolates Bb 23 and Ma 20, which were selected in the previous bioassays, were evaluated. Fifteen and 17 replicates were carried out for *B. bassiana* s.l. and *M. anisopliae* s.l. respectively. Control with the same number of replicates was done in Tween 80 (0.05%). Each weevil was individually placed in a petri dish and one uninfected greenhouse-grown cotton square (7–9 mm in diameter at the widest part of the flower bud and with intact bracteoles) was provided daily for 4 d. Unsealed punctures were considered as feeding punctures (Greenberg et al., 2005).

The number of feeding punctures in the squares and the change in the weight of females were recorded at 24, 48, 72 and 96 h. Once the insects died, fungal infection was confirmed. The feeding punctures data were compared with a control using repeated measures ANOVA and the weight gain at 96 h was analyzed by t-test.

2.4.4. Compatibility of preselected isolates with chemical insecticides

The in vitro compatibility of *B. bassiana* s.l. (isolates Bb 23, Bb 301, and Bb 302) and *M. anisopliae* s.l. (isolates Ma 20, Ma 49, Ma 50) with three insecticides commonly used for *A. grandis* control: Furia (zetamethrin 18%, FMC Latinoamerica S.A., Argentina), Atrion (Beta cypermethrin 10%, Chemotecnica S.A., Argentina), and Thionex L (Endosulfan 35%, Magan Argentina S.A., Argentina) were examined. The following procedure was modified from Lecuona et al. (2001a). The final concentrations of the insecticide suspensions were

Table 2
LC_{50} and LC_{90} (95% confidence intervals (CI)) and regression parameters a (*y*-intercept) and *b* (slope) estimated by the probit method for fungal isolates tested in adults of *A. grandis*.

Isolates	LC_{50} (conidia ml^{-1}) (CI)	LC_{90} (conidia ml^{-1}) (CI)	*b*	*a*	χ^2 [a]	df [c]	
Bb 23	8.63×10^7 C [d] (5.97×10^7–1.32×10^8)	9.86×10^8 B (5.06×10^8–2.84×10^9)	1.17	−4.27	6.26	4	NS[b]
Bb 286	1.45×10^8 C (9.41×10^7–2.53×10^8)	2.15×10^9 C (9.23×10^8–9.18×10^9)	1.07	−3.72	1.43	3	NS
Bb 301	4.47×10^7 BC (3.18×10^7–6.12×10^7)	3.27×10^8 B (2.18×10^8–5.65×10^8)	1.30	−4.99	4.72	4	NS
Bb 302	4.76×10^7 ABC (3.43×10^7–6.63×10^7)	3.55×10^8 B (2.17×10^8–7.44×10^8)	1.44	−6.08	5.10	3	NS
Ma 2	3.53×10^7 B (2.43×10^7–5.53×10^7)	3.54×10^8 BC (1.79×10^8–1.10×10^9)	1.28	−4.64	0.11	3	NS
Ma 7	3.30×10^7 B (2.35×10^7–4.83×10^7)	2.57×10^8 B (1.44×10^8–6.51×10^8)	1.27	−4.53	2.15	3	NS
Ma 20	1.20×10^7 A (8.82×10^6–1.62×10^7)	7.77×10^7 A (5.04×10^7–1.14×10^8)	1.39	−4.81	3.77	3	NS
Ma 36	1.92×10^7 AB (1.44×10^7–2.57×10^7)	9.58×10^7 AB (3.35×10^7–1.77×10^8)	1.43	−5.28	5.11	3	NS
Ma 49	1.71×10^7 AB (1.21×10^7–2.48×10^7)	1.54×10^8 AB (8.98×10^7–3.36×10^8)	1.18	−3.47	3.96	3	NS
Ma 50	1.13×10^7 A (9.26×10^6–1.80×10^7)	9.43×10^7 AB (5.95×10^7–1.84×10^8)	1.40	−4.93	0.98	3	NS

[a] χ^2 indicates data adjustment to the probit model.
[b] NS indicates non-significant lack of fit (5%) with df.
[c] df (degrees of freedom).
[d] LC_{50} values followed by the same letter are not different based on the failure of 95% CI to overlap.

adjusted to 10%, 50%, 100%, and 200% of the recommended field application rate (RFAR). Isolates were incorporated as 0.2 ml of 5×10^7 conidia/ml suspensions to a final mix volume of 50 ml in Erlenmeyer flasks. All treatments were compared with a control of conidia added to water without insecticide (0% of the RFAR). Three replicates of each treatment, including the control, were carried out. The mixtures were shaken at 200 rpm for 16 h at 27 °C. Each mixture was diluted 10,000 times and 0.1 ml was spread onto CMA or PDA plates kept at 26 °C. The cfu were counted 3 d later, and the percentage of survival was calculated. Four plates for each treatment were counted. The cfu data were analyzed with ANOVA and Tukey's test ($\alpha = 0.05$) and square-root transformed when the assumptions of ANOVA were not satisfied.

3. Results and discussion

3.1. Isolation of entomopathogenic fungi

We obtained five isolates of *M. anisopliae* s.l. (Ma 47, Ma 48, Ma 49, Ma 50, Ma 51) and two isolates of *B. bassiana* s.l. (Bb 302, Bb 303) from soil samples, and two of *B. bassiana* s.l. (Bb 301, Bb 316) from *Dyscinetus hidrophilides* and *A. grandis* (Coleoptera: Scarabaeidae) cadavers, respectively. The isolates obtained were used in the following selection assays (Table 1).

3.2. Pathogenicity bioassays

All *M. anisopliae* s.l. isolates were pathogenic against the boll weevil and between 32% and 100% mortality was obtained. Furthermore, 46% of the isolates caused more than 90% mortality (Table 1).

With regard to *B. bassiana* s.l. isolates, most of the isolates were nonpathogenic against the boll weevil or presented low virulence. Only Bb 301 caused 92% mortality (Table 1). We found that these isolates were less virulent than *M. anisopliae* s.l. isolates. Our results are in agreement with those of Domingues da Silva (2001), who screened 12 *B. bassiana* isolates of different origins and the range of mortality of boll weevil adults was between 15% and 83%. Again, only one isolate was able to cause more than 65% mortality. Lecuona et al. (2001b) evaluated 36 strains of *M. anisopliae* and 189 strains of *B. bassiana* against the boll weevil and found that when immersing insects in a conidial suspension (10^8 conidia/ml) of *M. anisopliae*, the highest mortality rate was 74%. In the present work, the assays resulted in higher levels of infection of *M. anisopliae* isolates, probably because of the higher inoculum used. However, in contrast to our results, the percentage of *B. bassiana* strains (53%) pathogenic against the boll weevil was higher than that of *M. anisopliae* strains (22%) (Lecuona et al., 2001b).

The isolates, that caused high mortality of boll weevil, were obtained from a wide variety of sources. These results are in agreement with those of Fernandes et al. (2011), who observed that several *B. bassiana* isolates obtained from naturally infected ticks were not significantly more virulent against *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) than isolates obtained from other arthropod orders. Some host range descriptions rely on laboratory bioassay studies (physiological host range) and do not necessarily reflect the true host range in nature. The true biological host range is determined by spatial and temporal factors as well as by physiological interactions (Castrillo et al., 2005).

Based on the bioassays, 4 isolates of *B. bassiana* s.l. (Bb 23, Bb 286, Bb 301, Bb 302) and 6 of *M. anisopliae* s.l. (Ma 2, Ma 7, Ma 20, Ma 36, Ma 49, Ma 50) were preselected. These isolates presented the lowest ST_{50} values, which ranged between 4 and 6 d for *M. anisopliae* s.l., and between 7 and 9 d for *B. bassiana* s.l. (Table 1). This result is consistent with those of Gindin et al. (2006), who found that *M. anisopliae* strains were more virulent and that the lethal time was shorter than that of *B. bassiana* on red palm weevil larvae.

3.3. Virulence bioassays

LC_{50} and LC_{90} values of the preselected isolates are shown in Table 2. Ma 20 presented the lowest values of LC_{50} and LC_{90} corresponding to 1.20×10^7 and 7.77×10^7 conidia/ml, respectively.

We observed that *B. bassiana* s.l. isolates were less virulent than *M. anisopliae* s.l. isolates. This fact, together with a shorter ST_{50} of *M. anisopliae* s.l. as compared to that of *B. bassiana* s.l., led us to suggest *M. anisopliae* s.l. as an efficient biocontrol agent against the boll weevil. Previous studies have shown the possibility to use *B. bassiana* in the control of the boll weevil (McLaughlin, 1962). Furthermore, a mycoinsecticide Naturalis-L based on this species has been previously tested against overwintered boll weevil populations and found to be active (Wright and Chandler, 1992; Wright, 1993). However, no mycoinsecticide has been formulated based on *M. anisopliae* for this pest control (Faria and Wraight, 2007).

3.4. Effect of fungal infection on feeding behavior

The weight gain (mg) by boll weevils was reduced when they were infected with Ma 20 (t-value = 4.01; df = 32; $p < 0.0003$) and Bb 23 (t-value = 3.17; df = 28; $p < 0.004$). The mean number of punctures on the square performed per infected female differed significantly from the control at 72 and 96 h after the treatment with fungi (Fig. 1a and b). There are numerous studies that investigated the feeding behavior of insect species infected with

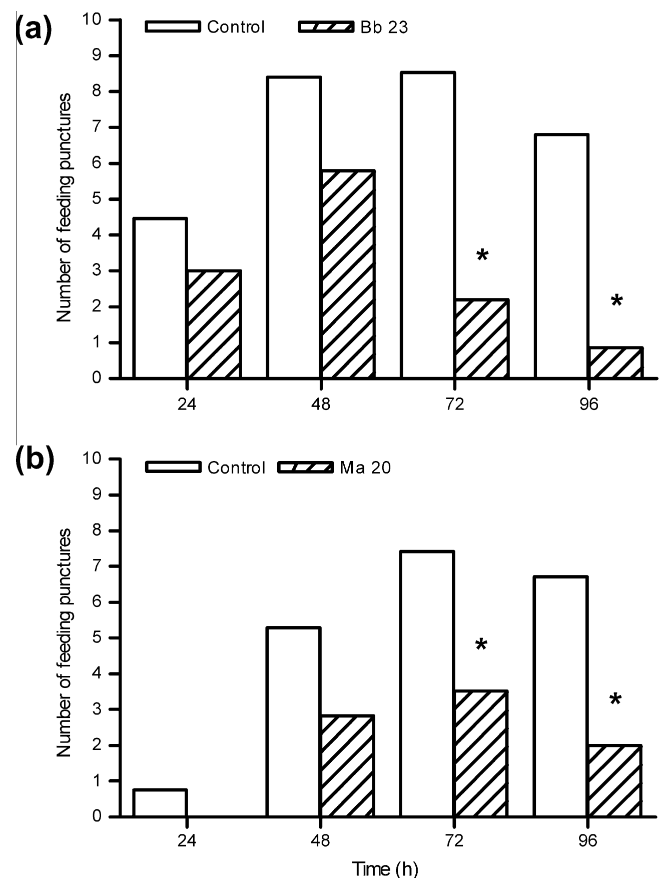

Fig. 1. Mean number of feeding punctures on a cotton square per female after 24, 48, 72 and 96 h of treatment with (a) Bb 23 and (b) Ma 20. *indicates significant differences between means (Repeated Measures ANOVA, $p < 0.001$).

Table 3
Colony forming units (cfu) per milliliter values from the six preselected isolates of *B. bassiana* and *M. anisopliae* versus five concentrations of insecticide.

% of the RFAR	Isolate					
	Bb 23	Bb 301	Bb 302	Ma 20	Ma 49	Ma 50
	Furia (Zetamethrin 18%)					
0	1.83×10^5 A[a]	2.46×10^5 A	2.64×10^5 A	4.58×10^5 A	2.86×10^5 A	1.42×10^5 A
10	1.90×10^5 A	2.20×10^5 A	3.05×10^5 A	3.95×10^5 A	2.58×10^5 AB	1.13×10^5 A
50	4.03×10^4 B	4.41×10^4 B	1.26×10^5 B	2.04×10^5 BC	1.76×10^5 BC	3.44×10^4 B
100	0.00 C	0.00 B	1.26×10^2 C	2.70×10^5 B	1.44×10^5 C	9.61×10^3 C
200	0.00 C	0.00 B	0.00 C	1.78×10^5 C	1.27×10^5 C	2.65×10^2 D
	Atrion (Beta-cypermethrin 10%)					
0	1.89×10^5 A	2.54×10^5 A	2.67×10^5 A	1.78×10^5 A	2.50×10^5 A	1.60×10^5 A
10	1.75×10^5 AB	2.33×10^5 A	2.38×10^5 A	1.32×10^5 AB	2.37×10^5 A	2.00×10^5 A
50	1.18×10^5 B	1.73×10^5 A	5.39×10^4 B	1.03×10^5 BC	2.00×10^5 A	1.22×10^5 AB
100	1.53×10^4 C	2.68×10^3 B	0.00 C	4.69×10^4 CD	1.55×10^5 A	7.37×10^4 B
200	0.00 D	0.00 B	0.00 C	1.63×10^4 D	1.68×10^4 B	1.43×10^3 C
	Thionex (Endosulfan 35%)					
0	1.45×10^5 A	2.42×10^5 A	2.1×10^5 A	1.19×10^5 A	2.59×10^5 A	1.73×10^5 A
10	0.00 B	2.30×10^4 B	1.08×10^5 B	7.13×10^4 B	2.06×10^5 A	9.35×10^4 A
50	0.00 B	0.00 C	0.00 C	1.54×10^4 C	8.18×10^4 B	0.00 B
100	0.00 B	0.00 C	0.00 C	1.05×10^4 C	2.27×10^4 C	0.00 B
200	0.00 B	0.00 C	0.00 C	5.49×10^2 D	7.55×10^3 D	0.00 B

[a] For each insecticide means followed by the same letter within the same column do not differ statistically ($p < 0.05$; Tukey's test).

Metarhizium sp. and *Beauveria* sp.. However, there are not studies about this effect on *A. grandis*. France et al. (2002) evaluated four native isolates of *B. bassiana* for food consumption over time in adults of the weevil *Asynonychus cervinus* (Coleoptera: Curculionidae), and found that food intake of infected insects decreases through time until death. Ekesi (2001), who evaluated antifeedant activity of *B. bassiana* against the cowpea leaf beetle *Ootheca mutabilis* (Coleoptera: Chrysomelidae), observed that daily food consumption between treated and control insects was significantly lower after 2 d of treatment. Similarly, Fargues et al. (1994) worked with fourth-instar Colorado potato beetles, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), infected with *B. bassiana* and found a significantly lower food consumption after 2 d of treatment. Tefera and Pringle (2003) studied the daily food consumption by the spotted stem borer, *Chilo partellus* (Lepidoptera: Pyralidae), and observed that this parameter decreased after 5 and 4 d of treatment with *M. anisopliae* and *B. bassiana* respectively. Additionally, they found that increasing the conidial concentrations resulted in a higher reduction in food consumption.

In the present work, we observed that control females began oviposition at 96 h, whereas females treated with both entomopathogenic fungi did not oviposit at this time. This may suggest a possible inhibition in the reproductive behavior. Gindin et al. (2006) found that treated females of the red palm weevil, *Rhynchophorus ferrugineus*, had a shorter oviposition period and three times lower fertility than the controls. However, these hypotheses concerning boll weevil require further exploration.

3.5. Assays of compatibility with insecticides

The effect of the insecticides on conidia varied depending on the isolate and the active compound (Table 3). Thionex (endosulfan 35%) was the most harmful insecticide for all the isolates evaluated. We found that none of the *B. bassiana* s.l. isolates grew with 50% of the RFAR, while *M. anisopliae* s.l. isolates were able to grow in spite of the reduction of conidia viability with the same dose. Particularly, Ma 20 and Ma 49 grew with the 50% of the RFAR (13% and 32% survival of conidia, respectively) (Table 3). Similarly, previous studies based on *B. bassiana* compatibility with different insecticides did not show viability of conidia when these were treated with endosulfan formulations (Alizadeh et al., 2007; Oliveira et al., 2003).

Beauveria bassiana s.l. isolates were not able to grow with 100% of the RFAR of Furia (Zetamethrin 18%). On the other hand, Ma 49 and Ma 20 presented a viability of conidia higher than 50% with this dose, and were compatible even with 200% of the RFAR (Table 3).

Atrion (Beta-cypermethrin 10%) was the most compatible with both *B. bassiana* s.l. and *M. anisopliae* s.l. With 50% of the RFAR all isolates were compatible, while with 100 and 200% of the RFAR, *B. bassiana* s.l. did not grow (Table 3). This is in agreement with the results by Lecuona et al. (2001a), who found that 100% and 200% of the RFAR cause total inhibition of all strains of *B. bassiana* s.l. and could be used in combination with the fungus at low doses. In addition, we found that this insecticide was less harmful to *M. anisopliae* s.l. isolates with 100% of the RFAR (Table 3).

Beta-cypermethrin and zetamethrin formulations could be considerated for being used in combination with the selected fungus at doses compatible with cotton IPM programs.

4. Conclusion

In this work, the Ma 20 and Ma 50 isolates caused the highest mortalities in boll weevil adults. In addition, they presented the lowest LC_{50} and ST_{50} values and were compatible with the pyrethroid chemical insecticides tested. Furthermore, Ma 20 showed a reduction in the feeding behavior, and this may reduce the damage on the buds.

This study highlights the importance of *M. anisopliae* s.l. as an essential species in boll weevil biocontrol. In summary, *M. anisopliae* showed the potential in the laboratory to control the boll weevil. However, further research is necessary with the main goal to formulate these isolates and to evaluate this mycoinsecticide in field for boll weevil control in crops.

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