Potential of *Metarhizium anisopliae* (Metsch.) Sorokin (Ascomycetes, hypocreales) in the control of *Bonagota salubricola* (Meyrick) (Lepidoptera, Tortricidae) and its compatibility with chemical insecticides

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(With 1 figure)

Abstract

Several insects are harmful to apples grown in Brazil, especially the leafroller *Bonagota salubricola* (Lepidoptera, Tortricidae), usually controlled with chemical insecticides. The purpose of the present study was to investigate the use of *Metarhizium anisopliae* strains in the control of the apple leafroller, by assessing their virulence to *B. salubricola* larvae in bioassays with suspensions of 2×10^5 to 2×10^9 conidia/mL as well as their relationship with protease expression. The most effective strain underwent a compatibility test with chemical insecticides. The *M. anisopliae* E6 strain showed a good performance, with up to 88% mortality and a LT_{50} of 1.66 days. The virulence was positively correlated with a higher enzymatic activity. The E6 strain was compatible with tebufenozide, evidencing its potential to control *B. salubricola*.

Keywords: entomopathogenic fungi, apple, biological control, leafroller.

Potencialidade de *Metarhizium anisopliae* (Metsch.) Sorokin (Ascomycetes, Hypocreales) no controle de *Bonagota salubricola* (Meyrick) (Lepidoptera, Tortricidae) e sua compatibilidade com inseticidas químicos

Resumo

Diversos insetos são nocivos à macieira no Brasil, destacando-se a lagarta-enroladeira *Bonagota salubricola* (Lepidoptera: Tortricidae), controlada com inseticidas químicos. Este trabalho objetivou avaliar linhagens de *Metarhizium anisopliae* passíveis de serem utilizadas no controle desta praga, determinando-se a virulência sobre larvas de *B. salubricola* em bioensaios com suspensões de 2×10^5 a 2×10^9 conídios/mL considerando-se mortalidade larval e pupal, bem como a sua relação com a expressão de proteases. A linhagem mais efetiva foi submetida a teste de compatibilidade com inseticidas químicos. A linhagem E6 de *M. anisopliae* destacou-se por proporcionar mortalidade de 88% com TL_{50} de 1,66 dias; tal virulência relacionou-se positivamente com maior produção enzimática. Esta linhagem foi compatível com tebufenozide, evidenciado seu potencial no controle de *B. salubricola*.

Palavras-chave: fungos entomopatogênicos, maçã, controle biológico, lagarta enroladeira da macieira.

1. Introduction

The *Bonagota salubricola* (Meyrick, 1931) (Lepidoptera: Tortricidae) leafroller, also known by a synonym, *Bonagota cranaodes* (Meyrick, 1937) (Brown and Razowski, 2003), is a primary pest in commercial apple orchards in South America.

The larvae feed mainly on the peel and pulp of the fruits, thus making them improper for commercialisation (Kovaleski et al., 1998).

The first leafroller attacks were identified in Brazil during the 1990s, and broad-spectrum chemical insecticides were used

to control this pest. Up to eight insecticide applications were carried out by harvest and yet losses in the field still stayed around 3-5% (Kovaleski et al., 1998), very much like in the control of *Cydia pomonella* (Linnaeus, 1758) (Lepidoptera: Tortricidae), which also damages apple orchards in other parts of the world (Lacey and Unruh, 2005).

Thereafter, pest-monitoring tools were developed, resulting in better management and fewer insecticide applications. Other products less toxic to the environment and to natural enemies such as growth regulators and modern molecules more efficient even at very low dosages were also developed. Both procedures – monitoring and use of less-toxic products – led to the reduction of pest population, which currently plays a less important economic role (Sugayama, R.L., personal observation).

The global market is currently inclined towards ecological products, leading fruit growers to adopt strategies as the Integrated Apple Production (IAP) (MAPA, 2006), reducing the use of agrochemicals or resorting to other alternatives (Way and Emden, 2000; Lacey and Unruh, 2005).

Thus, the use of natural agents such as entomopathogenic fungi can cut the use of chemical insecticides down to a minimum (Azevedo, 1998). These microorganisms produce epizootics in susceptible insects, with minimal effect on non-target species (Alves, 1998). Entomopathogenic fungi of the *Beauveria, Metarhizium* and *Paecilomyces* genera were already seen parasitising tortricidae (Humber and Hansen, 2005), indicating that it might be used for controlling *B. salubricola* (Robbs and Bittencourt, 1998). As to the potential of entomopathogenic fungi in pest control, several studies correlate enzymatic activity especially that of proteases with virulence, since they are directly involved in fungi ability in penetrating the host (Gupta et al., 1994; St. Leger et al., 1986; Tiago and Furlaneto, 2003).

Chemical insecticides and biological control by entomopathogenic fungi may be used alternatingly or simultaneously, if compatibility or – more rarely – synergism between them is identified (Alves, 1998; Silva et al., 2005). Thus, it is imperative to determine the compatibility between chemical insecticides and biological insecticides such as entomopathogenic fungi.

The purpose of the present study was to evaluate the potential of *Metarhizium anisopliae* (Metsch.) Sorokin in controlling *B. salubricola* as well as the compatibility of the more promising strain with chemical insecticides.

2. Materials and Methods

2.1. Metarhizium anisopliae strains and target insect

The study used strains E6, AL and CG46 of *M. anisopliae*. Strains E6 and AL were isolated from *Deois flavopicta* (Hemiptera: Cercopidae) from the Brazilian states of Espirito Santo and Alagoas, respectively (Rosato et al., 1981; Kava-Cordeiro et al., 1995). The CG46 (E9) strain, kindly provided by EMBRAPA, the Brazilian Agriculture and Cattle Research Institute, monosporic-culture, was isolated from *Deois incompleta* in the Brazilian state of Espirito Santo (EMBRAPA, 1996). Strains were kept in stock in medium with potato dextrose agar (PDA) and grown in dishes with PDA at 25ºC for 10-12 days for pathogenicity and virulence tests. *B. salubricola* insects were obtained from the breeding facilities of the Pest Control Laboratory of the Institute of Biotechnology of the University of Caxias do Sul, Brazil, where insects are bred in an air-conditioned room at 25 ± 1 ºC, 60 ± 10% RH and 14 hours photophase, using the methodology and artificial diet as described in Parra et al. (1995).

2.2. Virulence

Virulence tests used conidia from the 10-12-day old culture of the first subculture in PDA against second-instar caterpillars, except for the most virulent strain, whose tests were complemented with first and fourth-instar caterpillars. Each strain was assessed in the concentrations of 2×10^5, 2×10^6, 2×10^7, 2×10^8, and 2×10^9 conidia/mL, by wetting filter paper discs previously arranged in 9-cm ∅ Petri dishes with 2 mL of suspension. Conidia viability was around 100%. Caterpillars were kept in contact with the fungi for 24 h and were then put on artificial diet (Alves, 1998), and maintained at 25 ± 1 ºC and 60 ± 10% RH, with daily assessments. The control group underwent the same procedures, however without addition of microorganisms. Treatments consisted of five repetitions with 10 caterpillars each, with daily mortality assessments until the pupal stage.

2.3. Proteolytic activity

Strains were revitalised by subculture in PDA to obtain conidia. For each strain, a suspension of 5.6×10^6 conidia/mL was inoculated in 25 mL MM medium (Pontecorvo et al., 1953), without addition of nitrogen source and MM + CAS (MM plus 1% p/v casein solution). Glucose and $NaNO_3$ were removed from the liquid media.

Each strain was inoculated in triplicate, incubated at 28 ºC under agitation (180 rpm), and proteolytic activity of two samples of each replicate was assessed. In all, six repetitions were made for each period of time (0, 24, 48, and 72 hours).

The samples were centrifuged at 2240 *g* for 10 minutes. The supernatant was kept at –20 ºC and used as secreted fraction in the enzymatic assays. Subtilisin-like (Pr1) was determined using the specific synthetic substrate Scu-Ala-Ala-Pro-Phe-*p*-nitroanilide (Gupta et al., 1992). After induction at 28 ºC for 30 minutes, the reaction was stopped with 250 µL acetic acid (30%), the rate of substrate hydrolysis with release of *p*-nitroanilide was quantified by spectrophotometer (410 nm), and the results were expressed in nmols of *p*-nitroanilide in 30 minutes.

2.4. Compatibility between fungus and agrochemicals

The most virulent fungal strain (E6) was tested for compatibility with following chemical insecticides used in apple orchards: chlorpyrifos-ethyl (Lorsban 480BR – 72 g a.i./100 L), methidathion (Supracide 400CE – 60 g a.i./100 L), tebufenozide (Mimic 240SC – 21.6 g a.i./100 L), and fenitrotion (Sumithion 500 CE – 75 g a.i./100 L).

Compatibility was assessed in vitro by evaluating vegetative growth and sporulation of entomopathogens in the presence or absence of each insecticide. Products were added in culture medium (PDA) at 45 ± 5 ºC and poured onto Petri dishes in 25-mL aliquots. Entomopathogen inoculation was performed with a platinum loop at three equidistant spots. Twelve inoculations per insecticide were performed, with Petri dishes being kept at 26 ± 1 °C, 70 ± 10% RH, and 12-h photophase for ten days. The same procedure was used for the controls, however without addition of agrochemicals.

Vegetative growth was assessed by determining the average diameter of the colonies in three directions. The number of spores was estimated by removing a 15-mm ∅ disk at each inoculation spot, and then transferring it to a flask with Tween 80 aqueous solution (0.1% v/v), agitating it for 5 minutes, and then estimating spore concentration in a Neubauer chamber (Oliveira et al., 2002).

2.5. Data analysis

The mean rates of accumulated mortality were compared by analysis of variance (ANOVA) and means were grouped by Tukey's test at a probability of 95%. Virulence was assessed by the Probit model (Finney, 1971) by determining LC_{50} and LT_{50}, while the correlation between concentrations and mortality was assessed by Pearson's test.

The data obtained in the compatibility study between fungi and agrochemicals were compared with the compatibility factor proposed by Alves et al. (1998), which groups products in selectivity/compatibility classes by using the following equation:

$$T = 20\,[VG] + 80\,[SPO]/100$$

where: T = the corrected value for product classification; VG = percent of vegetative growth in relation to control, and SPO = percent of sporulation (conidiogenesis) in relation to control.

The following limits were established for "T": 0–30% = highly toxic; 31–45% = toxic; 46–60% = mildly toxic; > 60% = compatible.

3. Results

3.1. Virulence

At the highest fungal concentration, virulence to *B. salubricola* ranged between 38 and 88% on the tenth day (Table 1). Strain AL at highest concentration induced a 54% mortality rate (Table 1), with an average lethal concentration (LC_{50}) of 2.78×10^8 (2.10×10^8 to 3.27×10^8) conidia/mL, (χ^2 1.787) average lethal time (LT_{50}) of 8.42 (6.88 to 10.35) days (χ^2 4.725), and a positive correlation between concentration and mortality (PC = 0.794), which increased until the tenth day (Figure 1).

Strain CG46 at the highest concentration induced a 38% mortality rate (Table 1) with a merely numerical correlation between concentration and mortality (PC = 0.634), which also increased until the tenth day.

Strain E6 at the highest concentration induced an 88% mortality rate (Table 1), with LC_{50} of 2.68×10^7 (2.52×10^7 to 2.74×10^7) conidia/mL, (χ^2 0.336) conidia/mL and LT_{50} of 1.66 days (1.50 to 1.68) days (χ^2 3.718). A positive correlation between mortality and concentration (PC = 0.774) was observed, and mortality stabilised on the fifth day of the bioassay (Figure 1).

Strain E6 at the highest concentration induced an 82% mortality rate in first-instar larvae (Table 2), with a positive correlation between mortality and concentration (PC = 0.889). As for fourth-instar larvae, mortality rate was significant only at highest concentration (48%), with a high positive correlation being observed between concentration and mortality (PC = 0.997).

3.2. Proteolytic activity

All strains (Table 3) cultured in MM + CAS showed increasing rates of enzymatic activity in relation to culture time. Strains AL and CG46 expressed significant enzymatic activity at 72 hours, while strain E6 presented a significant increase at 48 hours and the highest rate observed (U 14.82) at 72 hours.

3.3. Compatibility between fungus and insecticides

According to *t*-test, strain E6 was compatible with tebufenozide and did not significantly differ from the control group with regard to vegetative growth and sporulation, having been considered compatible (Table 4).

Table 1. Accumulated mortality (%) of second-instar caterpillars (n = 50) of *Bonagota salubricola* inassays with different strainsand concentrations of *Metarhizium anisopliae.*

Strains	Concentration	Larval mortality		
		1st day	5th day	10th day
AL	Control	0^a	4^a	6^a
	2×10^5	0^a	6^a	10^a
	2×10^6	0^a	10^{ab}	18^{ab}
	2×10^7	4^a	12^{ab}	28^{abc}
	2×10^8	4^a	26^{ab}	40^{bc}
	2×10^9	2^a	36^b	54^c
CG	Control	0^a	6^a	6^a
	2×10^5	0^a	8^a	10^{ab}
	2×10^6	0^a	10^a	12^{abc}
	2×10^7	2^a	22^a	30^{abc}
	2×10^8	2^a	16^a	34^{bc}
	2×10^9	0^a	22^a	38^c
E6	Control	0^a	2^a	2^a
	2×10^5	0^a	0^a	4^a
	2×10^6	0^a	4^a	4^a
	2×10^7	0^a	38^b	40^b
	2×10^8	0^a	62^b	66^{bc}
	2×10^9	0^a	88^c	88^c

Averages followed by different letters indicate significance ($p < 0.05$) of mortality rate by concentrations of each strain (columns) and times (lines).

4. Discussion

In this study, all strains showed pathogenicity to *B. salubricola*, especially strain E6 (Table 1). This strain induced the highest mortality rates, lowest LT_{50} (1.66 days) with high enzymatic activity, indicating virulence (Silva, 2003), since enzymatic activity might be associated with a higher ability of penetrating the insect's cuticle (Gupta et al., 1994; St.Leger et al., 1986; Tiago and Furlaneto, 2003). High Pr1, E6 levels are related with the shortest mortality time, as observed by Gupta et al. (1994). Enzymatic activity either for subtilisin-like Pr1 and trypsin-like Pr2 was higher in MM + CAS than in MM, being related to the level of carbon and nitrogen sources, as well as the presence of inducer proteins in the medium (Morais et al., 2003; Campos et al., 2005).

The higher mortality of *B. salubricola* in the first and second instars in relation to the fourth instar is due in part to the cuticle's hydrophobicity, which in the first instars favours spore adherence and consequently increases host susceptibility to fungal propagules (Boucias et al., 1988).

Table 2. Accumulated mortality rate of first and fourth-instar *Bonagota salubricola* caterpillars (n = 50) in assays with strain E6 (*Metarhizium anisopliae*) at different concentrations.

Instar	Concentration	Confirmed mortality		
		1st day	5th day	10th day
First	Control	0^a	6^a	16^a
	2×10^5	0^a	16^a	20^a
	2×10^7	4^a	41^b	46^b
	2×10^9	6^a	74^d	82^d
Fourth	Control	0^a	0^a	0^a
	2×10^5	0^a	0^a	0^a
	2×10^7	0^a	4^a	4^a
	2×10^9	0^a	48^c	48^c

Averages followed by different letters indicate significance ($p < 0.05$) of mortality rate of each strain by concentration (column) and time (line).

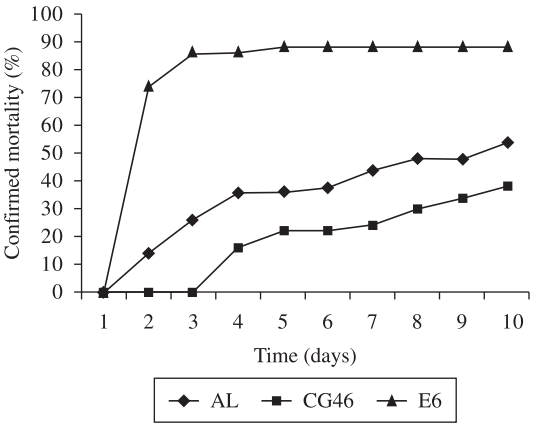

Figure 1. Accumulated mortality (%) of second-instar *Bonagota salubricola* caterpillars (n = 50) in assays with strains of *Metarhizium anisopliae* (◆AL ■CG46 ▲E6) in a concentration of 2×10^9 conidia/mL.

Table 3. Subtilisin-like (Pr1) proteolytic activity (U) of *Metarhizium anisopliae* in MM and MM + CAS.

Strain	Culture medium	Time			
		0 h	24 h	48 h	72 h
AL	MM	0.003 ± 0.005^a	0.060 ± 0.050^a	0.150 ± 0.145^a	0.076 ± 0.070^a
	MM + Cas	0.066 ± 0.058^a	0.166 ± 0.144^a	0.263 ± 0.196^a	8.870 ± 2.255^b
CG46	MM	0.014 ± 0.025^a	0.037 ± 0.034^a	0.077 ± 0.080^a	0.009 ± 0.009^a
	MM + Cas	0.104 ± 0.125^a	0.268 ± 0.252^a	0.277 ± 0.145^a	9.920 ± 0.585^b
E6	MM	0.000 ± 0.000^a	0.054 ± 0.078^a	0.062 ± 0.092^a	0.062 ± 0.092^a
	MM + Cas	0.112 ± 0.097^a	1.189 ± 0.866^a	9.286 ± 2.926^b	14.82 ± 1.925^c

U (nmol of p-nitroanilide.mL^{-1}.min^{-1}). *Averages followed by different letters indicate significance ($p < 0.05$) between U values, for each strain in relation to incubation time (line) and culture medium (column).

Table 4. In vitro compatibility of insecticides and *M. anisopliae* (E6 strain) according to vegetative growth (mm), spore number, compatibility factor "T" and respective classification.

Treatment	Vegetative growth	Qty. of spores	T %	Classification
Control	$35.08 \pm 3,19^a$	4.07×10^6	–	–
Tebufenozide	34.88 ± 1.59^a	4.49×10^6	108.22	Compatible
Methidathion	5.03 ± 0.2^b	6.20×10^4	4.08	Highly toxic
Fenitrothion	7.48 ± 0.66^b	4.20×10^4	5.09	Highly toxic
Chlorpyrifos	5.33 ± 0.78^b	1.50×10^4	3.41	Highly toxic

Averages followed by the same letter do not statistically differ ($p < 0.05$). *Highly toxic: between 0 and 30%; compatible: above 61% (Alves et al., 1998).

Other defense mechanisms include those performed by hemocytes, free cells circulating in the hemolymph, the number and types differ between species, age and stage of development (Gupta, 1985).

Results have shown that according to development stage of *B. salubricola* biological control with *M. anisopliae* might be an interesting alternative for *B. salubricola* control, since the mortality rate induced by strain E6 (82%) in first-instar larvae (Table 3) almost reached the 100% mortality rate of chemical insecticides chlorpyrifos-ethyl, methidathion, phosmet, trichlorfon, tebufenozide, fenitrotion, and carbaryl (Botton et al., 2000). With the fourth instar, mortality reached 48% (Table 3), a higher rate than that observed by Botton et al. (2000) with phosmet (27.7%), fenitrotion (22.2%) and methidathion (28.9%), and a lower mortality rate than with trichlorfon (80.6%), chlorpyrifos (88.8%), tebufenozide (83.3%), and carbaryl (55.6%). The selection of entomopathogenic fungi might ensure control of the apple leafroller in a more economical and safer way to humans and to the environment, as already shown with other pests and crops (Alves and Lopes, 2008), and as advocated by integrated management programmes (Way and Emden, 2000; Kovaleski and Ribeiro, 2003). In this respect, E6 emerges as the most virulent strain and the one with compatibility with tebufenozide. This compatibility allows their joint use in controlling the target pest more efficiently.

The use of an entomopathogen in the control of *B. salubricola* will also allow preserving known natural enemies, predators and parasitoids, thus maintaining the natural balance and decreasing those impacts routinely observed in orchards using the traditional management system on humans and the environment.

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