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The entomopathogenic fungus, *Metarhizium anisopliae* for the European grapevine moth, *Lobesia botrana* Den. & Schiff. (Lepidoptera: Tortricidae) and its effect to the phytopathogenic fungus, *Botrytis cinerea*

Juan Aguilera Sammaritano[1,2*†], María Deymié[1,2†], María Herrera[3], Fabio Vazquez[2], Andrew G.S. Cuthbertson[4], Claudia López-Lastra[1,5] and Bernardo Lechner[1,6*]

Abstract

The European grapevine moth, *Lobesia botrana* Den. & Schiff. (Lepidoptera: Tortricidae) and the gray rot fungus (*Botrytis cinerea*) are two important factors that cause elevated losses of productivity in vineyards globally. The European grapevine moth is one of the most important pests in vineyards around the world, not only because of its direct damage to crops, but also due to its association with the gray rot fungus; both organisms are highly detrimental to the same crop. Currently, there is no effective, economic, and eco-friendly technique that can be applied for the control of both agents. On the other hand, *Metarhizium anisopliae* belongs to a diverse group of entomopathogenic fungi of asexual reproduction and global distribution. Several *Metarhizium* isolates have been discovered causing large epizootics to over 300 insects' species worldwide. In this study, a simple design was conducted to evaluate the potential of native *M. anisopliae* isolates as one of biological control agents against *L. botrana* and as possible growth inhibitors to *B. cinerea*. Entomopathogenic fungal strains were isolated from arid soils under vine (*Vitis vinifera*) culture. Results suggest that the three entomopathogenic strains (CEP413, CEP589, and CEP591) were highly efficient in controlling larval and pupal stages of *L. botrana*, with mortality rates ranging from 81 to 98% (within 4–6 days). Also, growth inhibition over *B. cinerea* strains resulted in percentages ranged from 47 to 64%. Finally, the compatibility of the entomopathogenic strains, with seven commercial fungicides, was evaluated. The potential of the entomopathogenic fungal strains to act as control agents is discussed.

Keywords: Biological control, Entomopathogenic fungi, *Metarhizium*, *Lobesia botrana* , Gray rot fungus

Background

Agriculture is in a continuous search for intensification and expansion due in part to the ever expanding global population (FAO 2009). The importance of maintaining a sustainable agricultural system represents a serious challenge since this increase in food production is also directly associated with a greater requirement of resources such as water, land use, fertilizers, and pesticides (Foley et al. 2005). One of the major challenges in aiming to increase food production is that many crops are attacked by invertebrate pests, which reduce yields, generate physical crop damage, and limit exports. Thus, it impacts negatively the economy of large, medium, and small growers (Hanem 2012). Additionally, for many years, pest control techniques have been based only on

* Correspondence: aguijuan@gmail.com; blechner@bg.fcen.uba.ar
†Juan Aguilera Sammaritano and María Deymié contributed equally to this work.
[1]CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Buenos Aires, Argentina
Full list of author information is available at the end of the article

the application of chemical insecticides (Lima et al. 2012). Nevertheless, the indiscriminate use of these compounds has had negative consequences on the environment, agricultural workers' health, crop safety, and the associated growers' economy and has often led to increased pest problems (Cuthbertson and Murchie 2006). Negative impacts on the environment resulting from unnecessary pesticide applications include reduction of biodiversity along with the potential loss of key species such as bees and biological control agents, water and soil contamination, and even the generation of resistance in some invertebrate pest species (Cuthbertson 2004).

The European grapevine moth (*Lobesia botrana*) Den. & Schiff. (Lepidoptera: Tortricidae) is one of the most important pests in vineyards around the world. This moth is present in both North and South America and in many parts of Europe (Dagatti and Becerra 2015). *L. botrana* has 2–4 generations per year, depending on the latitude and prevailing climatic conditions (Martín-Vertedor et al. 2010). The first larval generation of the season usually attacks inflorescences, while the later generations cause damage to the fruits. The damage may be of two types. Direct damage is caused by larval feeding on the inflorescence or fruits, while an indirect damage occurs when larval feeding wounds are infected with fungi such as *Aspergillus*, *Alternaria*, *Rhizopus*, *Cladosporium*, *Penicillium*, and *Botrytis cinerea* Pers. (Helotiales: Sclerotiniaceae), all off which affect the quality of both fresh and wine grapes (SENASICA, 2014). *Botrytis cinerea*, the main agent of gray rot, has a broad host range and causes economic losses in both the fresh fruit and vegetable industries worldwide, causing serious losses before and after harvest. Most of the control strategies used to date have been based on the use of chemical products. However, the use of fungicides is increasingly discouraged due to problems of environmental pollution associated with high application rates and by the appearance of resistance in certain strains (Benito et al. 2000).

Studies on interactions between *L. botrana* and *B. cinerea* have demonstrated a mutualistic relationship between these organisms; both are simultaneously detrimental to the same crop (Mondy and Corio-Costet 2000). The larvae act as vectors of *B. cinerea*, disseminating conidia and opening wounds that serve as points of entry for the pathogen. These feeding wounds facilitate the rapid penetration and development of mycelium on grape berries (Fermaud and Le-Menn 1992).

In Argentina, *L. botrana* is a quarantine pest subject to official control (Heit et al. 2013 and Dagatti and Becerra 2015). Due to the impact of this pest, the competitiveness of the wine industry is at risk and can generate a crisis within important regional economies. In addition, fresh grapes destined for export must comply with internationally accepted quarantine treatments that, in some cases, increase the cost of production (Senasa (Servicio Nacional de Sanidad y Calidad Agroalimentaria) 2017). Although there are several effective techniques that aim to control adult reproduction, such as the pheromone release technique (Ioriatti et al. 2011), the costs associated with their use are too high for many local growers. The use of pheromones is expensive and only affects the adult stage of the moth. To date, there is no effective, economic, and ecologically safe technique to control the larval stages.

Entomopathogenic fungi (EPF) present an alternative solution for the pest. These organisms are important natural control agents that limit insect populations in many ecosystems, both natural and artificial (Cuthbertson and Audsley 2016). Many EPF attack eggs, immature stages, and adult life forms of many insect species (Hanem 2012); and there is a growing interest (Ali et al. 2017) to use them as biocontrol agents in integrated pest management programs (IPM).

The goal of the present study was to search for ecologically sustainable and highly effective alternative methods to control *L. botrana* larvae, using native EPF and to evaluate their effect as antagonistic agents to *B. cinerea*.

Material and methods

Insect rearing, fungal strains, and bioassays

To obtain newly emerged larvae of each instar, a breeding colony was established from *L. botrana* adults collected in Mendoza, Argentina (33°01′52″S, 68°46′34″O). Larvae were reared on an artificial diet (Herrera María et al. 2016) and were maintained in a growth chamber at a photoperiod of 16:8 (L: D), 25 ± 5 °C and a relative humidity ranging between 30 and 50%. This procedure allowed to produce high numbers of larvae of all instars for the individual bioassays.

EPF were isolated from arid soils under *V. vinifera* crops in San Juan, Argentina (31°65′67″S, 68°58′51″O). The soil sample technique followed that of Aguilera Sammaritano et al. (2016), and EPF were isolated, using the *Tenebrio molitor* larval baiting technique according to Meyling (2007). Three strains of *Metarhizium anisopliae* (Metsc.) Sorok. (CEP413, CEP589 and CEP591) were selected for the trials based on preliminary pathogenicity tests (Aguilera Sammaritano et al. 2017). All the strains were identified morphologically according to Bridge et al. (1993) and Liu et al. (2003) and are registered at the Fungal Entomopathogens Collection from the "Centro de Estudios Parasitológicos y de Vectores" (CEPAVE-CONICET, La Plata, Buenos Aires, Argentina).

For the bioassays, 20 individuals from each larval instar (L_2–L_5) and pupae (P_p) were used for each isolate, in three replicates for all cases. Sixty larvae per treatment (isolate/instar) were treated, and the replicates were run at different times.

The infection procedure was performed by placing individual larvae and pupae on 15 day sporulation cultures of *M. anisopliae* for 5 s and then placed on sterile 90 mm Petri dishes lined with filter paper, moistened with 1 ml of sterile distilled water. Five grams of the *L. botrana* artificial diet (Herrera María et al. 2016) was added to each Petri dish, sealed with Parafilm® to maintain the internal humidity. The dishes were incubated in a growth chamber at 27 ± 2 °C for 7 days in darkness. For the control, the larvae and pupae were placed in contact with PGA (potato 200 g, glucose 20 g, agar 15 g) Britania® only. Larval mortality was assessed daily for 7 days. Each dead individual with confirmed mycosis was aseptically removed from the Petri dish, placed in separated 2 ml sterile Eppendorfs, labeled, and stored at 4 °C. Abbott's equation (Abbott 1925) (Eq. 1) was used to obtain the corrected mortality (CM). Confirmation of death was made transferring spores from cadavers to individual Petri dishes with PGA. The colonies were observed after 10 days of growth at 27 °C in the dark.

$$\mathrm{CM} = \frac{\%\text{Treatment mortality} - \%\text{control mortality}}{100 - \%\text{control mortality}} \times 100 \quad (1)$$

Inhibition of Botrytis cinerea by Metarhizium anisopliae

For this trial, two strains of *B. cinerea* (B_{11} and B_{15}) isolated from vine grapes were used. These strains have previously been shown to be highly pathogenic to *V. vinifera* (Muñoz et al. 2012) and are preserved in the Mycological Collection of the Institute of Biotechnology (UNSJ-San Juan, Argentina). For each *B. cinerea* strain, a 5-mm disc of agar containing fresh mycelia from 10 days was placed in the center of a Petri dish containing 25 ml of PGA. Immediately, 3 discs from the same diameter of each EPF (from 15 days cultures) were placed carefully on the edges of the Petri dish forming a triangle around the *B. cinerea* disc. The Petri dishes were inverted to prevent conidia from either fungus falling on to the agar medium and were incubated at 28 ± 2 °C in darkness. Colony diameters (for all fungi) were measured in two perpendicular directions over the following 20 days under a stereomicroscope using a digital caliper. Three replicate plates were made for each *Botrytis* strain. There were two control treatments: the first (C_{BC}) was obtained by measuring the radial diameter of each *B. cinerea* on separate Petri dishes (i.e., potential growth) and the second (C_{EF}) comprised the three EPF strains arranged in a similar pattern to that used with the *Botrytis* discs. Three replicate plates were prepared for each control treatment. The inhibition percentage (Eq. 2) was estimated according to Jiang et al. (2014). Inhibition was considered positive when it reached > 40% (Table 1).

$$\%\text{Inhibition} = \frac{(\text{Control diameter-treatment diameter})}{\text{Control diameter}} \text{ x } 100 \quad (2)$$

Fungicide susceptibility

The susceptibility of the three EPF isolates to seven commercial fungicides was assessed. Fungicides were added directly to PGA at the rates provided by the manufacturer (Table 2) after autoclaving and cooling to 55 °C media sterilization and mixed thoroughly for 5 min before pouring into the Petri dishes. Then, 100 μl of a fungal spore suspension (3×10^3 c/ml) of each EF strain were carefully placed in the center of a 90-mm Petri dish containing 25 ml of PGA. Fungal strains were grown for 20 days at 25 °C in darkness, with a colony diameter measured in two perpendicular directions every 48 h, 2 days after inoculation. Three replicate plates were made for each strain/fungicide treatment.

Table 1 Inhibition trials of seven commercial fungicides to access compatibility with three entomopathogenic fungal strains

Fungicide	Strain	Inhibition (%) ± SD
Carbendazim	CEP413	55.98 ± 21.1 ^{A}
Carbendazim	CEP589	100 ± 0 ^{B}
Carbendazim	CEP591	100 ± 0 ^{B}
Copper oxychloride 36% WP	CEP413	80.67 ± 11.1 ^{A}
Copper oxychloride 36% WP	CEP589	73.86 ± 12.4 ^{A}
Copper oxychloride 36% WP	CEP591	73.10 ± 13.7 ^{A}
Cyprodinile - fludioxonile	CEP413	94.79 ± 4.23 ^{A}
Cyprodinile - fludioxonile	CEP589	96.95 ± 3.63 ^{A}
Cyprodinile - fludioxonile	CEP591	94.50 ± 5.17 ^{A}
Dicarboximide	CEP413	91.22 ± 5.71 ^{A}
Dicarboximide	CEP589	89.35 ± 8.10 ^{A}
Dicarboximide	CEP591	87.56 ± 11.5 ^{A}
Fenhexamide 50%	CEP413	79.17 ± 10.9 ^{C}
Fenhexamide 50%	CEP589	58.25 ± 21.8 ^{B}
Fenhexamide 50%	CEP591	41.04 ± 12.5 ^{A}
Iprodione	CEP413	89.24 ± 5.43 ^{C}
Iprodione	CEP589	61.59 ± 22.5 ^{A}
Iprodione	CEP591	74.83 ± 12.3 ^{B}
Miclobutanil 24% *p/v*	CEP413	98.72 ± 2.16 ^{A}
Miclobutanil 24% *p/v*	CEP589	97.09 ± 4.30 ^{A}
Miclobutanil 24% *p/v*	CEP591	97.88 ± 3.70 ^{A}

The inhibition (%) shows the cumulated percentage for the complete trial (288 h). Different letters indicate significant differences among strains within each fungicide (LSD Fisher 0.05) for three replicates ($n = 18$). Control treatment data (0% inhibition) are not shown

Table 2 Commercial fungicides used in trials (active ingredient and applied dose)

Commercial name	Active ingredient	Manufacturer	Dose	Target (genus)
Captan® Tomen	Dicarboximide	Cheminova	1.8 g/l	*Colletotrichum* spp. *Rhizoctonia* spp. *Plasmopara* spp.
Rovral® 50 PH	Iprodione	Pro Agro	1.5 g/l	*Rhizoctonia* spp. *Botrytis* spp. *Sclerotinia* spp.
Switch®	Cyprodinile - fludioxonile Copper oxychloride 36% WP	Syngenta	1 g/l	*Botrytis cinerea*
Teldor®	Fenhexamide 50%	Bayer	1 g/l	*Botrytis* spp.
Carbendazim® 50	Carbendazim	Nufarm	100 μL/l	*Penicilium* spp. *Fusarium* spp. *Botrytis* spp.
Systhane® Forte	Miclobutanil 24% *p/v*	Dow Agrosciences	100 μl/l	*Colletotrichum* spp. *Alternaria* spp.

Growth rate was estimated according to Kalm and Kalyoncu (2008). Control treatment for each isolate was made by adding 100 μl of the spore suspension to PGA media without fungicides.

Data analyses

The data were analyzed, using a one-way analysis of variance (Infostat 2017). In all data sets, normality and variance homogeneity were tested prior to analyses, where $p < 0.05$ was considered significant. Nonparametrical analyses were performed when certain data sets did not comply with the homoscedasticity assumption. The median lethal time to death, LT_{50}, was estimated, using parametric survival regression for combinations of fungal strains, hours of survival, and *L. botrana* larval stage. LT_{50} was performed only for the larval instars which had cumulative mortalities higher than 50%.

Results and discussion

Pathogenicity trials

There were significant differences in P_p stage in mortality levels caused by the 3 fungal strains tested ($H = 5.49$, $p = 0.03$) (Fig. 1). However, no differences were observed against L_2 ($H = 2.49$, $p = 0.32$), L_3 ($H = 1.16$, $p = 0.61$), L_4

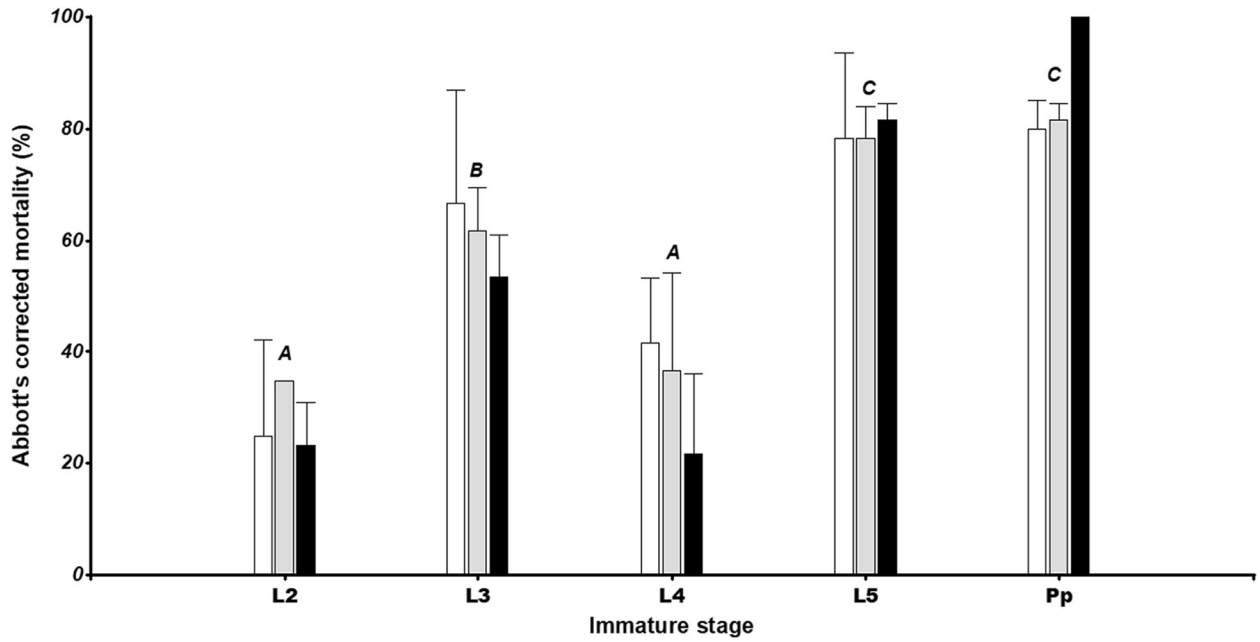

Fig. 1 Corrected mortality of *Lobesia botrana* larvae (L_2–L_5) and pupae (P_p) caused by the entomopathogenic fungus *Metarhizium anisopliae* after 7 days of exposure. Error bars represent standard deviation for three replicates. Different letters indicate significant differences only among immature stages. Columns represent corrected mortality caused by CEP413 (white), CEP589 (gray), and CEP591 (black)

($H = 3.62$, $p = 0.16$), or L_5 ($H = 0.82$, $p = 0.75$) immature stages. Among the larval instars, the CM ranged between 21.65 ± 14.43% (CEP591-L_4) and 81.6 ± 2.89% (CEP591-L_5). The highest CM (99.98 ± 0.0%) was registered for the P_p stage (CEP591). Mortality percentages among control treatments (not shown) ranged between 2% (L_3) and 7% (L_2, L_5). To our records, this is the first time that the susceptibility of larval stages of *L. botrana* to *M. anisopliae* has been demonstrated. Probit analysis for LT_{50} measured in hours showed that the highest larval instar (L_5) had lower lethal times for CEP413 (110), CEP589 (112), and CEP591 (113), when compared to L_3 instar (138, 139, 150), respectively.

All the three *Metarhizium* isolates infected and killed the larvae and pupae of the grapevine species. Additional studies to further define their potential role in integrated pest management programs for this pest seem warranted. The study presents a new evidence of demonstrating that some native strains of *M. anisopliae* derived from arid zones within Argentina were active against different stages of the vine moth, especially the older larval instars. It is widely accepted that among immature stages, eggs are more difficult to infect than larval stage (Skinner et al. 2014) and that pupae are typically very resistant to succumb to infection (Vestergaard et al. 1995). However, this is not always the case, and some biocontrol strategies effectively targeted pupal stage (Ansari et al. 2008).

The majority of biological control studies on the vine moth have focused on the use of *Bacillus thuringiensis* (Roditakis 1986 and De Escudero et al. 2007). However, Cozzi et al. (2013) tested 11 fungal strains belonging to *Fusarium* (3 strains), *Beauveria* (6 strains), *Paecilomyces* (1 strain), and *Verticillium* (1 strain) genera. They obtained a maximum mortality of 55% on *L. botrana* larvae with *Beauveria bassiana* under field conditions. Although the obtained results cannot be compared directly to those of Cozzi et al. (2013), additional fungal entomopathogens were identified that may be useful in the biocontrol of *L. botrana*. In addition, it is the first report to demonstrate the susceptibility of immature stages of grapevine moth to *Metarhizium*.

Botrytis cinerea growth inhibition

The ANOVA test showed that inhibition of strains B_{11} and B_{15} were first detected at 72 h post inoculation. No inhibition was detected prior to 48 h post inoculation (Figs. 2 and 3). There was no difference in the percent inhibition of strain B_{11} between 72 and 168 h post inoculation ($F = 0.67$, $p = 0.619$). However, differences were significant for B_{15} ($F = 5.28$, $p = 0.002$). There were no differences in the levels of *B. cinerea* inhibitions among the 3 EPF strains during 72–168 h post inoculation for B_{11} ($F = 0.14$, $p = 0.873$) and B_{15} ($F = 1.93$, $p = 0.163$). The level of inhibition ranged between 48 and 64% (B_{11}) and 47–62% (B_{15}).

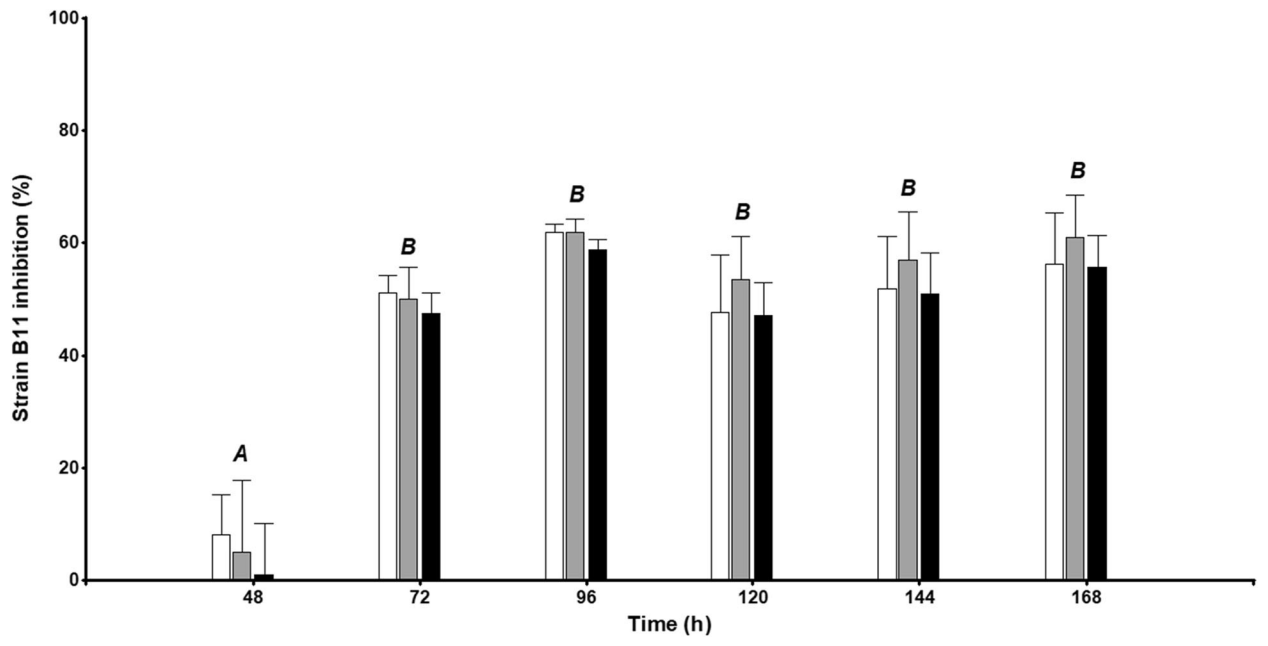

Fig. 2 Percent inhibition of *Botrytis cinerea* strain B_{11} caused by three entomopathogenic fungi. Error bars represent the standard deviation for three replicates. Different letters indicate significant differences among the time stages (LSD Fisher 0.05). Control treatments (not shown) represent 0% inhibition, and they were statistically different from the three fungal treatments. Columns represent inhibition percentage given by CEP413 (white), CEP589 (gray), and CEP591 (black)

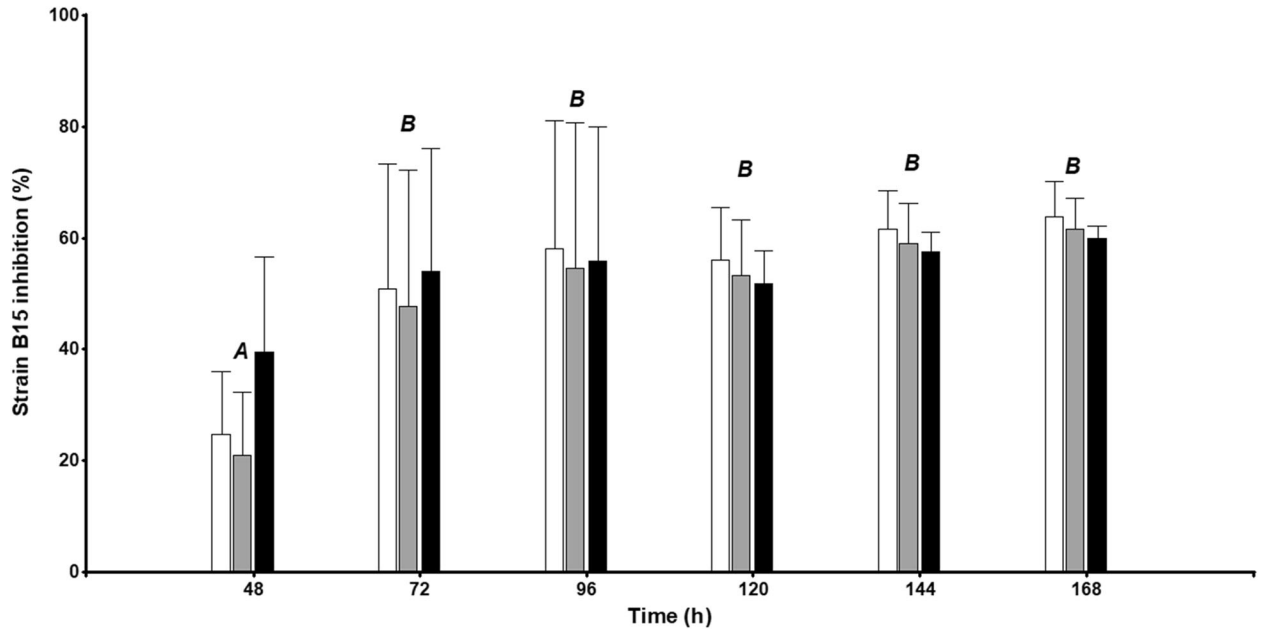

Fig. 3 Percent inhibition of *Botrytis cinerea* strain B_{15} caused by three entomopathogenic fungi. Error bars represent the standard deviation for three replicates. Different letters indicate significant differences among the time stages (LSD Fisher 0.05). Control treatments (not shown) represent 0% inhibition, and they were statistically different from the three fungal treatments. Columns represent inhibition percentage given by CEP413 (white), CEP589 (gray), and CEP591 (black)

With respect to the *B. cinerea* trials, the level of growth inhibition observed suggests that the same isolates provide additional benefits through their ability to inhibit growth of the pathogen in situ. The capacity of different entomopathogenic strains to suppress *B. cinerea* growth could improve the overall level of control obtained with selective fungicides. The tested strains had a similar inhibitory effect that reached 64%. Although there is no evidence from previous studies about using EPF to decrease the growth rate of the gray rot fungi, the study by Molina et al. (2006) proved that inhibitory effects on *B. cinerea*, using *Clonostachys* spp. provided similar results. According to Campbell (1989), *Botrytis* is highly vulnerable to competition for nutrients and substrate, which may in part explain the growth inhibition observed in the Petri dish assays. Recent studies (Hwi-Geon et al. 2017) found that *B. bassiana* and *M. anisopliae* can inhibit *B. cinerea* and control *Myzus persicae*. Therefore, there is a pre-existing antecedent, using *Metarhizium* as a potential fungicide/fungistatic agent.

Fungicide susceptibility

In general terms, the ANOVA test detected statistical differences among treatments (Table 1). On the one side, the 3 EPF strains were equally inhibited by *dicarboximide* (87–91%, $F = 0.78$, $p = 0.464$), copper oxychloride (73–80%, $F = 2.02$, $p = 0.143$), cyprodinile-fludioxonile (94–96%, $F = 1.68$, $p = 0.197$), and miclobutanil (97–98%, $F = 0.98$, $p = 0.384$). Nevertheless, some fungicides affected the strains differently. Carbendazim for example, completely inhibited strains CEP589 and CEP591. However, growth of CEP413 was inhibited by 56%. Similarly, the inhibitory effect caused by iprodione was higher for CEP413 (89%) than for CEP591 (74%) and CEP589 (61%). In the case of fenhexamide, growth inhibition was higher for CEP413 (79%) than for CEP589 (58%) and CEP591 (41%).

All of the EPF strains were highly sensitive to dicarboximide, copper oxychloride, miclobutanil, and cyprodinile—fludioxonile with inhibition percentages ranging from 73 to 98%. Therefore, the use of these fungicides together with the tested EPF strains is probably unadvisable. However, CEP413 in contrast to CEP589 and CEP591, was moderately resistant to carbendazim (56%). Equally, fenhexamide caused only a moderate inhibition on CEP589 and CEP591 (58 and 41%, respectively).

Conclusions

The entomopathogenic fungus, *Metarhizium* seems to be a good candidate for controlling *L. botrana* larval and pupal stages. Based on the obtained results, more work is required to demonstrate that EPF strains are sufficiently virulent, can control different pest life stages outside the laboratory, and can be produced and formulated in a fashion that makes it economically feasible to use one of these strains in an IPM strategy and to test compatibility with beneficial species, etc. On the other hand, the tested strains were also capable to produce a moderate antagonistic effect on *B. cinerea* and able to be combined with some fungicides.

Abbreviations
CM: Cumulative mortality; EPF: Entomopathogenic fungi; FAO: Food and Agriculture Organization; IPM: Integrated pest management; LT_{50}: Median lethal time; PGA: Potato Glucose Agar

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Availability of data and materials
Will be shared if needed.

Authors' contributions
ASJ and DM designed and conducted the study. VF, HM, and LB helped in review and proof reading and data analyses. CA and LLC helped in data collection and analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interest.

Publisher's Note

Author details
[1]CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Buenos Aires, Argentina. [2]IBT (Instituto de Biotecnología), UNSJ, San Juan, Argentina. [3]INTA (Instituto Nacional de Tecnología Agropecuaria), EEA Luján de Cuyo, Mendoza, Argentina. [4]Independent Science Advisor, York, UK. [5]CEPAVE (Centro de Estudios Parasitológicos y de Vectores), UNLP, La Plata, Argentina. [6]Universidad de Buenos Aires, FCEN, DBBE, InMiBo (Instituto de Micología y Botánica), Buenos Aires, Argentina.

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