Endophytic colonisation of tomato by the entomopathogenic fungus Beauveria bassiana: the use of different inoculation techniques and their effects on the tomato leafminer Tuta absoluta (Lepidoptera: Gelechiidae)

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Abstract
Fungal entomopathogens can naturally regulate populations of various insects. The entomopathogen Beauveria bassiana (Bals.- Criv.) Vuill. is also able to endophytically colonize different plants. Endophytic colonization by entomopathogens may provide a source of indirect interactions between fungi and insects and has been associated with the ability of the fungus to control insect pests. The tomato leaf miner, Tuta absoluta (Meyrick) is considered one of the most devastating pests of tomato (Solanum lycopersicum L.) and its difficult control is due to its miner habit, short life cycle, and high reproductive capacity. The aims of this study were: (i) to use three different techniques, i.e. leaf spraying, seed immersion and root dipping, for the endophytic inoculation of B. bassiana in tomato plants; and (ii) to assess the effect of B. bassiana on tomato leaf consumption and mortality of T. absoluta after inoculation. The percentage of colonization by B. bassiana was assessed 7, 14 and 28 days after inoculation. All inoculation techniques employed allowed the recovery of B. bassiana, although our results showed significant differences between techniques. Leaf spraying was the most effective, with the highest percentage of colonization recorded 7 days after inoculation. We also evaluated (i) the effect on the mortality of T. absoluta by direct contact with conidia of B. bassiana, and (ii) the effect on tomato leaf consumption and mortality by indirect contact through ingestion of inoculated plant tissues with B. bassiana. Mortality bioassays showed that B. bassiana infected T. absoluta, either by direct contact or indirectly, via ingestion of inoculated tomato leaves. Direct contact showed a higher percentage of mortality and a lower median survival time (MST) than indirect contact. Significant differences in the mortality percentages of T. absoluta after exposure with B. bassiana were found among the treatments and the control. Our results suggest that the endophytic inoculation of B. bassiana in tomato crops provides the basis for further investigation, which should focus on the virulence of the endophytic B. bassiana against T. absoluta.

Key words: Beauveria bassiana, biocontrol, endophytes, fungal entomopathogens, Solanum lycopersicum, Tuta absoluta

Introduction
Entomopathogenic fungi (EPF) are commonly found in diverse habitats and are known to infect many different taxa of arthropods. These fungi have also been found as rhizosphere colonizers in the surrounding environment of the host plant. In addition, recent evidence suggests that certain EPF species have the potential to engage in fungus-plant interactions, as fungal endophytes or plant disease antagonists, without causing any immediate negative effect or even promoting growth of host plants (Vega et al. 2008).
However, although many entomopathogenic fungal endophytes might not be very abundant in most plant species, some taxa like Beauveria bassiana (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales) have a wide range of plant hosts. The occurrence of EPF as natural endo-


trol of T. absoluta.

Materials and Methods

Fungal isolate

The fungal strain used was B. bassiana LPSC 1067, obtained from the culture collection of "Instituto Spe-
gazzini" (Universidad Nacional de La Plata), La Plata, Buenos Aires, Argentina. Selection of this fungal strain was based on its laboratory efficacy against other com-


Conidial suspension

Conidia were obtained from cultures grown on potato dextrose agar medium (PDA; Britania S.A., Buenos Aires, Argentina) after incubation for 10 days at 25°C in darkness. Conidia were harvested with disposable cell scrapers (Fisherbrand) and placed in test tubes containing 0.01% (v/v) Tween 80 (polyoxyethyl-


Tomato plants

Tomato seeds (Solanum lycopersicum cv. platense) were obtained from organic farms in the surroundings of La Plata city, Buenos Aires province, Argentina (34°57'17"S, 57°53'26"W). None of these seeds had received prior chemical treatment. Seeds were planted in pots filled with approximately 86 g of an equally ground mixture of perlite, vermiculite, and soil (1 : 1 : 1), which was sterilized in an autoclave for 1 hour over three consecutive days prior to use. The seedlings were maintained in a greenhouse at 24°C, with 70% relative humidity and under 12 : 12 h (L : D) photoperiod by using 400 nmol·m⁻²·s⁻¹ cold light lamps.
Inoculation techniques

Tomato seeds were surface sterilized by placing them in 70% ethanol for 2 min, then washed in sterile distilled water, followed by immersion in 0.5% sodium hypochlorite for 1 min, and rinsed again in sterile distilled water (Brownbridge et al. 2012). Seeds were placed on sterile filter paper to dry for 30 min and then divided into two portions. One portion was used for seed inoculation and the other was used for leaf spraying and root dipping after emergence. For seed inoculation, seeds (50 g) were immersed in 10 ml of a conidial suspension (1 × 10^8 conidia · ml⁻¹) of B. bassiana for 24 h, according to Brownbridge et al. (2012). Thereafter, seeds were dried on sterile tissue paper in a laminar flow cabinet for 30 min, sown in pots at a depth of 4 cm, and maintained in a greenhouse at 25°C under 12 : 12 h (L : D) photoperiod. Control seeds were immersed in a conidia-free solution of 0.01% Tween®80.

In the case of leaf spraying and root dipping, seeds were planted in pots filled with approximately 86 g of an equally ground mixture of perlite, vermiculite, and soil (1 : 1 : 1), which was sterilized in an autoclave for 1 h over three consecutive days prior to use. For the leaf spraying technique, leaves of 30 cm tall tomato plants (7 weeks old) were sprayed with 3 ml of a conidial suspension (1 × 10^8 conidia · ml⁻¹) using a glass hand sprayer (30 ml capacity). To avoid conidial runoff to the soil, the top of each pot was covered with aluminium foil (Posada et al. 2007). Control plants were sprayed with a conidia-free solution of 0.01% Tween®80.

For root dipping, we used seedlings three weeks after emergence. Each seedling was removed from the pot and rinsed three times with sterile distilled water. The ends of the roots were cut for better absorption and placed individually in test tubes with 2 ml of a conidial suspension (1 × 10^6 conidia · ml⁻¹) of B. bassiana for 1 h over three consecutive days prior to use. For the leaf spraying technique, leaves of 30 cm tall tomato plants (7 weeks old) were sprayed with 3 ml of a conidial suspension (1 × 10^8 conidia · ml⁻¹) using a glass hand sprayer (30 ml capacity). To avoid conidial runoff to the soil, the top of each pot was covered with aluminium foil (Posada et al. 2007). Control plants were sprayed with a conidia-free solution of 0.01% Tween®80.

Evaluation of the presence of Beauveria bassiana as an endophyte

Colonization of tomato seedlings by B. bassiana was assessed 7, 14 and 28 days after inoculation. To determine the potential presence of this fungus in the plant tissues, one leaf from each plant was randomly chosen and surface-disinfected by immersion in 0.5% sodium hypochlorite for 2 min, followed by 2 min in 70% ethanol and rinsed with sterile distilled water (Arnold et al. 2001). Leaves were dried on sterile paper towels in a laminar flow cabinet and their edges were cut to remove dead tissue ensuing from the sterilization process. Complete disinfection of leaves was checked by plating 100 ml of the last rinsing water of each sample onto PDA. In addition, subsamples of surface sterilized leaves were pressed against PDA to determine whether residual conidia retained the germinative potential (Gurulingappa et al. 2010). Leaves were cut into six sections of approximately 1 cm² using a sterile scalpel. For each of the inoculation techniques tested, six leaf sections from each of the 30 treated plants and six leaf sections from the control plants were used.

A 0.1% stock of 0.02 g of each antibiotic (tetracycline, streptomycin, and penicillin) dissolved in 10 ml sterile distilled water, followed by filter sterilization through a 0.2 µm filter (Syringe filter sterile, E-Chrom Tech, Taiwan) was prepared and then 1 ml of this stock was added to each litre of medium (Vega et al. 2008). Leaf sections were placed on dishes containing PDA with antibiotics. For each inoculation technique, we made 30 replicates for each treated seedling and 30 replicates for control plants. A total of 120 plants and 720 leaf sections were examined. The presence or absence of B. bassiana on the leaf sections was recorded after 10 days at 25°C. Data were expressed as frequency of colonization (number of colonized leaf sections/total number of leaf sections) × 100 (Petriini and Fisher 1986).

Insect rearing

The colony of Tuta absoluta was established in 2011 from plant material infested with larvae collected from crops without any history of pesticides, in fields surrounding La Plata city, Argentina (35°01’24.97”S, 58°03’34.69”W). The plant material was maintained under quarantine to discard the presence of potential diseases and parasites and then incorporated into the colony. Periodically, the colony was infused with wild stocks collected from the same geographical area, to maintain its genetic variability. Larvae were fed pesticide-free tomato seedlings (S. lycopersicum cv. platensis) whereas adults were fed a 15% honey-water solution. The maintenance of the insect colony and all bioassays was carried out in the laboratory under controlled conditions: 25±2°C, 70±5% of relative humidity and 14 : 10 h (L : D) photoperiod.

Bioassay 1: Mortality of Tuta absoluta by direct contact with Beauveria bassiana

To determine the infectivity of the selected strain against T. absoluta, 20 tomato leaf discs (2 cm diam.)
were cut. Ten of them were immersed in a 0.001% solution of Tween®80 of *B. bassiana* (1 × 10⁸ conidia · ml⁻¹) and the other 10 (controls) in a 0.001% conidia-free solution of Tween®80. The experiment consisted of four replicate test groups and four control groups, each group containing 10 2nd instar larvae. Leaf discs were placed in humid chambers with a second instar larva per disc. Every 24 h, larval mortality and larval instar were recorded. Experiments were repeated four times under comparable laboratory conditions. All bioassays were carried out in a growth chamber with controlled conditions [25±2°C, 70±5% of relative humidity and 16 : 8 h (L : D) photoperiod]. Larval mortality was evaluated daily for 10 days until the end of the experiment. Dead larvae were removed and immediately deposited in high-humidity chambers (sterile Petri dishes with filter paper dampened with sterile distilled water) and mycosis was confirmed under a stereoscopic microscope and by microscopical examination of the dead insects.

**Bioassay 2: Evaluation of leaf consumption and mortality of *Tuta absoluta***

Tomato plants from seeds sown in a sterile organic substrate and maintained in a greenhouse [24°C, 70% relative humidity and 12 : 12 h (L : D) photoperiod] were employed to evaluate the effect of *B. bassiana* as an endophyte on *T. absoluta*. Inoculation was performed by spraying the leaves with 30 ml of a 0.001% (v/v) conidial suspension in Tween®80 (1 × 10⁸ conidia · ml⁻¹) using a glass hand atomizer (35 ml capacity). In the case of controls, leaves were sprayed with 30 ml of a 0.001% (v/v) conidia-free solution of Tween®80. Tomato treated leaves were inoculated four days before starting the test. Twenty tomato leaf discs (2 cm diam.) were cut. Ten of them corresponded to inoculated discs and 10 to control discs. The experiment consisted of four replicate test groups and four control groups, each group containing 10 2nd instar larvae. Leaf discs were placed in humid chambers with a second instar larva per disc. The discs were scanned 24, 48 and 72 hours post-treatment and the area consumed (mm²) was calculated (in mm²) using the program ImageJ at 24, 48 and 72 hours post-treatment. Data were tested for normality using the Shapiro-Wilk test and analysis of data was performed either with Mann-Whitney’s test or Student’s t-test for two independent samples. The median survival time (MST) was calculated based on the Kaplan-Meier estimate of the survival distribution function (XLSTAT Life Software, 2014). Pairwise comparisons between survival curves were made by the log-rank test.

When the mortality in treated *T. absoluta* was 50% or higher, the MST was calculated based on the Kaplan-Meier Survival distribution function (XLSTAT Life Software, 2013). Pairwise comparisons between survival curves were made by the log-rank test.

**Results**

**Evaluation of Beauveria bassiana as an endophyte**

We evaluated the frequency of endophytic colonization of tomato plants by *B. bassiana* according to the inoculation technique (leaf spraying, root dipping, and seed immersion), post-inoculation time (7, 15, and 28 days) and the interaction between these two variables. Results showed significant differences between inoculation techniques (F = 13.9; df = 2; p < 0.001). Spraying yielded the highest frequency (10%), 27 inoculated plants, followed by root dipping (5.56%) and by seed immersion, which showed significantly lower
valuable (0.93%), 16 and 2 inoculated plants respectively. Differences of statistical significance were found in the colonization of B. bassiana between post-inoculation times (F = 36.06; df = 2; p < 0.001). The highest percentage of colonization was obtained 7 days after inoculation (13.89%) and the lowest after 28 days (0.56%). The interaction between inoculation techniques and post-inoculation times also showed significant differences (F = 17.97; df = 4; p < 0.001). The highest values were recorded 7 days after inoculation using both leaf spraying (mean values: 29.44%) and root dipping (mean values: 12.22%). The remaining values recorded for the interaction of these variables were significantly lower (Fig. 1).

Likewise, it was observed that the endophytic implementation, which was estimated by the percent of post-inoculation recovery of B. bassiana, decreased over time. Beauveria bassiana was not isolated from the control plants or found in washings from surface sterilized leaves (Gurunligappa et al. 2010). In all cases, the average viability of the conidia was over 95%.

Bioassays 1
Results from mortality bioassay showed that B. bassiana was able to infect T. absoluta by direct contact with conidia. Significant differences of mortality of T. absoluta were observed between treatment and control (F = 215.09; df = 1; p < 0.0001). Infection by B. bassiana, in all dead larvae was confirmed undera stereoscopic microscope and by microscopical examination.

Bioassays 2
Colonization percentages for the plants utilized in the test were 86.6% and B. bassiana was not detected in any of the control plants. Significant differences of mortality of T. absoluta were observed between treatments and control (F = 23.70; df = 1; p = 0.0028). Infection by B. bassiana, in all dead larvae was confirmed undera stereoscopic microscope and by microscopical examination. The leaf area consumed showed no significant differences between the bioasssay and the control at 24 and 72 hours post-inoculation, however, significant differences between treatments were observed after 48 h (Table 1). Beauveria bassiana was not isolated from the control plants or found in washings from surface sterilized leaves (Gurunligappa et al. 2010).

Results from mortality bioassays showed that B. bassiana was able to infect T. absoluta, either by direct contact with conidia or indirectly by ingestion of tomato leaves colonized endophytically by B. bassiana (Fig. 2). The highest percentage of mortality was recorded in bioassay 1 (87.5±5%), followed by bioassay 2 (72.5±20.6%), while the controls presented 5±10% mortality. The MST recorded in bioassay 2 (9.47±0.57 days) was higher than the results obtained in bioassay 1 (5.97±0.72 days), which shows a greater effectiveness of the conidia when they invade the host through the integument.

Table 1. Leaf consumed area by Tuta absoluta second instar larvae exposed to tomato leaf discs inoculated with Beauveria bassiana (bioassay 2) and to non-inoculated leaf discs (control)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Area consumed [mm²]</th>
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<tbody>
<tr>
<td></td>
<td>24 h*</td>
</tr>
<tr>
<td>Control</td>
<td>2.72 (±1.133) a</td>
</tr>
<tr>
<td>Bioassay 2</td>
<td>2.553 (±1.061) a</td>
</tr>
</tbody>
</table>

Mean values (±standard deviation) of area consumed (mm²) by second instar larvae of T. absoluta at 24, 48 and 72 hours after the beginning of the test. Different letters indicate significant differences between treatments; * t-Test for two independent samples; ** Mann-Whitney’s test.
Discussion

Fungal endophytes have evolved to inhabit both apoplastic and symplastic regions of plant tissues (Saikkonen et al. 1998), without causing visible harm or signs of disease in the host (Gimenez et al. 2007). Approximately 700 species from 90 genera of entomopathogenic fungi are known, including Acremonium, Beauveria, Cladosporium, Clonostachys and Isaria. Many of these genera are naturally found or proved fungal endophytes have evolved to inhabit both apoplastic and symplastic regions of plant tissues (Saikkonen et al. 1998), without causing visible harm or signs of disease in the host (Gimenez et al. 2007). Approximately 700 species from 90 genera of entomopathogenic fungi are known, including Acremonium, Beauveria, Cladosporium, Clonostachys and Isaria. Many of to be plant endophytes, but only 12 of them have been tested as biocontrol agents (Vega et al. 2008). In this work, we reported for the first time the ability of B. bassiana LPSC 1067 to colonize endophytically tomato plants. Other isolates of B. bassiana have been established previously as endophytes in various plants using different inoculation methods. Some examples are in potatoes by foliar spraying (Wagner and Lewis 2000), in tomatoes by stem injections (Bing and Lewis 1991) or coating seeds with B. bassiana conidial suspensions and in opium poppies after spraying leaves or coating seeds with B. bassiana conidial suspensions (Quesada-Moraga et al. 2006). However, some studies indicated that leaves are poor routes of entry for this fungus in some plants, such as coffee (Posada et al. 2007). Our results indicate that B. bassiana was effectively established as an endophyte in tomato plants when inoculated either by leaf spraying, root dipping or seed immersion, and was re-isolated from leaves 7, 14 and 28 days after its inoculation. The most effective inoculation technique was leaf spraying and the highest percentage of colonization was recorded 7 days after inoculation. It was observed that the endophytic colonization, estimated by the percentage of recovery of B. bassiana after inoculation, decreased over time. We demonstrated that the selected isolate of B. bassiana was able to colonize tomato plant tissues and was re-isolated from new leaves, which confirmed the establishment of the fungus in the plant tissues and its potential to move throughout them. This work showed that B. bassiana was able to infect T. absoluta by direct contact with conidia. Direct contact by leaf spraying showed a higher mortality rate and a lower MST value than indirect contact. Since T. absoluta is a leaf miner pest of tomato leaves, the endophytic colonization of tomato tissues by B. bassiana and its infective capacity of the pest T. absoluta provides the basis for further investigation, which should focus on the virulence of the endophytic B. bassiana against T. absoluta.

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References


Akello J., Dubois T., Gold C.S., Coyne D., Nakamura J., Pararu P. 2007. Beauveria bassiana (Balsamo) Vuillimin as an endophyte in tissue culture banana (Musa spp.). Journal of Invertebrate Pathology 96 (1): 34–42. DOI: 10.1016/j.jip.2007.02.004


Barrientos Z.R., Apablaza H.J., Norero S.A., Estay P.P. 1998. Temperatura base y constante termica de desarrollo de la polilla del tomate, Tuta absoluta (Lepidoptera: Gelechiidae) [Base temperature and constant thermal development of the tomato moth, Tuta absoluta (Lepidoptera: Gelechiidae)]. Ciencia e Investigacion Agraria 25 (3): 133–137.


Pelizza S.A., Cabello M.N., Lange C.E. 2010. Nuevos registros de hongos entomopatógenos en acridios (Orthoptera: Acri-
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