

ORIGINAL ARTICLE

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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Vol. 57, No. 4: 331–337, 2017

DOI: 10.1515/jppr-2017-0045

Received: July 14, 2017

Accepted: November 17, 2017

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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 endophytes also indicates that these fungi have complex life cycles, which can be completed in soil, invertebrates, and plants. *Beauveria bassiana* is an entomopathogenic fungus with worldwide distribution, which can live as a plant endophyte and usually does not cause visible damage to the host (Van Bael *et al.* 2005). It has been naturally isolated from several plant species, and artificially introduced into many others, such as tomato (*Solanum lycopersicum* L.) (Ownley *et al.* 2004), banana (*Musa paradisiaca* L.) (Akello *et al.* 2007), coffee (*Coffea arabica* L.) (Posada *et al.* 2007), sorghum (*Sorghum bicolor* Kuntze) (Reddy *et al.* 2009), pine (*Pinus radiata* D. Don) (Brownbridge *et al.* 2012), tobacco (*Nicotiana tabacum* L.), corn (*Zea mays* L.), wheat (*Triticum aestivum* L.) and soybeans (*Glycine max* L.) (Russo *et al.* 2015) by using various techniques. Colonization of plant tissues by *B. bassiana* has proved to provide protection against insect damage and inhibition of insect establishment and development (Ownley *et al.* 2004; Vega *et al.* 2008). In addition, protection against phytopathogens has been documented (Ownley *et al.* 2004). In Argentina, *B. bassiana* has been registered as entomopathogenic in Lepidoptera (Fresa 1979), Hemiptera (Toledo *et al.* 2008), Orthoptera (Pelizza *et al.* 2010), Diptera (Siri *et al.* 2005) and Coleoptera (López Lastra 1988).

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), was first described in Peru in 1917, and is now found throughout South America where it is considered to be one of the most devastating tomato pests (Barrientos *et al.* 1998). This pest was initially reported in eastern Spain in late 2006 and has subsequently spread throughout the Mediterranean Basin and Europe (Potting 2009). Since the time of its initial detection, it has caused serious damage to tomato crops in invaded areas and it is currently considered a major agricultural threat to European and North African tomato production (Abd El-Ghany *et al.* 2016). Although its main host is tomato, it also affects other Solanaceae, whether cultivated or not (Tropea Garzia *et al.* 2012). The control of *T. absoluta* is difficult due to its miner habit, short life cycle, and high reproductive capacity. The current management of *T. absoluta* is mainly based on the application of chemical insecticides. Nevertheless, a few active ingredients are effective against this pest without harming beneficial insects and pollinators at the same time. The use of synthetic insecticides, persistent or not, leads to resistant populations (Lietti *et al.* 2005) and to the disruption of natural biological control (Riquelme Virgala *et al.* 2006). Therefore, the

use of integrative control methods (cultural, biological and biotechnological) becomes imperative, as the continued use of chemical insecticides could harm non-target organisms and the environment. The endophytic inoculation of tomato plants with *B. bassiana* could be a promising alternative for the biocontrol of *T. absoluta*.

Materials and Methods

Fungal isolate

The fungal strain used was *B. bassiana* LPSC 1067, obtained from the culture collection of “Instituto Spaggiari” (Universidad Nacional de La Plata), La Plata, Buenos Aires, Argentina. Selection of this fungal strain was based on its laboratory efficacy against other common pest insects in Argentina (Pelizza *et al.* 2012a, b). The species was identified using both molecular (GenBank accession number KF500409) and morphological data, as described by Russo *et al.* (2015).

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 (polyoxyethylene sorbitan monolaurate) (Merck). Suspensions were vortexed for 2 min, filtered through four layers of sterile muslin, and adjusted to 1×10^8 conidia \cdot ml⁻¹ (Gurulingappa *et al.* 2010) after cell counting in a Neubauer haemocytometer. Conidial viability was assessed before every experiment (Goettel and Inglis 1997). This germination test was repeated for each stock suspension to maintain the constancy of the viability assessments. In all cases, the average viability of the conidia was over 95%.

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 \cdot m⁻² \cdot 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 seedling emergence. For seed inoculation, seeds (50 g) were immersed in 10 ml of a conidial suspension (1×10^8 conidia \cdot 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 \cdot 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 (1×10^8 conidia \cdot ml⁻¹) after Akello *et al.* (2007). Each tube was covered with aluminium foil. The roots of control plants were submerged in sterile distilled water. Control plants were incubated at 25°C, with 80% relative humidity and under 12 : 12 h (L : D) photoperiod for 24 h. Thereafter, both the control and treated plants were placed on sterile filter paper until completely dried and then replanted in the same pots. Seedlings in all treatments were watered as needed and maintained in a greenhouse at 24°C, with 70% relative humidity and under 12 : 12 h (L : D) photoperiod. For each of the inoculation techniques, 30 replicates were made for each treated seedling and 10 for the control.

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) \times 100 (Petrini 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. *platense*) 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* (1×10^8 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 (1×10^8 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 using the program ImageJ program. At 96 hours post-treatment, a foliolo or an untreated tomato leaf was added to each dish as food in order to continue the development of the different larvae. The larval mortality was evaluated daily under a stereomicroscope for 10 days and/or 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. To confirm the presence of *B. bassiana* in the internal tissues of each inoculated leaf disc, the remaining parts of the inoculated leaves used

to cut the discs were individually disinfected (Arnold *et al.* 2001) as previously explained. Leaf pieces were dried on sterile paper towels in a laminar flow cabinet and the edges were cut to remove dead tissue resulting from the sterilization process. Thereafter, leaf pieces were placed on PDA in a Petri dish and incubated in the dark at 25°C for 10 days. In addition, subsamples of surface sterilised leaves were pressed against PDA to determine whether residual conidia retained the germinative potential (Gurunligappa *et al.* 2010).

Data analysis

Inoculation techniques: An analysis of variance (ANOVA) was used to evaluate the differences between inoculation techniques, the time of permanence of the fungus in the plant and the interaction between the two variables. The frequency of colonization (expressed as percentage) was angular transformed to stabilize the variance. ANOVA was performed with the software InfoStat 2007 (InfoStat 2001).

Bioassays 1 and 2: ANOVA and a *posteriori* Tuckey's test were performed to analyse the mortality data, using the program XLSTAT (Addinsoft XLSTAT for Excel, Paris, France, 2009). The leaf area consumed 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

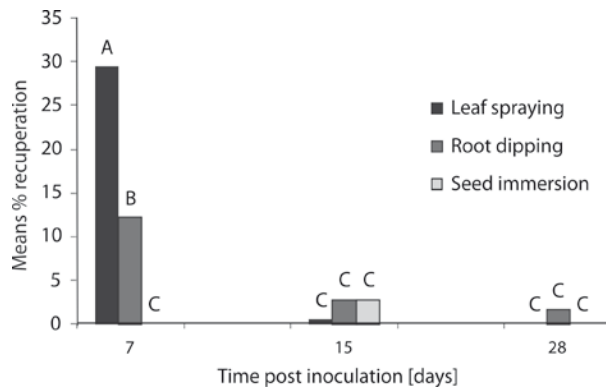


Fig. 1. Mean (\pm standard deviation) colonization (%) of tomato leaf sections 7, 15 and 28 days after inoculation with *Beauveria bassiana*, using different inoculation techniques (leaf spraying, root dipping and seed immersion). Bars with different letters indicate differences of statistical significance according to Tukey's test ($p < 0.05$)

values (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

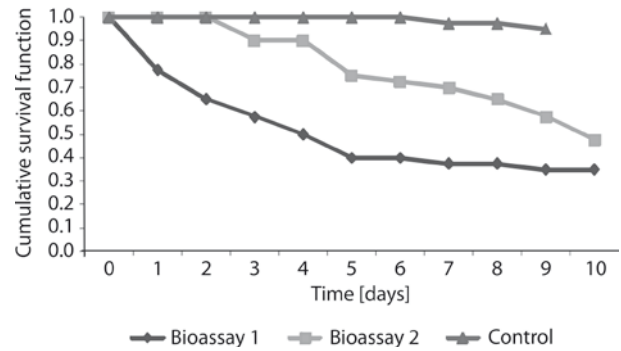


Fig. 2. Cumulative survival curves of *Tuta absoluta* in bioassay 1, bioassay 2 and control

($F = 215.09$; $df = 1$; $p < 0.0001$). Infection by *B. bassiana*, in all dead larvae was confirmed under a 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 under a stereoscopic microscope and by microscopical examination. The leaf area consumed showed no significant differences between the bioassay 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 ($87.5 \pm 5\%$), followed by bioassay 2 ($72.5 \pm 20.6\%$), while the controls presented $5 \pm 10\%$ 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)

Treatments	Area consumed [mm ²]		
	24 h*	48 h**	72 h*
Control	2.72 (± 1.133) a	8.451 (± 4.485) b	16.909 (± 8.024) d
Bioassay 2	2.553 (± 1.061) a	11.987 (± 6.300) c	20.3 (± 9.712) d

Mean values (\pm 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 noted 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*.

Acknowledgements

This research was partially supported by Fondo para la Investigación Científica y Tecnológica (FONCYT; PICT 2013-0543 and PICT 2014- 0677).

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