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Endophytic colonization of tomato plants by the entomopathogenic fungus *Beauveria bassiana* for controlling the South American tomato pinworm, *Tuta absoluta*

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Abstract

Background: The deployment of entomopathogenic fungi (EPF) for the control of crop pests is an important alternative to synthetic pesticides. Despite recent advances in EPF formulations and application techniques, their efficacy is still limited by abiotic and biotic factors. Entomopathogenic fungi naturally colonize plant tissues or they can be artificially inoculated, protecting the plants from insect attack. *Beauveria bassiana* is the most widely used fungal biological control agent and has potential as an endophyte to protect a range of crops. Although *B. bassiana* is known to be pathogenic to the South American tomato pinworm, *Tuta absoluta* (Meyrick), this fungus could be more efficient when deployed as an endophyte to protect tomato plants from attack.

Methods: Three *B. bassiana* isolates were screened for virulence against *T. absoluta* larvae by application of conidia to leaf surfaces. Following confirmation of virulence, tomato plants were then exposed to conidial suspensions using a forced uptake technique. Only one isolate, LPP139, colonized the leaves. Seedlings were then inoculated with LPP139 and the presence of the fungus in different plant tissues was monitored over 30 days. Possible effects of *B. bassiana* endophytic colonization on plant development were also evaluated. Following confirmation of endophytic colonization of leaf tissues, *T. absoluta* 2nd or 3rd instar larvae were offered leaves and survival was monitored over a 7-day period.

Results: All three *B. bassiana* isolates were virulent to *T. absoluta*, with approximately 90% mortality over 10 days when conidia had been applied to leaf surfaces. Various plant inoculation techniques were tested but only seedling inoculation was successful. LPP139 successfully colonized all of the plant tissues. High percentages of colonization were observed in roots, stems and leaves up to 30 days after inoculation, with no negative effects on plant growth. When *T. absoluta* larvae were exposed to *B. bassiana* colonized leaves, survival was reduced to zero over a 7-day period.

Conclusions: The endophytic colonization of tomato plants with EPF is a promising method of controlling the South American tomato pinworm. The fungus was detectable for up to 30 days, longer than has been previously observed

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for tomato plants. Seedling inoculation could be a viable commercial option for marketing pre-colonized tomato plants.

Keywords: Biological control, Insect, Pest, Crop damage, Fungus, Pathogen, Virulence

Background

The demand for more sustainable pest control measures is being driven by the ever increasing levels of insecticide resistance now observed in many pest populations and concerns for environmental safety (Silva et al. 2011; Bacci et al. 2019). The use of biological control is an alternative to synthetic insecticides. The application of insect pathogens, such as entomopathogenic fungi (EPF), is a highly promising alternative for the protection of crops against pest insects (Charnley and Collins 2007; Maina et al. 2018). Entomopathogenic fungi can be deployed against phytophagous insects by conventional spraying techniques aiming to cause mortality by direct contact of the inoculum to the host or indirectly when the insect comes into contact with the inoculum on the plant surface. Entomopathogenic fungi normally infect insects by penetration of the integument, followed by proliferation in the hemocoel and subsequent colonization of host tissues, resulting in host death (Charnley 1984).

When selecting a microbial control method, it is very important to not only understand the biology of the target insect but also the biology and ecology of the microbial control agent. Entomopathogenic fungi are living organisms and therefore require more stringent storage and application procedure than synthetic insecticides. The efficiency of EPF can be seriously compromised by biotic and abiotic factors. Exposure of EPF to high levels of UV radiation, low relative humidity or unfavorable temperatures (lower than 20 °C or higher than 35 °C) can drastically reduce virulence (Lindow 2006; Saunders et al. 2010). However, formulation techniques have been shown to increase EPF viability under stressful environmental field conditions. For example, emulsifiable oil-based formulations increased fungal tolerance to high temperatures (Oliveira et al. 2018), whilst addition of vegetable oils to conidial suspensions increased protection against the harmful effects of UV (Kaiser et al. 2018).

Considering alternative ways of protecting EPF from adverse conditions, the incorporation of EPF in plants as endophytes would appear to be a highly interesting and promising approach (Vega 2018; Branine et al. 2019). Furthermore, some insect pests have evolved feeding habits which make them more difficult to control by conventional methods. Leaf-miners and stem-borers are examples of such pests (Guedes et al. 2019).

Worldwide tomato cultivation suffers huge losses due to insect attack, reducing the quality of the fruits and transmitting plant pathogens. The South American tomato pinworm, *Tuta absoluta* (Lepidoptera: Gelechiidae), is one of the main insect pests of tomato (*Solanum lycopersicum*). Larvae of this moth are leaf-miners, damaging leaves, stems and terminal buds (Biondi et al. 2018). The mining behavior makes this pest very difficult to control and even systemic insecticides result in low levels of pest control, causing contamination of the fruits, environmental poisoning and human health problems (Guedes et al. 2019). Alternative strategies using EPF have been proposed to control *T. absoluta*. Giustolin et al. (2001) demonstrated that all larval stages of *T. absoluta* were susceptible to *B. bassiana* infection, although 1st instar larvae were more susceptible than 4th instar larvae. This could have been due to the longer exposure times of 1st instars, which remain on the leaf surface for 20–45 min before penetrating the mesophyll. A variety of *M. anisopliae* and *B. bassiana* strains/isolates have been screened against *T. absoluta* eggs and larvae, with different levels of virulence. The infection of different stages of *T. absoluta* lifecycle is important factor that could further reduce crop damage (Pires et al. 2010; Zekeya et al. 2019).

The term "endophyte" was first used by De Bary in 1884 (Vega et al. 2008), and is defined as a microorganism that is present asymptomatically within plant tissues. Several genera of EPF have been characterized as natural endophytes, however EPF also have the potential to be introduced into plants as "innate" biological control agents (Vega 2018). The introduction of an EPF as an endophyte could protect the pathogen from adverse environmental conditions, reducing the negative effects of ultraviolet radiation and limitations of low humidity on development. The deployment of endophytic EPF could be the basis of a biological control strategy against specific pests, especially those with life cycles involving perforating and feeding inside the leaves, stems, rhizomes, roots and seeds, behaviors that reduce the exposure of the pest to synthetic insecticides and other control methods (Resquín-Romero et al. 2016).

Reduced insect performance on plants containing EPF as endophytes can result from feeding inhibition (Vega et al. 2008; Christian et al. 2020). Experiments have shown reduced feeding of *Ostrinia nubilalis* (Lepidoptera; Crambidae) on corn plants with endophytic *B. bassiana* (Bing and Lewis, 1991; Vega et al. 2008). The corn borer *Sesamia calamistis* also showed reduced levels of feeding on corn plants colonized with endophytic

B. bassiana (Cherry et al. 2004). Studies on maize indicated that the establishment of *Beauveria* sp. in the plants offered systemic protection against defoliating insects (Bing and Lewis, 1992; Resquín-Romero et al. 2016). Temporary endophytic colonization has been reported in alfalfa, melon and tomato plants following foliar spraying of *Metarhizium brunneum* (Ascomycota; Hypocreales) and *B. bassiana*. These fungi established themselves as endophytes for up to 96 h after inoculation (Resquín-Romero et al. 2016).

Beauveria bassiana and *M. brunneum* have been reported as endophytes in *Capsicum annum* peppers, establishing themselves within 17 days following inoculation (Jaber and Araj 2018). The endophytic association of *Beauveria brongniartii* and *M. brunneum* in *Vicia faba* was evaluated using different fungal strains inoculated through foliar spraying (Jaber and Enkerli 2017). The efficiency of colonization techniques can differ between plant species, which could be related to genotype and this could also be a determining factor in the persistence of the fungus in the plant. A range of artificial inoculation procedures of EPF in plants have show variable levels of efficiency. Inoculation methods include leaf spraying, injection through the stem, soil irrigation and seed treatments (Bing and Lewis 1992; Quesada-Moraga et al. 2006). Tomato plants have been artificially colonized by EPF (Klieber and Reineke 2016; Allegrucci et al. 2017) with low persistence of the fungi as endophytes and efficiency when considering *T. absoluta* control.

The aim of the current study was to investigate techniques to colonize tomato plants with *B. bassiana* for the control the larval phase of *T. absoluta*, thus potentially reducing the need for excessive pesticide applications.

Methods

Fungal isolates and conidial production

All of the isolates used here were obtained from the collection maintained at the Universidade Estadual do Norte Fluminense-Insect Pathology laboratory. Two of the isolates, LEF140 and LEF141, where originally found infecting adult *Pachymerus* sp. (Coleoptera: Chrysomelidae: Bruchinae) and one isolate (LPP139) was found infecting an adult coffee berry borer *Hypothenemus hampei* (Coleoptera; Scolytidae).

Prior to testing these isolates against *T. absoluta*, all of the fungi were first cultivated on Sabouraud Dextrose Agar (SDA) and after incubation for 2 weeks at 27 °C, conidia were harvested under sterile conditions by scraping the surface of the cultures using a metal spatula and re-inoculating the conidia onto solid rice media for mass production. The rice media was prepared using 50 g of parboiled rice presoaked in 30 mL of water, placed in conical flasks and then autoclaved for 20 min at 121 °C. After 15 days incubating at 27 °C, conidia were harvested by suspending them in sterile distilled water + Tween 20 (0.01% v/v). The suspensions were centrifuged at 5000 rpm and the supernatant discarded. This process was repeated a total of three times. The conidial pellet was then resuspended in Tween 20 and the concentration was adjusted to 1×10^8 conidia mL^{-1} by serial dilution with the aid of a Neubauer hemocytometer.

Insect rearing

The insect colony was maintained using methods similar to those published by Silva et al. (2011). The colony was initiated using insects collected in the field from tomato plantations on a farm near the city of São Fidelis (coordinates: Latitude: −21.6431, Longitude: −41.7578, 21° 38′ 35″ South, 41° 45′ 28″ West), Rio de Janeiro State. Insects were maintained in the laboratory (25 °C; 75% RH ± 5; 12L:12D) by feeding on tomato plants (Santa Clara variety), cultivated from seed under greenhouse conditions (25–40 °C; 50–60% RH; approximately 12L:12D). Groups of four cages (height: 38 cm; diameter: 19 cm) were used for rearing, each with one tomato plant. The first cage was designated for adult oviposition, the second for maintaining larvae from 1st to 2nd instar, the third for 3rd to 4th instars and the fourth cage for maintaining the pupae and emerging adults.

Fungal virulence bioassays

The survival of *T. absoluta* larvae following exposure to conidia of three fungal isolates was evaluated using an indirect contact bioassay applying fungal suspensions to tomato leaves. Leaves from 30 to 40 day old plants were used here. Prior to inoculation, the leaves were surface sterilized using 0.5% sodium hypochlorite for 2 min, 70% ethanol for 2 min and then rinsed 3 times in sterile distilled water. The tomato leaves (with stems attached) were then immersed in conidial suspensions (1×10^8 conidia mL^{-1} in Tween 20 0.01% v/v) of each isolate for 1 min before being left to dry on sterilized filter paper for approximately 20 min under ambient conditions. Tomato leaves were then placed in sterile plastic Petri dishes (9 cm diameter). Five leaves were treated with conidial suspensions of each isolate (15 leaves in total) and five control leaves were treated with Tween 20 (0.01% v/v) only. Ten 2nd–3rd instar larvae were then placed on each leaf (upper side) using a fine paintbrush, with a total of 50 larvae evaluated per isolate. Larval survival was evaluated on a daily basis for 10 days and dead larvae were removed and placed in Petri dishes with damp filter paper to evaluate conidiogenesis. Fresh (non-treated) leaves were placed in the Petri dishes every 2 days. The experiments were carried out at 25–27 °C 50–70% RH and 12L:12D. All tests were performed three times The Log-rank

(Mantel-Cox) test was used for survival curve comparison analysis and Kaplan–Meier analysis for median survival time (S_{50}) calculation (GraphPad Prism software).

Colonization of tomato plants with *Beauveria bassiana*

Tomato plants were cultivated from seeds ("Italian tomato" variety from Topseed Ltd., São Paulo, Brazil) using commercial plant substrate under greenhouse conditions (peat, pine bark, vermiculite, enriched with macro and micro nutrients supplied by Mogifertil Flores Ltd., São Paulo, Brazil). The seeds were certified free of fungicides and insecticides by the manufacturer. Before use, the substrate was autoclaved twice for 30 min over two consecutive days. When the plants had started to produce large numbers of leaves (approximately 1 month after seeding), stems with leaves were removed from the main tomato plants and water stressed for 4 h (no water) with the aim of stimulating fungal uptake. After this treatment, the detached stems with leaves were placed in test tubes containing conidial suspensions (1×10^8 conidia mL^{-1}) of each isolate (LEF140, LEF141 and LPP139). Control detached stems with leaves were placed in Tween 20 (0.01% v/v). All detached stems were maintained in the suspensions for 24 h. Five detached stems with leaves were used for each treatment and control group.

Colonization of the leaves was evaluated following a 24 h exposure to the fungal suspension. Firstly, the leaves were surface sterilized by dipping in 0.5% sodium hypochlorite for 2 min, 70% ethanol for 2 min and then rinsing the leaves 3 times in sterile distilled water. Water from the final rinse was plated out to check for the presence of contaminants. The leaves from the detached stems were then cut into small fragments (approximately 0.5 cm in length) using a sterile dissecting blade. The fragments of were placed in Petri dishes containing SDA and incubated at 27 °C 12L:12D for 7 days.

When fungal development was observed at the interface of the plant tissue with the surface of the culture media, fungal structures were re-isolated onto fresh SDA media and cultured until the appearance of conidiophores, which were used for identification (Lacey 2012). Re-isolated fungi confirmed as *B. bassiana*, were cultured on rice media (as described above) for 7 days in order to produce large quantities of conidia for use in the following experiment.

Evaluation of endophytic colonization of tomato seedlings with *Beauveria bassiana*

Colonization of *Solanum lycopersicum* was carried out by inoculating seedlings with *B. bassiana* conidia from isolate LPP139 only. The seeds were surface sterilized in 0.5% sodium hypochlorite for 2 min, 70% ethanol for 2 min and then washed 3 times in sterile distilled water. The sterilized seeds were placed in Petri dishes lined with sterile filter paper moistened with 1 mL of sterile distilled water and placed in a humidity chamber (100% RH) for 6 days to stimulate germination (see Additional file 1: Fig. S1). Then a suspension of *B. bassiana* conidia (1 mL) was added to the dishes at a concentration of 1×10^8 conidia mL^{-1} + Tween 20 (0.01% v/v). Two days after fungal inoculation, the seedlings were transferred to sterile substrate (as previously stated) in 50 mL plastic cups. Controls seeds were surface sterilized as stated above and treated with 1 mL of Tween 20 (0.01% v/v) 6 days after germination.

For each experiment, forty plants (20 fungus treated seedlings and 20 control seedlings) were evaluated for the presence or absence of *B. bassiana* at 7 days post-inoculation (DPI), 14 DPI, 21 DPI and 30 DPI. All plants were cultivated in a Fitotron growth chamber at 27 °C and a photoperiod of 12hL:12hD. Prior to sectioning, the plants were surface sterilized as previously described. For each plant, 3 parts (roots, stems and leaves) were evaluated for the presence of endophytic fungi. For each time point (DPI), five 0.5 cm pieces of each part of the plant were placed in Petri dishes with SDA culture media. The sections were scored for presence or absence of fungi after a 7 day incubation at 27 °C by visual observation using a stereomicroscope. Data were presented as the mean percentage of each plant section which was scored positive for the fungus per time point. The experiment was carried out three times.

Survival of *Tuta absoluta* larvae exposed to *Beauveria bassiana* colonized tomato plants

Leaves from tomato plants that had been colonized with *B. bassiana* as described above were offered to *T. absoluta* larvae. Leaves were removed from plants at 30 DPI, a time at which the previous experiment had confirmed 100% colonization of leaves. Leaves were placed in 9 cm Petri dishes and ten (2nd or 3rd instar) larvae were carefully transferred to the leaves in each Petri dish. The survival rate of 50 larvae was evaluated on a daily basis for 7 days and fresh (non-fungus treated) leaves were placed in the Petri dishes every 2 days. Dead larvae were removed on a daily basis and placed in dishes with moistened filter paper to evaluate conidiogenesis. Insect survival rate data were analyzed using the GraphPad Prism 7 statistical program as stated above. The experiment was carried out twice. Control larvae were offered non-fungus treated leaves.

Plant growth parameters

In order to evaluate the possible effects of fungal colonization on plant development, five tomato plants were

chosen on a random basis for the measurement of the following parameters: stem diameter (mm; using Vernier calipers); plant height (cm; using a steel ruler); number of leaves. These parameters were estimated at 20, 25 and 30 DPI. Five control plants were also monitored during the same time periods. The mean values for plant growth data were analyzed for statistical differences using one way ANOVA (SPSS software). This experiment was carried out twice, for both treated and control plants with a total of 20 plants evaluated.

Results

Virulence of three *Beauveria bassiana* isolates to *Tuta absoluta* larvae

This bioassay demonstrated that all of the *B. bassiana* isolates tested here were virulent to 2nd and 3rd instar *T. absoluta* larvae following contact of the insects with leaves that had been treated with fungal suspensions. There were some differences in virulence when comparing isolates although these were not significant. Log-Rank curve comparisons for all of the survival data generated $X^2 = 97.06$ ($df = 3$; $P < 0.0001$), demonstrating that the treated groups were significantly different to the controls. Although each isolate had different S_{50} values (median survival time in days), there were no statistical differences between survival curves ($X^2 = 5.29$; $df = 2$; $P = 0.071$). The most virulent isolate was LEF141 with a S_{50} value of 6 days, whilst S_{50} for LEF140 was 7 days. The lowest virulence was seen when testing LPP139, with a S_{50} of 8 days. However, the final percentage survival rate after 10 days was similar for all three isolates, with approximately 10% of the larvae remaining alive at the end of the experiment (Fig. 1). The control group survival rate after 10 days was 71%. All three isolates were used in the next set of experiments. Conidiogenesis was observed in > 90% of cadavers of insects that had been exposed to the fungus. No conidiogenesis was observed in the controls.

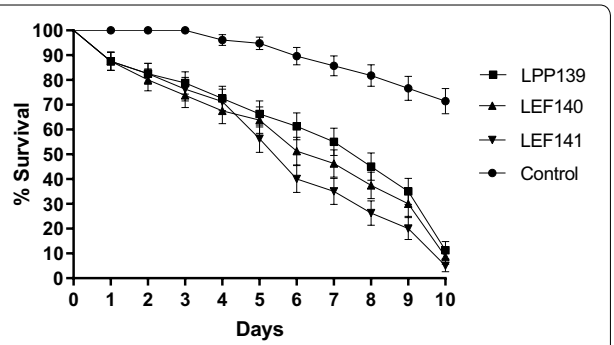

Fig. 1 Survival curves of *Tuta absoluta* 2nd and 3rd instar larvae following exposure to conidia of three isolates of *Beauveria bassiana* on tomato leaves. Isolates used here (LPP 139; LEF 140; LEF 141) were applied to tomato leaves at a concentration of 1×10^8 conidia mL^{-1}. Survival was evaluated on a daily basis. Results were the means of three repetitions per isolate (error bars: SEM). Fifty insects were used per experiment and per isolate. Control leaves were treated with Tween 20 (0.01% v/v) only

Artificial colonization of tomato plants with *Beauveria bassiana*

Four well documented colonization techniques were tested here without success (methods and results not shown here; for detailed descriptions of these standard techniques see review by Vega 2018). Root drenching, non-germinated seed treatment, stem injection and foliar application of *B. bassiana* did not result in plant tissue colonization as determined by plating out sections of plant material from roots, stems or leaves at different times after fungal inoculation. The only technique that gave positive results for one of the isolates was a "water stress" technique as described above. Using this technique it was only possible to re-isolate *B. bassiana* LPP139 from the leaves of fungus treated plants. Therefore, LPP139 that had been "passaged" through tomato plants was utilized in the following experiments.

Endophytic colonization of tomato seedlings

These experiments were carried out using tomato seedlings that had been colonized by LPP139. The root tissues and stems displayed the highest percentages of colonization at 7 DPI, with 80% of the tissue samples being positive for *B. bassiana*, whilst at this time only 46% of the leaf tissue samples were positive (Table 1). At 14 DPI 86% of the root and stem samples were positive, with positive counts for the leaves rising slightly to 53%. By 21 DPI, all root and stem samples were positive for *B. bassiana* colonization, with a continued rise in leaf section colonization (66%). At 30 DPI all tissue samples from all parts of the plants were 100% positive for fungal colonization. The results for the controls are not shown in the table as all samples were negative for *B. bassiana* colonization.

Table 1 Presence (%) of *Beauveria bassiana* in three parts of tomato plants at different times following seedling inoculation

Part of the plant	7 DPI	14 DPI	21 DPI	30 DPI
Roots	80 ± 20	86.6 ± 11.5	100	100
Stems	66.6 ± 11.5	86.6 ± 11.5	100	100
Leaves	46.6 ± 11.5	53.3 ± 11.5	66.6 ± 11.5	100

Results are shown as mean percentages of positive scores for three experiments (± SD). Five sections of each part of the plant (root; stem; leaf) were tested for the presence of the fungus at each time point. All control plants were negative for *B. bassiana* and therefore the results were not shown

DPI days after plant inoculation

Survival rates of *Tuta absoluta* larvae exposed to *Beauveria bassiana* colonized tomato plants

Leaves from tomato plants 30 DPI (seedlings treated with *B. bassiana* LPP139) were offered to 2nd and 3rd instar *T. absoluta* larvae. The survival rates of the larvae are shown in Fig. 2. There were significant differences in the survival rates of controls and treatments (Log-Rank $X^2 = 169.5$; df 3; $P < 0.0001$). In both experiments (Exp. 1 and Exp. 2), where larvae were exposed to leaves colonized by LPP139, the S_{50} was 4 days and all of the larvae had died by the 7th day of evaluation. No conidiogenesis was observed on the cadavers of dead larvae that had been exposed to *B. bassiana* colonized leaves.

Effect of endophytic colonization on plant development

No significant effects on plant development (stem diameter, plant height and number of leaves) were observed following colonization by LPP139. The results for these experiments are shown in Additional file 1: Fig. S2.

Discussion

Entomopathogenic fungi can be found colonizing a range of plant species and may provide a form of natural protection against herbivorous insects. It is also possible to artificially "infect" plants with entomopathogenic. This option has important applications for crop protection and to date *B. bassiana* has been the species most often employed in such studies (Vega 2018).

There is a range of techniques which are used to artificially inoculate plants with entomopathogenic fungi. These have been well reviewed by McKinnon et al. (2017) and Vega (2018). Some techniques may be effective for certain plant species, whilst for other species, they are inefficient. The following conventional methods, previously described to artificially inoculate tomato plants with *B. bassiana*, leaf soaking, soil/root drenching and direct injection of fungal suspensions into the stems were initially tested in the current study. However, none of these methods was successful; no fungi were isolated from tomato leaves following inoculation (results not shown). The first positive result was obtained here by water stressing tomato plants and then placing the detached stems with leaves in *B. bassiana* suspensions. However, only one of the three isolates used here (LPP139) was subsequently re-isolated from leaf tissues. There has been little work carried out to compare the endophytic properties of different isolates of the same species of fungus (McKinnon et al. 2017; Vega 2018). Although it has been stated that endophytic colonization by *B. bassiana* is influenced not only by the inoculation methods used but also the fungal isolate or plant species in question (Posada et al. 2007; Parsa et al. 2013). Interestingly, *B. bassiana* which was discovered as a natural endophyte in wild tomatoes, more successfully colonized cultivated tomato plants than two soil isolates of *B. bassiana* (Qayyum et al. 2015). The mechanisms responsible for these differences were not investigated. Our results indicated that LPP139 quickly adapted to the novel environment within the plant and was subsequently able to colonize tomato plants via a more conventional route (seedling inoculation).

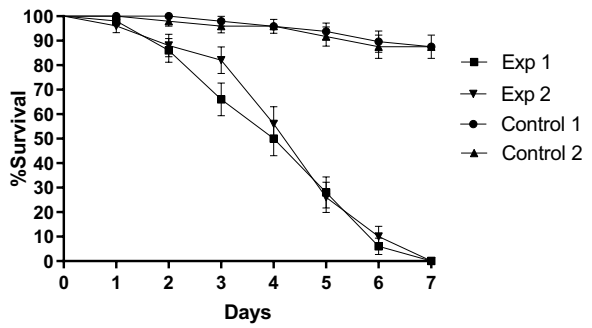

Fig. 2 Survival curves of *Tuta absoluta* 2nd and 3rd instar larvae following exposure to tomato leaves colonized by endophytic *Beauveria bassiana* (LPP 139). Survival was evaluated on a daily basis. The survival curves for two replicate experiments (Exp 1 and Exp 2) are shown as separate curves. Results are mean survival % (error bars: SEM). Controls insects were exposed to plants that had not been treated with *Beauveria bassiana*

No endophytic colonization of plants was detected here by soaking seeds in fungal suspensions. Seed treatment with *B. bassiana* conidial suspensions was also found to be of low efficiency in the endophytic colonization of tobacco, corn, wheat, soybeans (Russo et al. 2015) and tomato (Allegrucci et al. 2017). Alternatively, other workers have used tomato seed inoculation methods to successfully colonize plants (Ownley et al. 2008; Powell et al. 2009; Sánchez-Rodríguez et al. 2015; Shrivastava et al. 2015).

Although seed inoculation would appear to be a very similar technique to that of seedling inoculation, the current study showed that only seedling inoculation was efficient. Seedling inoculation resulted in relatively rapid and lasting colonization of tomato plants by LPP139. The results demonstrated initial colonization of the roots and stems and then eventually the leaves. Root dipping of tomato seedlings was demonstrated to be the most suitable inoculation method for establishing *B. bassiana* as an endophyte with the aim of protecting tomato plants from *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Qayyum et al. 2015).

Endophytes can remain detectable within the plant tissues for different times following inoculation and colonization. Here *B. bassiana* was detected for up to 30 days in all tissues (roots, stems and leaves). As

expected, this parameter is highly variable when comparing different studies. Allegrucci et al. (2017) showed that in the case of tomato plants colonized by application of fungal suspensions to the leaves (most efficient method as stated by the authors), the highest levels of colonization (10%) occurred on day 7. However, by day 28 the fungus was hardly detectable (0.5%) in the leaves. Gurulingappa et al. (2010) also documented a rapid decline in endophyte levels over time in a range of plants. The decline in percentage colonization over time may be caused by competition from other endophytes in the plant (Posada et al. 2007). McKinnon et al. (2017) pointed out the need for more stringent sterilization protocols to be adopted for quantification of endophytic colonization and called for a greater understanding of the antagonistic mechanisms involved in plant–insect interactions.

The results for reduction of survival rates of *T. absoluta* when larvae were offered *B. bassiana* endophytically colonized tomato leaves was very encouraging. The survival curves (Fig. 2) showed the rapid mortality of *T. absoluta* larvae over a 7-day period. It would appear that *B. bassiana* was more effective at killing *T. absoluta* when in the endophytic form than when conidia were applied directly to leaf surfaces (Fig. 1). This could have been due to the mining behavior of the larvae, reducing the exposure time to fungi applied to the surface of the leaves in the first experiment. However, it is very difficult to compare results for conventional fungal pathogenesis with those of endophytic antagonism. El-Deeb et al. (2012) observed that the mortality of whiteflies, *Bemisia tabaci* (Hemiptera: Aleyrodidae), was highest when offered tomato leaves endophytically colonized by *B. bassiana* (90% mortality) compared to mortality rates (10%) observed following exposure to fungi applied to the leaf surface. The opposite was the case when Allegrucci et al. (2017) and Klieber and Reineke (2016) observed the survival rates of *T. absoluta* offered tomato leaves colonized by endophytic *B. bassiana* when compared to survival rates following exposure of *T. absoluta* to conidia on tomato leaves. These studies showed that endophytes were less effective at reducing survival of *T. absoluta* than fungi directly applied to the leaves. The fact that larval survival rates were rapidly reduced following exposure/feeding on endophytically colonized tomato leaves when compared to larvae placed on leaves that had been dipped in fungal suspensions, indicates that endophytic fungi act more efficiently within the plant than when on the plant surface. This result could be interpreted in a variety of ways: the fungus secreted toxin(s) which killed the insects or the fungus stimulated the plant to produce defensive compounds (induced resistance).

Research on the antagonistic mode of action of endophytic entomopathogens requires attention and caution is needed when observing the so called "pathogenesis" of endophytes that may not actually produce infective propagules in the internal environment of the plant. A possibility that has yet to be investigated is whether entomopathogenic fungi are capable of producing blastospores during colonization of plants. Blastospores are highly infective propagules and can penetrate the insect integument, rapidly colonizing and killing their hosts (Alklhaibari et al. 2016).

The question of how fungal endophytes cause inhibition of insect development, repellency or insect death, is debatable. A range of possibilities have been suggested, and the least probable would appear to be infection (mycosis) per se (Vega 2018). Studies have shown the presence of fungal hyphae within plant tissues (Wagner and Lewis 2000; Sakulkoo et al. 2018), however hyphae are not known to cause infection, even following ingestion. Leckie et al. (2008) demonstrated the toxic effects of dried mycelia on *Helicoverpa zea* (Lepidoptera: Noctuidae) larvae via ingestion. Although this was an in vitro experiment, it is possible that ingestion of living hyphae/mycelia could have negative effects on insect herbivores. Another possibility is that the toxins secreted by entomopathogenic fungi could negatively affect insects. *Beauveria* and *Metarhizium* produce a range of toxic compounds, including the cyclic peptides beauvericin, bassianolide and destruxin (Strasser et al. 2000; Wang and Xu 2012; Golo et al. 2014). It is believed that fungal toxins play an important role in the pathogenicity process during infection of the host insect (Samuels et al. 1988), however they could also be expressed during endophytic colonization, protecting the plants from herbivores (Tan and Zou 2001; Christian et al. 2020). Destruxin A was detected in *Spodoptera littoralis* larvae (Lepidoptera: Noctuidae) and *B. tabaci* nymphs fed on tomato and melon leaves endophytically colonized by *M. brunneum* (Resquín-Romero et al. 2016; Garrido-Jurado et al. 2017). Here no conidiogenesis was observed on the cadavers of *T. absoluta* larvae that had fed on endophytically colonized tomato leaves. This would indicate that the insects did not succumb to a fungal infection but death was due to other causes. However, it is not uncommon for fungi to kill their hosts without subsequently sporulating on the cadavers (RIS: personal observation). The absence of mycosis was also observed on cadavers of *S. littoralis* and *Plutella xylostella* L. (Lepidoptera: Plutellidae) exposed to *M. brunneum* and *B. bassiana* colonized tomato and cauliflower leaves (Gautam et al. 2016; Resquín-Romero et al. 2016).

Another factor which could result in antagonistic effects on herbivorous insects is the induction of plant

defense compounds following colonization by endophytic fungi. These compounds could be produced by the plant as a reaction to fungal infection, resulting in a collateral effect on insect pests. In the case of tomato plants colonized by *B. bassiana*, significantly higher levels of two monoterpenes (δ-2-carene, sabinene) and three sesquiterpenes (δ-elemene, (E)-β-caryophyllene, α-humulene) were detected when compared to control plants (Shrivastava et al. 2015). The development of the beet armyworm, *Spodoptera exigua*, was negatively effected following feeding on the endophytically colonized plants.

Many studies have shown that endophytes have either no negative effects on plant growth or they can actually stimulate plant growth (Vega 2018). Here, plant development was monitored over a 30 day period post-inoculation with *B. bassiana* at the seeding stage. There were no differences in stem diameter, plant height and number of leaves when comparing endophytically colonized tomato plants and control plants. This is an important result when aiming to convince farmers to adopt alternative pest control strategies. The commercialization of tomato plants pre-colonized with *B. bassiana* is feasible given the growing market for organic produce. Further studies are now being performed to monitor the presence of *B. bassiana* in tomato plants up to the stage when they produce fruits. The colonization of other varieties of tomatoes is also being investigated.

Conclusions

This study describes an effective method for colonizing tomato plants with the entomopathogenic fungus *B. bassiana*, which acting as an endophyte, causes no adverse effects on the plant and protects the plant from attack by *T. absoluta*, a notoriously difficult to control tomato pest. This approach is highly promising for the reduction of the damage caused by insect pests and will also reduce the need for applications of highly toxic insecticides.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s43170-020-00002-x.

Additional file 1. Additional figures.

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Authors' contributions
ACL carried out the experiments and analysed the results. PHNA helped with insect colony maintenance. ATC helped with experimental procedure and data analysis. RIS and AGS supervised the research. RIS wrote the manuscript. All authors read and approved the final manuscript.

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