RESPONSE OF *TUTA ABSOLUTA* TO ENDOPHYTIC FUNGAL ENTOMOPATHOGENS IN TOMATO

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ABSTRACT

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Tuta absoluta is a leaf miner pest devastating tomato production. Use of broad-spectrum pesticides to control this pest leads to the development of insecticide resistance, food contamination, poor health and environmental pollution. Recent knowledge of entomopathogenic fungi colonizing plants and, thus, antagonizing insect pests and plant diseases, sparked appreciable enthusiasm in using these capabilities. Therefore, this study aimed to assess the abilities of entomopathogens to endophytically colonize tomato plants, and to determine the ability of fungal species to modulate the behavior of T. absoluta. The two inoculation procedures (seed treatment and direct injection) allowed Metarhizium anisopliae and Beauveria bassiana strains to endophytically colonize tomato plants and thus have influenced the preference of T. absoluta oviposition. In Beauveria- and Metarhizium-colonized leaf mesophyll, damage by T. absoluta was significantly lower compared to control plants. This was attributed to the reduced number of eggs (7-8 plant^{-1}) on fungus-colonized plants as opposed to 19 plant^{-1} on control plants in seed-treated assay. Reduced size of mines in Beauveria-injected plants has also contributed to the decrease in damage area by leaf miner larvae. The outcomes of this study suggest that seed inoculation is an effective method to be adopted by plant nurseries for commercial production of pre-colonised with entomopathogenic endophytes tomato plantlets.

Keywords: *Tuta absoluta,* biological control, endophytic entomopathogens, tomato plants, inoculation.

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INTRODUCTION

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is a serious pest of solanaceous crops such as tomato, potato, aubergine and tobacco. Originated from Peru, this pest was introduced into Europe in 2006 and rapidly spread throughout Africa, Middle East and Asia (Desneux et al., 2011; Ibrahim et al., 2017). Management of *T. absoluta* is difficult due to its short life cycle, high reproductive capacity and mesophyll-feeding habits. The intensive use of insecticides against this pest leads to rapid development of insect resistance (Lietti et al., 2005), environmental imbalance and destruction of natural biological control. Therefore, development of more benign methods to control the pests is a major goal and challenge for modern agriculture. Entomopathogenic fungi (EPF) *Beauveria bassiana* (Balsamo-Grivelli) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales) are well known and widely used as biological control agents for managing many insect-pests and insects of veterinary importance. Nevertheless, entomopathogenic fungal species were reported to possess the ability to colonize the plant tissues (Vega, 2018) without causing any external symptoms (Wilson, 1995). The role of endophytic colonisation of plants by EPF is proven to enhance tolerance to pest invasion and subsequent damage (Vega, 2018), to provide protection against phytopathogens (Jaber & Salem, 2014) and plant parasitic nematodes ((Hallmann & Sikora, 1996), to improve tolerance to droughts (Lugtenberg et al., 2016), to enhance plant growth (Jaber and Enkerli, 2017), and to have a significant part in bio-remediation of heavy metals (Nair & Padmavathy, 2014).

Traditionally, EPF is applied onto field crops by foliar applications (Shah & Pell, 2003). This method, however, has its disadvantages since spraying fungi directly onto plants makes them susceptible to high temperatures, low humidity, UV and other abiotic factors and, thus, reduces their effectiveness against targeted pests. Biswas et al. (2013) have suggested that efficacy of EPF could be enhanced through endophytic colonization of plant tissues where they would be protected from some abiotic stresses and remain available throughout the crop growth period. Therefore, current study aimed to evaluate the potential of two fungal species, *M. anisopliae* and *B. bassiana,* to colonize endophytically tomato plant tissues using two inoculation methods, seed inoculation and direct injection into plant conductive (stem) tissue and to determine the ability of tested endophytes to modulate the behavior (oviposition) of *T. absoluta* leaf miner on endophyte treated *vs* non-treated plants.

MATERIALS AND METHODS

Origin of fungal isolates, plants and insects

One isolate of *M. anisopliae* and one isolate of *B. bassiana* pathogenic to many pests (Ibrahim et al., 2011; Ibrahim et al., 2017) were used in this study. Single spore (SS) cultures were prepared to allow repeatability of results for each experimental test. Once a pure culture has been obtained, it was maintained on Potato Dextrose Agar (PDA, commercial powder containing 20 g dextrose, 15 g agar, and 4 g potato starch) at 5º C or at -20º C in cryovials containing 10% w/w glycerol (stock cultures).

Tomato (*Solanum lycopersicum***)** seeds variety "Baladi" obtained from an organic farm (Tery village, south Lebanon) were sown in seed trays. Seedlings with 1-2 true leaves were transplanted into plastic pots (10 cm diameter and 15 cm deep) containing unsterilized mixture of peat-moss and sand (1:1 proportion) amended with NPK (12-12-17, S + 2MgO) growth medium. The potted plants were maintained at 25±3°C, 60±5% relative humidity (RH) and 16h/8h (Light/Dark) photoperiod and irrigated daily.

Stock colony of *T. absoluta* was established from a population collected from tomato field located in the south Lebanon (Jiyeh region) and maintained on potted tomato plants held in rearing wooden cages (70 cm × 50 cm × 50 cm) under laboratory conditions (25°C, 60% RH and photoperiod of 16h light/8h dark). The insects were reared as described previously (Abou-Fakhr Hammad et al., 2019). Briefly, 100 unsexed adults were released onto fresh tomato plants for oviposition. 72 h post-release, plants containing eggs were removed and placed into new cages and maintained there until emergence of 2nd generation of adults. Adults of 2nd generation were used for attaining the 3rd generation of adults. To maintain its genetic variability, 2nd generation of *T. absoluta* colony was also pervaded with a wild population of leaf miners (Krechemer & Foerster, 2015). Adults of 3rd generation were used in two tests described below.

Preparation of inoculum

Conidia obtained from 14-day-old sporulating cultures were suspended in 0.03% (v/v) Tween 20 solution on a rotary shaker at 330-350 rpm for at least 30 min. The resultant suspension was filtered through 4 layers of sterile muslin and adjusted to 1×10^7 conidia ml^{-1} using hemocytometer. Conidial viability was tested (Ibrahim et al., 2017) for each stock suspension to maintain the constancy of the assessments.

Effect of inoculation procedure on endophytic colonization, tomato leaf miner infestation and leaf damage

Test 1 - seed inoculation method

Prior to inoculation with EPF, seeds were soaked in sterile distilled water (dH_2O) for 15 min and then surface-sterilized by immersion in 3% sodium hypochlorite and 70% ethanol for 2 min, respectively, with three rinses with dH_2O after each submersion. Sterilized seeds were stored overnight at 5°C for synchronization of seed germination and growth. The effectiveness of the sterilization method was checked by placing 20 randomly selected seeds onto PDA media (Greenfield et al., 2015), incubated at 25 °C in darkness and examined regularly at 2 days intervals for 10 days. If fungal growth did not appear during examination period, the disinfection was considered successful. Refrigerated sterilized seeds were allowed to adapt to room temperature and then immersed into respective conidial suspension for 2 h. Control group of seeds was immersed into sterile 0.03% Tween 20. After drainage, one set of treated and control seeds was placed in Petri dishes lined with moist sterile filter paper and incubated at 25ºC and 16light:8dark h photoperiod for 10 days to assess the effect of inoculation procedure on seed germination. Resultant seedlings then were checked for fungal colonization using culture dependent method (CDM) described in Klieber and Reineke (2015). Disinfected seedlings were air-dried, cut into root, stem and cotyledon parts using sterile scalpel. Obtained plant segments were placed onto PDA media amended with antibiotic (20 mg l^{-1} Amoxicillin, Co-Amoxiclav 250/125mg Tablets BP), incubated at 25°C for 4 weeks and inspected regularly to observe fungal outgrowth. Percent colonization (RC) of different seedling's parts by the respective inoculated fungus was calculated following the Fisher and Petrini (1987) formula. There were 4 replicates for each treatment with 10 seedlings in each replicate. Second set of treated and control seeds was sown in seed trays and then transplanted into plastic pots containing unsterilized growth peat-moss and sand mixture. Potted plants were maintained under polyethylene tunnel (5m x 1m) conditions (25±5 ºC and 14day/10night h) until they reached 4th leaf stage. Fifteen plants per each fungal isolate and 15 control plants were randomly placed into individual clear plastic cages (20 cm dim. x 30 cm high) sealed on top with mesh to prevent adults from escaping. Twelve plants of each treatment were exposed to three adults (including one male) for 72 h to allow copulation and subsequent oviposition of eggs to occur. The caged plants were maintained under polytunnel conditions. Three days post infestation the adults were removed, and the number of eggs was recorded on each leaf using a hand lens (20× magnification). Egg hatching, larval development, mine development and their morphology were observed over the period of 3 weeks at regular intervals. Upon completion of the experiment, the measurements of large (>1.0 cm x ≥0.3 cm) and small

(≤1.0 cm x ≤0.3 cm) mines were taken manually, and total damage per plant (a sum of all measured mines (length x width, cm^2), Gharekhani and Salek-Ebrahimi, (2014) was calculated.

Test 2 – inoculation by injection method

Plants grown from sterilized untreated seeds were used for this method of inoculation. Origin and maintenance of potted plants were identical to those described in Test 1 section. At 4th leaf stage, tomato plants were injected using a disposable sterile syringe (3 ml/cc) and 200 µl of 10^7 conidia ml^{-1} of respective conidial suspension (treatment) or 0.03% Tween 20 solution (control) under the petiole of each leaf (at node point) with 3 injections per plant (2 x 10^6 conidia $plant^{-1}$). A week post injection, three *T. absoluta* adults were introduced to the injection-inoculated plants and the impact of colonized tomato leaves were determined using experimental design, methods and techniques described for Test 1.

Assessment of the internal colonization

The endophytic colonization of plants by *B. bassiana* and *M. anisopliae* were determined by random sampling of 2 leaves from 3 plants per treatment 4 weeks post seed inoculation (Test 1) and 7 days post injection (dpi) (Test 2). The sampled leaves were then subjected to surface sterilization using method of Klieber and Reineke (2015). Fungal outgrowth from the plated plant samples were identified according to colony morphology, and microscopic examination of conidia (Humber, 1997). Colonization rate (CR) for each treatment was evaluated using Fisher and Petrini (1987) formula.

Statistical analysis

All experiments were arranged in a complete randomized design. All data sets were analyzed with the statistical program IBM SPSS Statistics for Windows, Version 23.0 (2015) using one-way ANOVA after checking the assumptions for normality and the homogeneity of variance (Levene's test). Colonization of different seedling parts with *M. anisopliae* and *B. bassiana*, fungal colonization recovery of both strains from leaves in addition to the number of eggs, larvae, pupae, mines, area of mines tunneled by *Tuta absoluta* and leaf yellowing were subjected to one-way ANOVA with fungal strain as a main factor. Analysis of data for Test 1 (seed inoculated) and Test 2 (stem injected) were performed separately. When a significant F test was obtained at $P = 0.05$, separation of treatment means was performed using Tukey Test.

RESULTS

Effect of inoculation procedure on endophytic colonization of tomato seedlings and plants

Inoculation with *B. bassiana* and *M. anisopliae* isolates neither reduced seed germination nor seedling growth and did not result in the development of root, stem or cotyledon diseases. EPF tested were successfully recovered from root radicals, stems and cotyledons (Figure 1, Test 1). Marginally higher ($F_{2,9}$ = 4.5, P = 0.044) colonization (50%) was observed in stems of 7-days-old seedlings treated with *M. anisopieae* suspension (Figure 1, Test 1). However, recovery of endophytes (12.5% and 14.4%) from the cotyledons ($F_{2,9}$ = 3.00, P = 0.1) and roots ($F_{2,9}$ = 1.286, P = 0.323) were similar regardless of treatment (Figure 1, Test 1). No fungal growth was recorded in control seedlings.

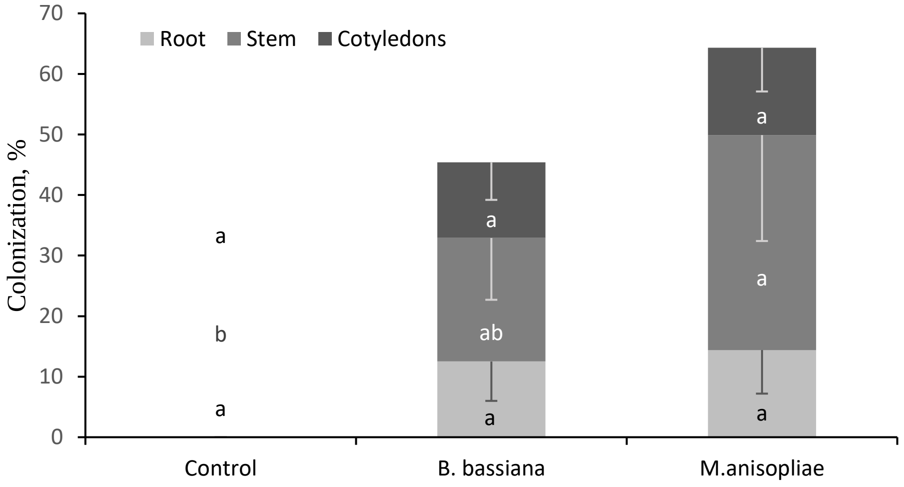

Figure 1. Colonization (% ± SE) of roots, stems and cotyledons of 7-days-old tomato seedlings by entomopathogenic fungi (*Bassiana bassiana*, *Metarhizium anisopliae* and the control) emerging from treated seeds. Bars with different letters differ significantly among treatments at *P*≤0.05 (Tukey's test, one-way ANOVA).

Inoculation of seeds (Test 1) also provided successful colonization of tomato leaves of 4 weeks old plants (Figure 2; $F_{2,15}$ = 6.887, *P* = 0.026). As 75% of leaf tissue sampled from leaves treated with *M. anisopliae* and 66.7% of those treated with *B. bassiana* were positive for endophyte presence, this corresponded to a successful endophytic establishment of the tested fungal species in tomato plants. The movement of the fungi within the plant tissues 7 dpi (Test 2, Figure 2) was confirmed by recovery of each fungus from the leaf tissues distant from the initial point of inoculation. However, *B. bassiana* isolate was more efficient in colonizing 100% of sampled leaves ($F_{2,15} = 17.5$, $P < 0.0001$) compared to *M. anisoplie* entering 67% of tested leaf blades (Figure 2, Test 2).

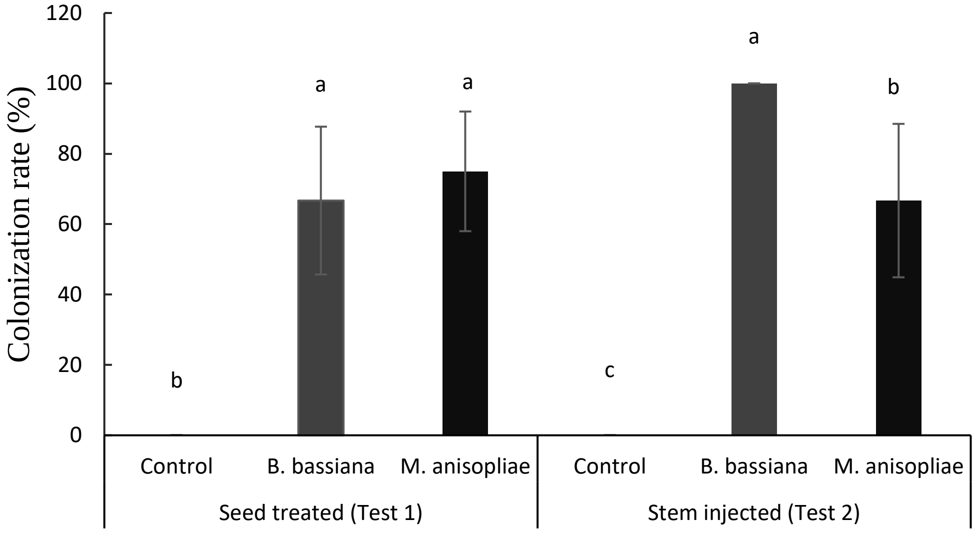

Figure 2. Colonization rate (%) of tomato leaves from seed treated plants (Test 1, 4 weeks post inoculation) and from directly injected stems (Test 2, 7 dpi) by *B. bassiana* and *M. anisopliae*. In total 60 discs from each treatment (2 leaves per plant x 3 plants per treatment) were observed. Bars with different letters differ significantly among treatments within each test at $P \leq 0.05$ (Turkey's test, one-way ANOVA).

Effects of endophytic entomopathogenic fungi on *Tuta absoluta* life cycle and feeding

In general, there was a negative effect of tomato plant colonization with *B. bassiana* and *M. anisopliae* on the oviposition and subsequent hatching of *T. absoluta* eggs (Table 1). Treating seeds with conidial suspensions resulted in more than 2-fold fewer eggs ($F_{2,33} = 3.453$, $P = 0.05$) and 3-fold fewer larvae ($F_{2,33} = 4.08$, $P = 0.026$) on the fungus-colonized leaves in comparison to uncolonized leaves. Besides, last instar larvae on control plants were observed feeding, whereas those on treated plants have all pupated. Yet, the tunneling (The number and the morphology) by the leaf miner on both, colonized and control leaves, was somewhat similar across treatments ($P \geq 0.05$).

Furthermore, a mean damaged area on control leaves was larger (2.8 cm^2 $plant^{-1}$) and about twice as that (0.9-1.3 cm^2 $plant^{-1}$) on fungus-colonized leaves ($F_{2,33} = 4.158$, $P = 0.012$). At the end of the bioassay, the unusual yellowing of the leaves was recorded for *B. bassiana* and *M. anisopliae* colonized plants. Significantly higher number of yellow leaves (0.40 and 0.26) was detected among plants grown from seeds treated with both endophytic species when compared to control plants ($F_{2,33} = 4.0$, $P = 0.026$).

Where colonization of leaves was achieved through stem injections (Test 2), leaf miner infestation of *B. bassiana* leaves was significantly higher compared to the influx of moth individuals on uncolonized/control leaves or leaves colonized with *M. anisopliae* (Table 1). Higher mean number of emerged larvae (29.1 $plant^{-1}$, $F_{1,33} = 3.458$, $P = 0.05$) was a direct reflection of the number of eggs (32.9 $plant^{-1}$; $F_{1,33} = 4.077$, $P = 0.026$) recorded on *B. bassiana*-colonized leaves. *Beauveria*-colonized leaves also affected larval development and behavior. Significant differences ($F_{1,33} = 6.336$, $P = 0.005$) were obtained between mean number of small mines developing on *Beauveria*-colonized and uncolonized leaf blades. Overall damage (measured as mean area of mines) caused by *T. absoluta* larvae to fungus-injected tomato plants, however, did not differ significantly among the treatments ($F_{1,33} = 0.490$, $P = 0.617$).

In addition, the mean number of *T. absoluta* pupae that completed their development in colonized leaves was negligible, regardless of isolate or method of inoculation applied (Table 1). However, pupation on fungus-colonized plants started 3-5 days earlier than on control plants.

DISCUSSION

Current study demonstrates successful establishment of the introduced fungal entomopathogens, *B. bassiana* and *M. anisopliae*, in tomato plants with two different inoculation methods, seed submersion and direct injection of fungal conidia into stem tissues. In recent review (Vega, 2018) it was pointed out that establishment of entomopathogenic endophytes would persist in root, stem and leaf tissues for a short period of time following transient colonization (i.e., their recovery would decline with time). For example, Bamisile et al. (2019) have reported of two *B. bassiana*, BB Fafu-16 and BB Fafu-13, strains being able to sustain their presence in *Citrus limon* colonised seedlings for only 8- and 12-weeks post-inoculation, respectively. An attempt to study vertical transmission of entomopathogenic endophytes to coffee seeds through spraying inflorescences with conidial suspension (Vega, 2018) resulted in negative outcome (e.i., *B. bassiana* was not detected in the resultant coffee berries). However, it appears that success of plant colonization would likely depend on plant species tested, age of the plants, plant growth conditions, fungal species used, concentration of fungal inoculum and the method of inoculation (Vidal & Jaber, 2015). The highest re-isolation rate (50%) of fungal endophyte in the present study was achieved from stems of seedlings emerging from *M. anisopliae* treated seeds. Four weeks post treatment, both isolates, *B. bassiana* and *M. anisopliae*, were recovered at equal rates if original entry into leaf tissue had occurred through treated seeds. In contrast, full (100%) fungal recovery was achieved from colonized leaves when stems of tomato plants were injected with *B. bassiana* conidia. The high successes of *B. bassiana* colonization of tomato leaf tissue could be explained by tissue specificity. For example, *Metarhizium* spp. were shown to be more compatible with plant root systems (Bruck, 2010), whereas *B. bassiana* preferred to colonize above ground parts rather than roots of sorghum plant (Tefera & Vidal, 2009). The preference for colonization of specific plant tissue displayed by both species in this study could be also explained by biochemical content of plant tissue, its physiological condition and the differences in the biochemistry and physiology of fungal stain (Ibrahim et al, (unpublished observations); Jaber and Enkerli, 2017).

Results presented here illustrate that inoculation method would not only directly affect the success of colonization but also indirectly affect the ability of pests to recognize the plant host. Usually, tomato plants emit a complex mixture of volatile organic compounds (VOCs) such as β-phellandrene, limonene, 2-carene, (E), β-caryophyllene and a few minor compounds and blend proportions that are easily recognized by *T. absoluta* (Proffit *et al*., 2011). These particular infochemicals play an important role in identification of a suitable host for *T. absoluta* oviposition (Terzidis et al., 2014). Inoculating tomato seeds with entomopatogenic endophytes in our study discouraged *Tuta absoluta* from oviposition on colonized host plants. Direct injection, in contrast, has attracted the leaf miner adults to lay more eggs. On one hand, endophytes result in a positive response of the plant against insect pest, as in case of mining fly (*Liriomyza huidobrensis*) (Akutse et al., 2013), on the other, they contribute to the attraction of some insects such as *M. persicae* (Aragón et al., 2016). This finding, in turn, supports the idea that changes in VOC content due to fungal presence may protect the plant from pest invasion as well as it may harm the same plant by attracting the herbivores (Halitschke et al., 2008). Moreover, a recent study of McKinnon et al. (2018) has suggested that damaging maize plant would induce changes in its defense response through enhancement of systemic susceptibility of plant rhizosphere to *Beauveria* as indirect strategy against insect resulting in plant colonization.

So, how do the fungal endophytes used in this study confer these beneficial or negative effects on the tomato plant? While *T. absoluta* larvae fed upon control and treated tomato plants with very few remaining alive until pupation, plants subjected to fungal colonization were observed to change their morphological appearances such as yellowing. Besides volatiles, plant surfaces provide many insects with visual cues (Juma *et al*., 2016) for their behavior. For example, *Euphydryas aurinia* moth oviposit on the greenest leaves which act as a guaranty of nutritional provision for her progeny (Stefanescu et al., 2006). In addition, it would have been possible that tomato plants in our study also reacted to the *T. absoluta* damage by synthesizing more pigments (e.g. carotenoids or tannins) that masked the chlorophyll in *B. bassiana* and *M. anisopliae* colonized plants showing yellowing. Leaf yellowing is frequently indicative of a nitrogen, iron and sulphur deficiency. Tall and Meyling (2018) suggested that fungal inoculation under low nutrient conditions could cause mineral deficiency in plants. When nutrients become limited, an endophyte possibly becomes a parasite that consumes most of the host plant nutrients (Wilson, 1995). In addition, high number of necrotic mines observed in tomato plants colonized by *B. bassiana* (data is not presented here) is a probable result of dual defense response to endophyte and insect invasion resulting in melanization and lignification (Ibrahim et.al., 2001).

CONCLUSION

Seed submersion in conidial suspension and direct plant injection, two inoculation methods used in the present study, resulted in successful establishment of *M. anisopliae* and *B. bassiana* entomopathogens as endophytic fungi. Systemic colonisation of tomato seedlings and leaf tissues of mature plants by both spp. was detected 7 and 28 days post-seed-treatment, respectively. Both endophytes were also recovered from the leaf tissues distant from the initial point of stem injection confirming the endophytic movement of the fungi within the tomato plant. Colonization of tomato plants has occurred under polytunnel conditions and in non-sterile substrate. The presence of endophytic entomopathogens inside tomato plants has affected *T. absoluta* oviposition, the morphometric parameters of mines and significantly reduced larval damage. These outcomes could have been a result of antixenotic repellence of leaf miners, phenomena that warrant further studies to unravel the underlying mechanisms (e.g. possible release of fungal metabolites and/or plant volatiles which could have effects on *T. absoluta*). This study also provides the basis for further investigation, which should focus on an application technology in the field, long-term establishment of endophytes in mature plants, and side effects on non-target organisms. Meanwhile, we suggest commercializing the production of *B. bassiana* and *M. anisopliae* pre-colonized tomato plantlets in the plant nurseries, from where Lebanese tomato growers regularly purchase their stock plantlets.

Table 1. Effects of endophytic *Beauveria* and *Metarhizium* colonizing leaves of seed-treated and stem-injected plants on a number of eggs, larvae, pupae, mines, area of mines (damage, cm^2) tunneled by *Tuta absoluta* and leaf yellowing (leaf plant^{-1}).

Treatment	Eggs*	Larvae*	Pupae*	Large mines*	Small mines*	Damage* (cm^2)	Leaf yellowing
Seed-treated							
Control	18.9±5.5a	16.8±5.4a	0.00±0.00** a	6.3±2.4a	12.5±5.7a	2.8±0.5a	0.00 0.00 b
B. bassiana	7.3±1.7b	5.3±1.3b	0.25±0.13a	3.8±0.8a	6.5±1.6a	1.3±0.2b	0.40 0.13 a
M. anisopliae	8.3±2.3b	5.2±1.4b	0.08±0.0.8a	2.5±0.8a	11.1±4.0a	0.9±0.2b	0.26 0.11ab
Stem-injected							
Control	17.8±3.1b	17.3±3.1ab	0.8±0.1a	4.6±0.8a	13.8±2.5ab	1.73±0.2a	-***
B. bassiana	32.9±7.2a	29.1±6.5a	0.0±0.0a	3.2±0.9a	31.3±6.9a	1.28±0.3a	-
M. anisopliae	21.6±3.5ab	13.4±3.3b	0.9±0.1a	4.5±1.3a	9.8±2.9b	1.29±0.4a	-

*Means (± SE, standard error) followed by different letters within a column differ significantly among treatments at $P \leq 0.05$ (Tukey's test, one-way ANOVA)

** Note that there was no pupation of late instar larvae observed yet (delayed pupation)

*** Not observed

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