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ENDOPHYTIC COLONIZATION OF TWO ENTOMOPATHOGENIC FUNGI ON TOMATO PLANT AND THEIR MORTALITY EFFECTS AGAINST THE SOUTH AMERICAN TOMATO PINWORM, *Tuta absoluta* (MEYRICK) (LEPIDOPTERA: GELECHIIDAE)

Zeynep Diker Taşçı [1], Murat Muştu [1, *]

[1] Erciyes University Faculty of Agriculture, Department of Plant Protection, Kayseri, Türkiye

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Abstract
In this study, the endophytic colonization on tomato plant of the entomopathogenic fungi Beauveria bassiana (Balsamo) Vuillemin (Sordariomycetes: Hypocreales) and Isaria farinosa (Holmsk.) Fries (Sordariomycetes: Hypocreales) using different inoculation techniques and their mortality effects on South American tomato pinworm, Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) larvae were determined. Experiments were conducted in a climate-cabinet under conditions at 25 ± 1 ˚C, 16L:8D and 60 ± 2% RH. While it was observed that the colonization rate of the plants treated with B. bassiana or I. farinosa increased as the weeks progressed, the highest fungal colonization for both isolates was obtained in the leaf spraying method as %100 and %96 respectively. It was determined that there was no statistical difference between the colonization rates of both fungal isolates inoculated to tomato plants with the same inoculation method on the leaves examined in the same weeks. When the mortality effects of different inoculation methods on T. absoluta larvae were examined, there was no statistical difference between the fungal isolates, while the highest larval mortality rate was obtained in the seed treatment of B. bassiana with 64%. At the end of the study, promising findings were obtained that both entomopathogenic fungi can be used in integrated pest management of T. absoluta.

Keywords: Beauveria bassiana, endophytic colonization, entomopathogenic fungi, Isaria farinosa, T. absoluta

1. INTRODUCTION

The South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), one of the most important pests of tomato (*Solanum lycopersicum* L.), is the main pest of tomato, which is cultivated under cover and in the open field worldwide. *T. absoluta* was first detected in Peru in 1917 and has since been found in almost all of South America (Biondi et al., 2018). The pest, which is thought to have been transmitted to the European continent due to globalization and tomato trade, was first detected in Spain in 2006 and spread first in Europe and then in Africa and Asia (Campos et al., 2017; Biondi et al., 2018). *T. absoluta* larvae cause damage by entering the leaves, stems, stalks and fruits of the plant (Miranda et al., 1998; Desneux et al., 2010). Cherif and Verheggen (2019) reported that *T. absoluta* feeds on 44 cultivated plants and weeds belonging to different families, mainly Solanaceous plants. This number increased to 52 with the recently added host plants (Colmenarez et al., 2022).

*Corresponding author, E-mail address: mmustu77@hotmail.com

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Due to the fact that tomato moth larvae feed on plant tissue, chemical control against the pest sometimes fails to achieve the desired success, so producers frequently use insecticides against the pest (Desneux et al., 2010). However, due to the unbalanced use of pesticides, the pest's susceptibility to pesticides has recently decreased and it is reported to have gained resistance to many insecticides (Guedes and Picanço, 2012; Roditakis et al., 2018; Guedes et al., 2019). However, in addition to the negative effects of intensive insecticide use on the environment and human health, the negative effects on natural enemy populations are also a very important problem (Biondi et al., 2018; Pandey et al., 2023). Therefore, alternative control methods that will reduce the use of chemical control in this pest control are emphasized (Biondi et al., 2018; Colmenarez et al., 2022; Pandey et al., 2023).

One of the most important of these methods is biological control and entomopathogenic fungi have an important use in biological control. Entomopathogenic fungi spores enter directly through the cuticle of pests and germinate there, reproduce in the insect body and hemolymph and cause death in insects (Zacharuk, 1981; Vega et al., 2012, Joop and Vilcinskas, 2016). Entomopathogenic fungi are usually applied by direct spraying on pests, just like chemical insecticides. However, many studies have shown that entomopathogenic fungi have the potential to colonize plant tissues and thus be effective against plant pests (Vega et al., 2008; Gurulingappa et al., 2011; Jaber and Ownley, 2018). Since endophytic entomopathogenic fungi are found inside the plant, they can be protected from adverse environmental conditions (Resquin-Romero et al., 2016). In the literature, there are few studies investigating the colonization of tomato plants by endophytic entomopathogenic fungi or their effects on *T. absoluta* (Klieber and Reineke, 2016; Allegrucci et al., 2017; Agbessenou et al., 2020; Silva et al., 2020; Ibrahim et al., 2021; Agbessenou et al., 2022). In this study, the colonization of *B. bassiana* and *Isaria farinosa* (Holmsk.) Fries (Sordariomycetes: Hypocreales) isolates inoculated on tomato plants using different inoculation methods and their lethal effects against *T. absoluta* larvae were investigated.

2. MATERIALS AND METHODS

Growing tomato plants

The tomato (*Solanum lycopersicum*) (SC 2121) plants used in the study were grown in an acclimatization room with 25±1 ˚C, 60±10% humidity and 16:8 hours light-dark period. For tomato production, seeds without any pesticide treatment were sown in pots and the pots were placed in cages covered with gauze. Germinated tomato plants were left in the climate room until they had 7-8 leaves.

Tuta absoluta culture

Tuta absoluta was collected from tomato production areas in Afyonkarahisar province, Türkiye. The adults brought to the laboratory were released into cages containing healthy tomato plants. Petri dishes containing cotton wool soaked in a mixture of 15% honey and water were prepared for the adults to feed and mate, and the petri dishes were placed in the cages. Pupae were placed in a separate cage and monitored until they became adults. The emerging adults were released on tomato plants grown in another cage to ensure the continuity of the stock culture. *T. absoluta* stock culture was reared in an acclimatization chamber at 25±1˚C, 60±10% humidity and 16:8 (light: dark) hour light period.

Fungi cultures

The fungal isolates used in the study were isolated from *Eurygaster* spp. adults in the project numbered 15L0447005 supported by Ankara University Scientific Research Projects Unit. These

*Corresponding author, E-mail address: mmustu77@hotmail.com

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isolates are *Beauveria bassiana* Esk-3 and *Isaria farinosa* N-19/2 and are kept in the fungal stock culture in the biological control laboratory of Erciyes University, Faculty of Agriculture, Department of Plant Protection (Figure 1). The size of conidia of *B. bassiana* Esk-3 isolate is 1.5-2.5x 1-2.1µm (width-length, min-max). Development in PDA is creamy-white, dim and rapid (Gül, 2016). The size of conidia of *I. farinosa* N19/2 isolate is 1.5- 3x 1-2 µm (length-en, min- max). Development in PDA is white, dim and rapid. Besides, its colonies are distributed all over Petri dish (Gül, 2016). In order to produce these fungal isolates used in the study, fungal spores were inoculated into PDA (Potato Dextrose Agar) medium in Petri dishes and Petri dishes were covered with parafilm. Isolates were left to grow in an incubator at 25±1˚C for 2 weeks. Conidial suspensions were prepared from the developing isolates. The fungal isolates were stored in the refrigerator at +4 ˚C for new inoculations.

Figure 1. Growth of Beauveria bassiana Esk-3 (a) and Isaria farinosa N19/2 (b) isolates in PDA

Preparation of Conidial Suspension

In the preparation of conidial suspension, 10 ml of distilled water was added to the petri dishes in which entomopathogenic fungi were grown and the spores were allowed to pass into the water. The spores in the Petri dishes were scraped and filtered through 2 layers of sterile cheesecloth into the beaker to remove the mycelial structure. Tween 80 (0.02%) was added to the fungal suspension in the beaker and vortexed for 2 min to obtain a homogeneous suspension. The suspension to be used in the experiments was prepared using a light microscope and Thoma slide at a spore density of $1x10^8$ conidia/ml for each isolate.

Colonization of tomato leaves by entomopathogenic fungi *Bauveria bassiana* and *Isaria farinosa* inoculated on tomato plants by different inoculation methods

The seeds used in the experiment were surface sterilized in 70% ethanol for 2 minutes and 0.5% sodium hypochlorite for 1 minute. They were then rinsed three times in a row with distilled water. The seeds were placed on blotting paper and allowed to dry in a laminar cabinet. Three different inoculation methods were used in the experiments; ⅓ of the dried seeds were used for seed inoculation and ⅔ were used for leaf spraying and root dipping after seedling emergence. All inoculation methods were modified from Allegrucci et al. (2017).

Seed inoculation

Sterilized seeds were immersed in 10 ml $1x10^8$ conidial suspension for 24 hours. At the end of this period, the seeds in the suspension were removed and dried on blotting paper for 30 minutes in a laminar flow cabinet. The dried seeds were sown in pots containing soil sterilized in autoclave at 121˚C for 20 minutes and tomato plants were grown in a climate chamber with 25±2˚C, 60±10% humidity and 16:8 hours light-dark conditions.

Root inoculation

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The sterilized seeds were planted in pots containing sterilized soil for use in the root dipping method. In this method, 21 day-old seedlings after germination were used. Seedlings were removed from the pots and rinsed 3 times with distilled water. The root tips of the plants were cut to ensure absorption of the conidial suspension. The cut plant roots were placed separately in tubes containing 3 ml ($1x10^8$ conidia/ml^{-1}) conidial suspension. The roots of the control plants were placed in 3 ml of distilled water + 0.02% Tween 80. Both inoculated and control plants were kept in the acclimatization chamber for 24 hours. At the end of this period, the plants immersed in conidial suspension and distiled water were kept on blotting paper to remove excess water from the roots. The dried roots were planted in the same pots and the plants were placed in the climate chamber with 25±2˚C, 60±10% humidity and 16:8 hours light-dark conditions.

Leaf inoculation

The sterilized seeds were sown in pots containing sterilized soil for use in the leaf spraying method. In this method, when the tomato plants reached 30 cm in height (7 weeks old), 3 ml of ($1x10^8$ conidia/ml^{-1}) conidial suspension was sprayed on the leaves with a spray pump. During this process, the soil surface was covered with aluminum foil to prevent the transfer of conidial flow from the leaves to the soil. Control plants were sprayed with 3 ml of distilled water containing 0.02% Tween 80. The plants were placed in the climate chamber with 25±2˚C, 60±10% humidity and 16:8 hours light-dark conditions.

For the detection of endophytic fungal colonization in tomato plants inoculated with different inoculation methods, a leaf was randomly selected from each seedling to determine the presence of fungi in the plants. The selected leaves were surface sterilized by immersion in 0.5% sodium hypochlorite for 2 minutes and 70% ethanol for 2 minutes. They were then rinsed three times with distiled water. The sterilized leaves were dried on filter paper in a laminar flow cabinet and the dead tissues caused by the sterilization process were removed. Before placing the leaves on PDA medium, leaves from fungus inoculated tomato plants and control plants were cut into 5 pieces each. The dissected leaf sections were placed in petri dishes containing PDA. Petri dishes were incubated for 10 days at 25±1˚C, 60±10% humidity, 16:8 hours light-dark period and the presence of fungi on the leaves was checked. The development of endophyte entomopathogenic fungi was monitored weekly over a 4-week period, the last 2 weeks for the seed inoculation method, 3 weeks for the root inoculation method and 4 weeks for the leaf inoculation method. The rate of fungal endophyte colonization (%) of host plant leaves was calculated as follows:

$$\text{Colonization (\%)} = \frac{\text{Number of colonized leaf sections}}{\text{Total number of leaf sections}} \text{ X } 100$$

Mortality rates of *Tuta absoluta* larvae fed on tomato plants containing endophytic entomopathogenic fungi *Bauveria bassiana* and *Isaria farinosa*

Leaves from tomato plants colonized by the entomopathogenic fungi *B. bassiana* and *I. farinosa* using different inoculation methods were placed in petri dishes containing moistened filter paper on the bottom with 1-2 leaves and 10 2^{nd}-3^{rd} instar *T. absoluta* larvae in each petri dish. The viability of the larvae in the petri dishes was evaluated at the end of seven days. During this period, entomopathogenic fungi were continued to be fed to the larvae from uninoculated leaves in order to maintain larval nutrition. Dead larvae were removed from the petri dish daily and recorded. Larval mortality rate (%) was calculated as follows:

$$\text{Larval mortality rate (\%)} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \text{ X } 100$$

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Statistical analysis

Angle transformation was applied to the endophyte entomopathogen fungus colonization values of different incubation periods. t-test for dependent groups was used to compare the means of the two groups. In the comparison of more than two group means, Repeated measures ANOVA was applied and the different groups were determined by Bonferroni test. Endophytic entomopathogenic fungal colonization values between different fungal species obtained for each week were subjected to One-way analysis of variance (ANOVA) and differences between group means were determined by Tukey multiple comparison test.

In the analysis of the data on tomato moth mortality rates, angle transformation was applied to the mortality rates obtained, these values were subjected to One-way analysis of variance (ANOVA) and the differences between group means were determined by Tukey multiple comparison test.

3. RESULTS AND DISCUSSIONS

Colonization of tomato leaves by entomopathogenic fungi *Bauveria bassiana* and *Isaria farinosa* inoculated on tomato plants by different inoculation methods

Both entomopathogenic fungi inoculated on tomato plants with three different inoculation methods were found to colonize the plants endophytically, while no fungal presence was detected in any of the control groups (Table 1). In the seed inoculation method, there was a statistical difference between the colonization rates of both *Bauveria bassiana* ($t = -4.811$; $df = 9$; $p = 0.001$) and *Isaria farinosa* ($t = -3.857$; $df = 9$; $p = 0.004$) inoculated plants at weeks 3 and 4. In the root dipping method, the colonization rate of *B. bassiana*-treated plants increased as the weeks progressed ($F = 11.477$; $df = 2$; $p = 0.001$), while fungal colonization reached 40% at the end of the 4th week. While a similar situation was observed for *I. farinosa* ($F = 7.789$; $df = 2$; $p = 0.004$), the colonization rate at the end of the 4th week was 32%. In the leaf spraying method, the endophytic colonization rate was found to be quite high. In *B. bassiana*-treated plants, the colonization rate increased as the weeks progressed ($F = 17.513$; $df = 3$; $p < 0.001$), and fungal colonization reached 100% at week 4. Similarly, the colonization rate of *I. farinosa* increased as the weeks progressed ($F = 30.992$; $df = 3$; $p < 0.001$), reaching 96% at week 4.

Table 1. Colonization of tomato leaves by entomopathogenic fungi Bauveria bassiana and Isaria farinosa inoculated on tomato plants using different inoculation methods (%)

Fungi	Inoculation method	Time after inoculation (week)			
		1	2	3	4
Bauveria bassiana	Seed	-	-	4.00 ± 2.67b *C **	28.00 ± 5.33aBC
	Root	-	16.00 ± 2.67bB	26.00± 3.06abB	40.00 ± 4.22aB
	Leaf	74.00 ± 5.21cA	84.00 ± 2.67bcA	94.00 ± 3.06abA	100.00 ± 0.00aA
Isaria farinosa	Seed	-	-	4.00 ± 2.67bC	22.00 ± 3.59aC
	Root	-	12.00 ± 3.27bB	20.00± 0.00abB	32.00± 5.33aBC
	Leaf	60.00 ± 2.98cB	72.00 ± 3.27bA	86.00 ± 3.06abA	96.00 ± 2.67aA
Control	Seed	-	-	0.00 ± 0.00C	0.00 ± 0.00D
	Root	-	0.00 ± 0.00C	0.00 ± 0.00C	0.00 ± 0.00D
	Leaf	0.00 ± 0.00C	0.00 ± 0.00C	0.00 ± 0.00C	0.00 ± 0.00D

**Different lowercase letters in the same row are statistically different according to T test for dependent groups or Bonferroni test ($p \leq 0.05$).*

***Different capital letters in the same column are statistically different according to Tukey's test ($p \leq 0.05$).*

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When different fungus and control treatments were compared on a weekly basis, it was found that there was a statistical difference ($F = 156.197$; $df = 2$; $p < 0.001$) between the treatments 1 week after the application in plants treated with entomopathogenic fungi by leaf spraying method, and the colonization rate of *B. bassiana* was higher than *I. farinosa*. When the colonization rates of different fungi were examined 2 weeks after the application, it was determined that there was no statistical difference between the two fungi applied with the same method and both methods were different from the control. However, the colonization rate of the leaf spraying method was higher than that of the root dipping method for both fungal species ($F = 108.059$; $df = 5$; $p < 0.001$).

No statistical difference was found between the fungal isolates inoculated with the same method 3 weeks after the application. Three weeks after inoculation with different methods, the highest colonization rate was 94% for *B. bassiana* leaf spraying method and the lowest colonization rate was 4% for both fungi in seed inoculation method ($F = 147.263$; $df = 8$; $p < 0.001$).

Similar to week 3, 4 weeks after the application, there was no statistical difference between the fungal isolates inoculated with the same method, but the highest colonization rate in terms of application method was reached in the leaf spraying method, followed by root dipping and seed inoculation methods, respectively ($F = 175.874$; $df = 8$; $p < 0.001$). It was determined that there was no statistical difference between the seed inoculation methods of both fungi and the root dip method, and the lowest colonization rate was 22% in the *I. farinosa* seed inoculation method.

Klieber and Reineke (2016) reported that a commercial preparation of *B. bassiana* ATCC 74040 (a.i. in product Naturalis®) inoculated from tomato leaves was 100% present on all tomato leaves after 18 days. Allegrucci et al. (2017) evaluated fungal colonization of tomato seedlings 7, 14 and 28 days after inoculation with *B. bassiana*, and the highest values were 29.44% for leaf spraying and 12.22% for root dipping 7 days after inoculation. Agbessenou et al. (2020) inoculated tomato plants with 15 isolates of entomopathogenic fungi belonging to 7 different genera (*Beauveria* (7), *Fusarium* (1), *Hypocrea* (1), *Metarhizium* (3) and *Trichoderma* (3)) using seed inoculation method and reported that 12 isolates colonized the roots, stems and leaves of tomato plants at different rates 4-5 weeks after inoculation. Silva et al. (2020) reported that *B. bassiana* colonized the roots and stems of tomato seedlings 21 days after inoculation and 100% colonized the whole plant after 30 days. Ibrahim et al. (2021) inoculated *B. bassiana* and *Metarhizium anisopliae* to tomato plants by seed inoculation and injection methods and reported that the colonization rate on tomato leaves 4 weeks after seed inoculation was 75% for *M. anisopliae* and 66.7% for *B. bassiana*. Agbessenou et al. (2022) reported the colonization rate of seed inoculated *Trichoderma asperellum* M2RT4 isolate on roots, stems and leaves of tomato plants as 95, 90, and 85%, respectively, 4-5 weeks after inoculation. As seen in the literature, there are great differences in the colonization rates in the plant according to the type of entomopathogen fungus inoculated, isolate differences, inoculation method and the time elapsed after the application. Similar results were obtained in the present study and significant differences were found especially according to inoculation methods. Moreover, the highest colonization rates were obtained in the leaf inoculation method for both entomopathogenic fungal species.

Mortality rates of *Tuta absoluta* larvae fed on tomato plants containing endophytic entomopathogenic fungi *Bauveria bassiana* and *Isaria farinosa*

Although the highest mortality rate of *T. absoluta* in tomato plants inoculated with *B. bassiana* using different inoculation methods was obtained in the seed inoculation method (64%), the mortality rates obtained from all three methods were not statistically different from each other ($F = 2.832$; $df = 2$; $p = 0.076$). (Table 2). A similar situation was found for *I. farinosa*, and although the

*Corresponding author, E-mail address: mmustu77@hotmail.com

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highest mortality rate (58%) was obtained from the seed inoculation method, there was no statistical difference between the methods (F = 1.833; df = 2; p = 0.179).

Table 2. Mortality rates of Tuta absoluta larvae feeding on tomato plants inoculated with Bauveria bassiana and Isaria farinosa (%)

Fungi	Inoculation method		
	Seed immersion	Root dipping	Leaf spraying
Bauveria bassiana	64.00 ± 4.52a*A**	51.00 ± 3.48aA	57.00 ± 3.67aA
Isaria farinosa	58.00 ± 7.42aA	44.00 ± 3.71aA	49.00 ± 4.58aA
Control	7.00 ± 1.53aB	6.00 ± 1.63aB	10.00 ± 1.49aB

**Different lowercase letters in the same row are statistically different according to Tukey's test ($p \leq 0.05$).*
***Different capital letters in the same column are statistically different according to Tukey's test ($p \leq 0.05$).*

When the different treatments were examined in terms of seed inoculation method, it was determined that there was no statistical difference between the two fungus species, but larval mortality in fungus-treated plants was different from the control (F = 40.015; df = 2; p < 0.001). Similar results were obtained for both root dipping method (F = 59.702; df = 2; p < 0.001) and leaf spraying method (F = 53.150; df = 2; p < 0.001).

Klieber and Reineke (2016) reported that 28.6-55.5% mortality occurred in larvae of *T. absoluta* at different stages feeding on tomato plants inoculated with *B. bassiana* for 19 days after inoculation. Allegrucci et al. (2017) reported that the highest mortality rate of *T. absoluta* feeding on *B. bassiana*-inoculated tomato plants was 75.5 ± 20.6% at the end of 10 days, while the mortality rate was 5 ± 10% when the larvae were fed on control plants. Silva et al. (2020) reported that the S_{50} ($survival_{50}$) period of 2nd and 3rd instar *T. absoluta* larvae feeding on the leaves of tomato plants inoculated with *B. bassiana* LPP139 isolate 30 days ago against tomato moth was 4 days and all larvae died on the 7th day of the study. Agbessenou et al. (2020) reported that the average viability rate of *T. absoluta* F1 larvae grown on tomato plants inoculated with *Trichoderma* sp. F2L41, *B. bassiana* ICIPE 35(4), *B. bassiana* ICIPE 35(15) and *F. proliferatum* F2S51 ranged from 15.6-24% on the 5th day after emergence, while the viability rate in the control was 58.6%. Previous studies have shown significant differences in the mortality rates of *T. absoluta* larvae fed on tomato plants inoculated with endophytic entomopathogenic fungi. However, it is seen that many endophytic entomopathogenic fungi are more or less effective on the pest, albeit for different periods of time. In our study, although the highest larval mortality rate occurred in the *B. bassiana* seed inoculation method (64%), there was no statistical difference between the treatments, but the mortality rates in all treatments were found to be different from the control.

4. CONCLUSIONS

According to the data obtained, it was concluded that this entomopathogen fungus isolate belonging to two species, which can show endophytic character, has the potential to be used in biological control. Entomopathogenic fungi are microbial agents that are emphasized because they have almost negligible negative effects on the living and non-living environment compared to pesticides. However, from time to time, various environmental factors, especially humidity, can limit their usability. The endophytic character of these fungi by inoculating them into plants may limit adverse environmental conditions and pave the way for their use in different ecosystems. In this way, they will be able to make significant contributions to a sustainable pest control by finding a place in integrated control programs, even if they are not alone.

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*Corresponding author, E-mail address: mmustu77@hotmail.com