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Trigonelline as an anti-diabetic metabolite increased in inoculated fenugreek by *Trichoderma*

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Abstract

Trigonelline has been known as an anti-diabetic substance extracted from plant sources. The Trigonelline content and growth factors of two *Trigonella foenum* ecotypes (Hamedan and Bandarabbas) treated by two *Trichoderma harzianum* isolates (chit4215MK and T8-7MK) were studied in a greenhouse. Different growth factors were observed in two ecotypes affected by *Trichoderma* strains. Hamedan ecotype had more stem length and the Bandarabbas ecotype showed more lateral branches and pod numbers. The results indicated that the *Trichoderma* strains had a positive effect on the growth of the Hamadan ecotypes stem, and the T8-7MK strain showed better effects than the chit4215MK. No shoot length differences were observed in the Bandarabbas ecotype between the control and *Trichoderma* treated plants. No significant difference was observed in peroxidase activity and total soluble carbohydrate content between the ecotypes, treatments, and the interaction effects of treatment and ecotypes. The highest Trigonelline content (4 mg g-1 DW) was obtained in the Bandarabbas ecotype treated with the *Trichoderma* strain T8-7MK, which was 1.6-fold higher than the control plants. Also, the Hamedan ecotype treated by the *Trichoderma* strain chit4215MK produced a higher content of Trigonelline (3.5 mg g^{-1} DW) which was 1.3-fold more than the control plants. The amount of Trigonelline in the treated Hamedan ecotype was lower than the treated Bandarabbas ecotype. Our results revealed that Fenugreek growth factors and Trigonelline biosynthesis can be affected by *Trichoderma* strains.

1. Introduction

Fenugreek, *Trigonella foenum-graecum*, is one of the most widely used medicinal plants in traditional medicine (Baquer et al., 2011). Enhancing flavour, colour, and texture of food is another advantage of using fenugreek seeds (Yadav & Baquer, 2013). Recent researchers have identified several health benefits and physiological attributes from the use of fenugreek in experimental animals and human clinical trials. It has medical properties such as carminative, gastric stimulant, anti-diabetic, and galactagogue effects

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and as a hypocholesterolemic, antioxidant, hepatoprotective, antifungal, antibacterial, antiulcer, antilithigenic, and anticarcinogenic (Yadav & Baquer, 2013; Baquer et al., 2011). The anti-diabetic effect of fenugreek has been reported in different articles, and researchers have shown that many components in the seeds create hypoglycemic effects (Allred et al., 2009; Yoshinari et al., 2009; Hirakawa et al., 2005).

Trigonella foenum-graecum contains many chemical constituents such as fibers, saponins, flavonoids, fixed oils, and alkaloids, namely Trigonelline and choline (Toppo et al., 2009; Shailajan et al., 2011). The plant seeds are economically attractive because of their specific properties as they contain 1–2% steroidal sapogenins, in particular diosgenin (Kaufmann et al., 2007). Trigonelline is a suitable bioactive marker to establish the quality of seeds because it has estrogenic, anti-diabetic and anti-invasive activites. (Shailajan et al., 2011). The quantity and quality of seeds can be significantly increased through suitable management of cultivation, irrigation, and harvesting.

Treating plants with different stimuli can stimulate many defenses responses in plants. One of these reactions is the production of secondary metabolites (Cheong & Hahn, 1991; Hanania & Avni, 1997; Zhang, 2019). Some species of the genus *Trichoderma* have been shown to have a symbiotic relationship with plants and are used as biopesticides and biofertilizers (Harman et al., 2004; Vinale et al., 2008; Kowsari et al., 2014). *Trichoderma* is one of the most abundant fungi in the soil bed. It is also found in the rhizosphere of many plants (Hermosa et al., 2004; Kowsari et al., 2014). *Trichoderma* is a fungal genus also found in different climates and ecosystems. Resistance to disease and tolerance to abiotic stresses can occur when *Trichoderma* strains interact with a plant's roots leading to increased plant growth potential (Hermosa et al., 2012; Kowsari et al., 2016). However, there has been little research on *Trichoderma* strains and their medicinal plant species. A field investigation was conducted by Arpana and Bgyaraj (2007) to establish the influence of inoculation with *Glomus mosseae* and *T. harizanum* on growth and yield of *Andrographis paniculate*. Their results showed that inoculation with these fungi improved the growth, biomass yield, P nutrition, and andrographolide concentration of *A. paniculate*. Research results have shown that secondary metabolites accumulation has been promoted by specific *Trichoderma* strains. For instance, Sumithra and Selvaraj (2011) indicated that inoculation of *Sphaeranthes amaranthoides* with *Glomus walker*, *Bacillus subtilis*, and *Trichoderma viride* enhanced its growth, biomass, nutrition, and secondary metabolites. As another example, Thomas and Rajeshkumar (2014) reported the increased biomass for *Strobilanthes ciliatus* in a pot culture that was significantly higher in plants inoculated with *G. aggregatum*, *B. coagulans*, and *T. harzianum*.

Based on our knowledge, there is no published research results introducing *Trichoderma* as an elicitor to increase Trigonelline content and growth characteristics of Fenugreek. The main objectives of this research were to investigate the effect of two strains of *Trichoderma* on growth characteristics and trigonelline accumulation of two Fenugreek genotypes and to find possible plant-fungus interactions.

2. Materials and methods

2.1. Plant materials

The fenugreek seeds were supplied by two provinces in Iran (Hamedan and Bandarabbas). The seeds were planted at a depth of 2 cm in pots which were filled with soil (50%), peat (25%), and perlite (25%). The pots were placed in a greenhouse for 10 days in suitable temperature and relative humidity. The mean temperature measured 24°C during the day and 18°C at night. Humidity was approximately 70% at night and

45% during the day. The light program was about 800 mmol m-2 s-1 and 16:8h light:dark. When the roots were approximately 10 cm long (after 30 days), the roots of each ecotype were immersed in a solution containing one *Trichoderma* isolate for 30 min while it was on the shaker (50 rpm). The control plants were shaken with an un-inoculated solution (without *Trichoderma* isolates). The inoculated and not-inoculated plants with fungi were transferred to pots (with a 20 cm diameter) filled with soil containing *Trichoderma* isolates (50%), peat (25%), and perlite (25%). The pots were then placed in a greenhouse. The stem length (cm), root length (cm), stem, and root dry weights (g) were measured for ten plants from each treatment and ecotype. When 50% of the plants were in flowering phase,. the plants were also treated with a 5 ml solution containing *Trichoderma* isolates (2×10^7) conidia/ml that injected by syringe into the plant soil.

The following growth characteristics were measured for the inoculated and not-inoculated plants for each treatment after 60 days in the flowering stage: Stem length, root length, root dry weight, stem dry weight, amount of chlorophyll, frequency of lateral branches, number of pods per plant, total soluble carbohydrate, peroxidase enzyme activity. The shoot length, pod length, frequency of pod per lateral branches, frequency of pod per the main shoot, the number of lateral branches, the abundance of seeds per pods, and weight of 1000 seed (1000SW) were recorded at the time of plant maturity (almost 14 weeks). The seeds were harvested after the pods of the plants were dried.

2.2. Fungal strains

T. harzianum isolates, ABRIICC chit4215MK and ABRIICC T8-7MK, were provided by the Agricultural Biotechnology Research Institute (ABRII) Gene Bank. The isolates were separated from soil collected from different areas of Iran. Fungal isolates were grown on PDA for 5 days in dark conditions at 25°C. The culture plates were moved into a new environment with light and the same temperature and grown for 2 days under these conditions. The fungus colonies on the plates were then cut into 50 mm plugs (Papavizas, 1985; Watts et al.,1988; Kubicek et al., 2003).

2.3. Extraction and quantitative analysis of Trigonelline

The powdered fenugreek seeds (50 mg) were extracted by ultrasonication with 2.5 ml of methanol. The supernatant was collected after centrifugation at 4 ºC. The residue was re-extracted with methanol and then ultrasonicated. The two supernatants were mixed and then evaporated until dried completely. The residue was immersed in 5 ml methanol and kept in the dark at 4 °C. A high-performance liquid chromatography (HPLC) was used for the quantitative determination of Trigonelline (Rongjie et al., 2010; Zheng & Ashihara, 2004; Koshiro et al., 2006; Mehrafarin et al., 2012). A Knauer liquid chromatography equipped with a Knauer injector with a 20 µl loop, a Nucleosil C18 (150 mm × 4.6 mm I.D, 5 µm) column, Knauer K2600A UV detector, and Chromgate software was used , and methanol (95%) was used as the mobile phase. The elution time and flow rates were 20 min at 1 ml min-1, and peaks were detected at 267 nm. Detection was done by comparison of retention times (Rt) with the standard of Trigonelline. The Trigonelline content was expressed as mg g-1 DW and accomplished using a known concentration of standard and peak areas. The mean of at least three replicates was given as data. The standard of Trigonelline was from Sigma.

2.4. Determination of total soluble carbo-hydrate

Leaf tissue (0.02 g, 15 leaves from the stem tip) was taken from the plants to measure the total soluble sugars content. Soluble sugars content was measured according to Dubois et al. (1956).

2.5. Assay of antioxidant enzyme and protein

The chlorophyll index was determined by the SPAD-502Plus. The SPAD index was determined by a Chlorophyll meter (Minolta, Japan).

2.6. Determination of Chlorophyll index

The chlorophyll index was determined by the SPAD-502Plus. The SPAD index was determined by a Chlorophyll meter (Minolta, Japan).

2.7. Root colonization

The roots were collected from a depth of 5 to 10 cm. Then the rinsed roots were transferred to a Falcon containing KOH (10 %) in a water bath at 70 °C for 20 min. Next, the roots were washed and kept in a cotton blue solution container (24 hours, room temperature). Finally, the roots were washed again with distilled water, and the longitudinal and transverse slices were prepared. Then, microscopic slides were prepared and observed under a microscope (Nikon ECLIPSE E600, X20) (Gamalero et al., 2003).

2.8. Statistical analysis

The experiment was done in a completely randomized design which was repeated twice. In this experiment, two ecotypes of fenugreek (Hamedan and Bandarabbas) were treated with two different isolates of *Trichoderma* (chit4215MK and T8-7MK). Each treatment had three replications. Data were subjected to analysis of variance with SAS statistical software. Differences among treatments were further analyzed using Duncan's Multiple Range tests.

3. Results and discussion

3.1. Effects of *Trichoderma* strains on growth characteristics

At ten days, both non-treated ecotypes (Hamedan and Bandarabbas) showed a difference in primary stem height. As shown in Figure 1, The Hamedan had more stem length after ten days. There were no significant differences between the root length or root and shoot dry weight in either ecotypes.

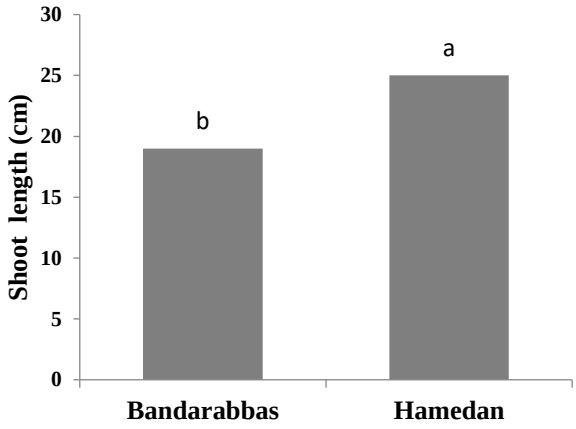

Figure 1. The shoot length of two fenugreek ecotypes (Bandarabbas and Hamedan) in 10-day non-treated plants. Values are means of triple results, and the same letters showed no significant difference.

The statistical analysis showed that the ecotype played an important role on shoot length, the number of pods, and lateral branches after 60 days (in the flowering stage). The figure also shows that the interaction of *Trichoderma* treatment and ecotype + *Trichoderma* strains had a significant effect on shoot length. Several previous studies have shown that some *Trichoderma* strains can stimulate plant growth, increase crop production, and disease resistance (Harman et al., 2004).

The results of this study demonstrated that the shoot length in the Hamedan (treated and non-treated) ecotype was higher than the Bandarabbas ecotype. As can be seen from Figure 2, the highest shoot length was observed in plants (Hamedan) treated with T8-7MK strain (50 cm) which was 1.18- fold that of the control (non-treated plants) (48 cm) and chit 4215MK treated plants (48 cm). These results corroborate the findings of previous works in this field (Sumithra. and Selvaraj, 2011; Thomas & Rajesh Kumar, 2014).

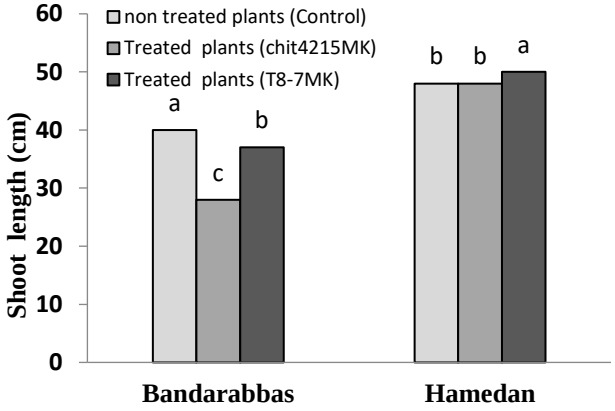

Figure 2. Effects of *T. harzianum* T8-7MK and *T. harzianum* chit4215MK on shoot length in the two fenugreek ecotypes in the flowering stage (after 60 days). Values are means of triple results, and the same letters showed no significant difference.

Figure 3 presents the number of lateral branches and pods per plant in the different ecotypes after 60 days. As we can see, the highest number of lateral branches (14) and pods per plant (1.9) were observed in the Bandarabbas ecotype, which was 2.33 and 1.72 higher than the Hamedan, respectively. There were no significant differences between root length, root and shoot dry weight, and chlorophyll index between the two ecotypes and the ecotype + treatment interaction.

In the final samples (at the time of plant maturity), significant differences were found between the two ecotypes in shoot length, pods length, the average number of pods per lateral branches, the average number of pods per the main shoot, weight of 1000 seed, and the number of lateral branches. Moreover, the *Trichoderma* treatment and ecotype + *Trichoderma* strains interaction had significant effects on shoot length. At this stage of the sampling, we see that the shoot length in the Hamedan (treated and non- treated) ecotype was higher than the Bandarabbas ecotype. As shown in Figure 4, the highest shoot length was obtained in plants (Hamedan) treated with the T8-7MK strain (108 cm), which was 1.18- fold that of the control (non-treated plants) (91 cm) and chit 4215MK treated plants (90 cm). This finding is in agreement with Thomas and Rajeshkumar (2014), which showed the plant height of *Strobilanthes ciliatus* in a pot culture was significantly higher in plants inoculated with *T. harzianum*. There was no significant difference between the control and chit4215MK treated plants.

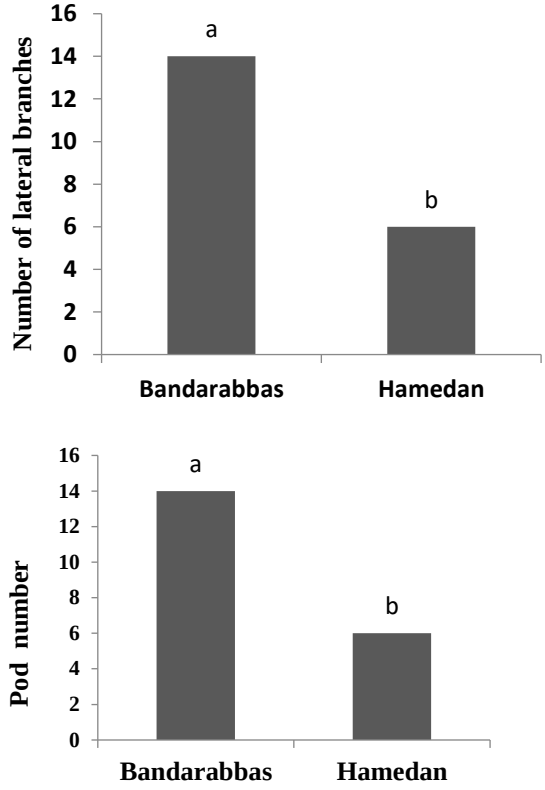

Figure 3. The number of lateral branches and pods number in the two fenugreek ecotypes (Bandarabbas and Hamedan) in the flowering stage (after 60 days). Values are means of triple results, and the same letters showed no significant difference.

Table 1 shows that pod length (7 cm) and weight of 1000 seeds (4 g) in the Hamedan plants were higher than the Bandarabbas plants. While, lateral shoots length (2 cm), number of lateral branches (1.4), average number of pods per lateral branches (4.5), average number of pods per the main shoot (1.9) of the Bandarabbas plants were higher than the Hamedan plants (Table 1). The findings showed that the Hamadan ecotype producing more stem length and the Bandarabbas ecotype producing more lateral branches and pod numbers. The results showed that *Trichoderma* strains had

different effects on the growth of the Hamadan ecotypes stem, with the T8-7MK strain producing a longer stem than the chit4215MK. The control and *Trichoderma* treated plants in the Bandarabbas ecotype had no differences in shoot length. The findings can be examined from different perspectives. There are reports on the effect of *Trichoderma* on the increase of root mass and growth of hairy roots (Bjorkman et al., 1998; Harman et al., 2004b). Arabidopsis seedlings were treated by two species of *Trichoderma* and the role of auxin investigated by Contreras-Cornejo et al. (2009). Their results confirmed the role of auxin in plant growth promotion by *Trichoderma*.

Table 1. The growth characteristics and yield of two fenugreek ecotypes (Bandarabbas and Hamedan) at the time of plant maturity. Values are means of triple results and ± SD.

Ecotype	Number of lateral branches	Number of pods on lateral branches	Number of pods on main shoot	Pod length (cm)	Length of lateral branches(cm)	Weight of thousands seeds (g)
Hamedan	1.2±0.3	3.7±0.11	1.5±0.10	7±0.08	1.3±0.08	14±0.10
Bandarabbas	1.4±0.25	4.5±0.18	1.9±0.09	5±0.11	2±0.09	11±0.11

The results of other studies have shown that some *Trichoderma* isolates can directly affect plant pathogens. In addition, research findings show that changes in phytohormones can play an important role in plant growth and plant resistance to stresses. Martínez-Medina et al. (2014) studied biocontrol activity of *Trichoderma* strains against *Fusarium oxysporum*. Their results indicated that plant growth was correlated to the abscisic acid, ethylene, and cytokinin trans-zeatin riboside.

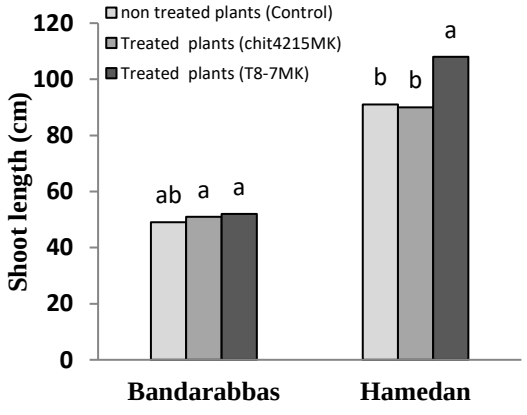

Figure 4. Effects of *T. harzianum* T8-7MK and *T.harzianum* chit4215MK on the shoot length of both fenugreek ecotypes at the time of plant maturity. Values are means of triple results, and the same letters showed no significant difference.

3.2. Effects of *Trichoderma* strains on peroxidase activity and total soluble carbohydrate content

To obtain further evidence of the involvement of a fenugreek defense responsive to *Trichoderma* treatment, the peroxidase activity and total soluble carbohydrate content of the leaf and root tissues were determined in the flowering stage 6 weeks after the second treatment with *Trichoderma*. The results showed that there were no significant differences between treatments and ecotypes, and no correlation between treatments and ecotypes for the total soluble carbohydrate content and peroxidase activity. Research results showed that after *Trichoderma*-plant interactions, plant responses begin immediately via ion fluxes and an oxidative burst, then calluses appearance and polyphenol synthesis occur (Shoresh et al., 2010). Subsequently, salicylic acid (SA) and jasmonate/ethylene (JA/ET) pathways activate, which induces tolerance in plants. Research findings indicated that treatment of plants with *Trichoderma* can induce SAR responses (Segarra et al., 2007; Yoshioka et al., 2012).

3.3. Root colonization

Some *Trichoderma* species can colonize the root surface of the plant or are even able to establish an endophytic relationship with the plant. This interaction can alter plant metabolism (Harman et al., 2004). To determine root colonization, the roots of two treated and non-treated ecotypes were investigated at the end of the growing season. Figure 6 illustrates the fungus symbiosis with the roots of both ecotypes (Hamedan and Bandarabbas). These results showed that *Trichoderma* spp. can colonize plant roots in both external and internal forms (Mukherjee et al., 2012). The interaction between *Trichoderma* and the plant has been investigated by electron microscopy (Yedidia et al., 1999, Kowsari et al., 2014). These observations have shown that this interaction limits the plant epidermis and outer root cortex, which stops the colonization by *Trichoderma*, probably due to callus formation (Yedidia et al., 1999). The findings show that the relationship of *Trichoderma* with plants is not parasitic, but a double symbiotic relationship (Vinalea et al., 2008) (Figure 5).

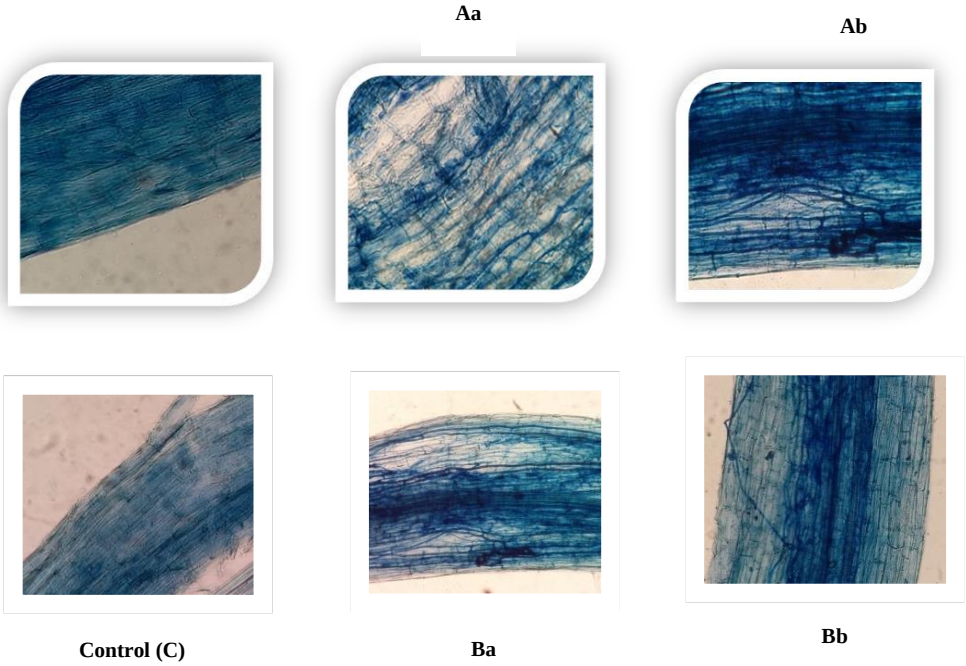

Figure 5. Root colonization of two fenugreek ecotypes (Bandarabbas (A) and Hamedan (B)) at the time of plant maturity with two *Trichoderma* strains (Chit4215mk (a) and T8-7MK (b)) and control (non-treated(c)).

3.4. Influences of *Trichoderma* strains on Trigonelline accumulation

Trigonelline content was determined in seeds collected from mature plants. The results showed that no significant differences were found between the ecotypes (Hamedan and Bandarabbas). The most important finding of these studies was the significant difference between treatments (by two *Trichoderma* strains) in each ecotype. The maximum amount of Trigonelline (4.11 mg g^{-1} DW) was obtained in the *Trichoderma* strain T8-7MK treated Bandarabbas ecotype, which was 1.55-fold higher than the control (2.64 mg g^{-1} DW). The maximum amount of Trigonelline (3.68 mg g^{-1} DW) was obtained in the *Trichoderma* strain chit4215MK treated Hamedan ecotype, which was 1.40-fold higher than the control (2.61 mg g^{-1} DW). The amount of Trigonelline in the treated Bandarabbas ecotype was higher than the Hamedan ecotype (1.11 times more) (Figure 6). These results emphasize the positive effect of

Trichoderma application in stimulating the production of secondary metabolites. The data of the current study are in accordance with those of other researchers who found that inoculation with these fungi improved secondary metabolites production (Thomas and Rajeshkumar, 2014; Sumithra & Selvaraj, 2011; Arpana & Bgyaraj, 2007). This result may be explained by the fact that many *Trichoderma* spp. in the soil can colonize around the root of the plant and penetrate into the root tissues (Harman et al., 2004). The Rhizosphere Microbes and their interaction with the host can activate the cascade of genes, which activates hormonal signals within the plant such as Jasmonic acid (JA), SA and ethylene (ET). JA, found in jasmine flowers (Jasminum grandiflorum), is known as a key signaling molecule that can regulate cellular activities (Memelink, 2009) and induce secondary metabolites (Afrin et al., 2015). SA has also has been shown to play key roles in plant metabolism, regulation of plant growth, development, and flowering (Hayat et al., 2010; Dučaiová et al., 2013). ET is also stimulated by stresses (Wang et al., 2002). The production of JA and ET induce various defense responses starting an induced systematic resistance in the plant. Correlation between JA and ET pathways causes plants to optimize their defense strategies.(Baldwin, 1998; Zhao et al., 2004). The results of several studies have shown that *Trichoderma* is effective in the production of ions and secondary metabolites, which are important mechanisms in biological control.

Evaluation of *Trichoderma's* effect on the roots of cotton (*Gossypium hirsutum*) seedlings indicated that *Trichoderma virens* motivate defense responses by stimulating the synthesis of terpenoids. The results also indicated that terpenoid synthesis and peroxidase activity were increased in treated plants (Howell et al., 2000).

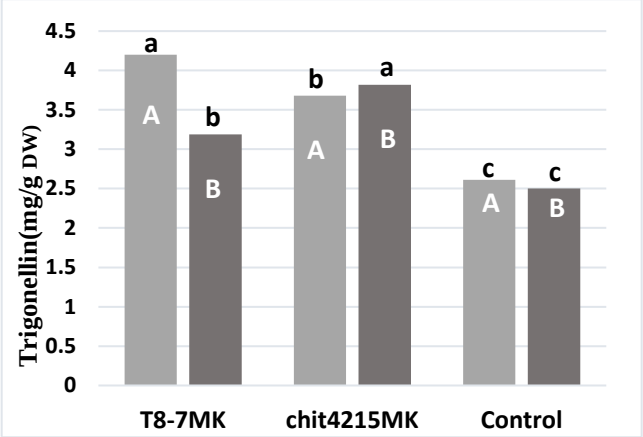

Figure 6. Trigonelline content (mg g^{-1} DW) in *Trichoderma* strains (T8-7MK and Chit4215mk) in treated and non-treated Fenugreek ecotypes (Bandarabbas (A) and Hamedan (B)) at the time of plant maturity. The Trigonelline was determined with HPLC. Values are means of triple results, and the same letters show no significant difference.

Secondary metabolite synthesis and accumulation in cell or hairy root cultures can be triggered by the application of fungal elicitors to the culture medium. Ajmalicine accumulation increased by about 3-fold when *Catharanthus roseus* cell cultures were treated with *A. niger*, *F. moniliforme*, and *T. viride* (Namdeo et al., 2002). Hasanloo et al. (2013) reported enhancement of silymarin production in hairy root cultures of *Silybum marianum* (L.) Gaertn using fungal elicitors. The findings of this study indicate that some *Trichoderma* strains can increase silymarin accumulation in the hairy roots of *S. marianum*. The results also suggested the presence of H and oxidative burst induced by *T. harzianum* as a signaling pathway. In order to have quality agricultural products, we have been forced to use chemical fertilizers that cause environmental pollution. The use of natural fertilizers is an opportunity to reduce this pollution while producing a high quality product. Different soil fungi can colonize plant roots and may have beneficial effects on the plant (Hermosa et al., 2012). Therefore, the identification and introduction of useful bacteria and fungi in the soil of each region are essential.

4. Conclusion

According to the results of this study, there is an increase in *Trigonelline* accumulation associated with *Trichoderma* treatment. It could be concluded that *Trichoderma* acts as a powerful inducing factor for secondary metabolite production. *Trichoderma* spp. can colonize root intercellular spaces. Therefore, it is important to recognize and introduce colonization mechanisms in important economic plants such as fenugreek.

Further studies to establish a precise understanding of the relationship between *Trichoderma* and plants would be beneficial in biofertilizer production, and the increase in the production of metabolites by *Tricoderma* could be one of the important results in these studies.

Conflict of interest

The authors declare that there is no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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