Caspian Journal of Environmental Sciences
Online ISSN: 1735-3866
Print ISSN: 1735-3033

Effects of interaction between ectomycorrhizal fungal and mycorrhiza helper bacteria on *Picea abies* seedlings growth

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ABSTRACT

Early growth and establishment success of *Picea abies* is poor, so ectomycorrhizal (ECM) fungi can be used to promote survival, establishment, as well as the growth of seedlings. This study aimed to evaluate the effect of valuable ECM fungi symbiosis and mycorrhiza helper bacteria (*Bacillus cereus*) on the growth of *P. abies*. The treatments included *B. cereus*, ECM fungi of *Cantharellus cibarius*, *Amanita caesarea*, *Boletus edulis*, three combined treatments (*Bacillus* + ECM fungi), and control. 2-month-old seedlings were inoculated with fungi and bacteria. Then root mycorrhization percent, morphological traits, chlorophyll content and nutrient uptake were evaluated in 15 months after plant inoculation. The results indicated that mycorrhization was significantly influenced by applied treatments. The highest (50.33%) and lowest (27.67%) mycorrhizations were obtained by *B. edulis* + *B. cereus* and *C. cibarius*, respectively. The combination treatments dramatically increased the plant height, stem and root dry weight, total chlorophyll, absorption of potassium and nitrogen compared to the control. The combined inoculation with *B. cereus* + *B. edulis* also resulted in an increase in K and Ca uptake as well as chlorophyll a compared to control. The lowest values for all evaluated traits except Ca uptake were obtained by control. Moreover, the highest values for all traits were observed in plants co-inoculated with *B. cereus* + *B. edulis*. In conclusion, the results suggested that the ECM fungi, especially *Boletus*, had a symbiosis with Norway spruce. The symbiosis was also improved by *B. cereus*.

Keywords: Colonization, Growth, Mycorrhizal fungi, Norway spruce, Nutrient absorption.
Article type: Research Article.

INTRODUCTION

Pines are the most conventional trees in the landscape and forests, which have symbiosis with ectomycorrhizal (ECM) fungi (Berg & McClaugherty 2008). *Picea abies* (L.) H. Karst., commonly referred to as Norway spruce, is a large evergreen coniferous tree belonging to the Pinaceae family and native to Europe. It is often used as a Christmas tree and is recognized as an important industrial tree in the world (Farahmand 2015). The establishment success of planted trees is associated with the rooting ability of the seedlings. Generally, in conifer seedlings and especially in Norway spruce, root growth continues longer than shoot growth (Kaakinen *et al*. 2004). Moreover, the early growth of Norway spruce is particularly slow (Nilsson *et al*. 2019), which can influence its establishment. In this case, ECM fungi have been reported to play an important role in seedling survival, establishment and growth (Menkis *et al*. 2011; Lazarević & Menkis 2018; Domínguez-Núñez *et al*. 2019). ECM is known to provide water and nutritional benefits for their hosts (Akhzari *et al*. 2018) and protection against unfavorable abiotic and biotic stress factors (Menkis *et al*. 2011; Domínguez-Núñez *et al.* 2019). Therefore, they are of considerable significance for seedling performance in the field (Menkis *et al*. 2011). These associations contribute significantly to the sustainability of these ecosystems through nutrient cycling and carbon sequestration (Garcia *et al*. 2015; Al- Abbasi *et al.* 2021; El-Sayed *et al.* 2022).

Several studies have been conducted to examine the development of ECM symbiosis with coniferous trees, indicating that the coexistence improves the growth parameters of the studied plants (Garbay *et al*. 1992; Ahangar *et al*. 2012; Wu *et al*. 2012). ECM symbiosis has been found to be improved by mycorrhizosphere bacteria (Deveau & Labbé 2016), called "mycorrhiza helper bacteria" (MHB; Mediavilla *et al*. 2016). Bacteria can increase the establishment of symbiosis by stimulating mycelial extension, increasing the root-fungus contacts and colonization, and reducing the impact of adverse environmental conditions on the mycelium of the mycorrhizal fungi (Frey-Klett *et al*. 2007). Several species have been reported as MHB, belonging to different genera of many bacteria (Duponnois & Plenchette 2003). The mycorrhiza helper *Pseudomonas fluorescens* strain BBc6R8 can promote the establishment of ECM symbiosis between Douglas fir and ECM fungi, such as *Laccaria bicolor* S238N (Frey-Klett *et al*. 1997). It has also been shown that *B. cereus* HB12 and HB59 can increase the mycorrhizal establishment of *B. edulis* in *Pinus thunbergii* (Wu *et al*. 2012). Moreover, the interaction of microbes between the root and rhizosphere plays an undeniable role in plant development. The microbes may provide the plant with physiologically accessible nutrients and phytohormones that improve plant growth. They also may suppress phytopathogens, or help the plants withstand abiotic stresses, such as heat, salt, and drought (Liu *et al*. 2019). The golden chanterelle (*C. cibarius*) is widely recognized in Europe (Pilz *et al*. 2003). It is one of the most important wild-type fungi in northern Iran found in the pine and oak forests (Olfati *et al*. 2009). The genus *Amanita* is one of the largest basidiomycetous genera. It has over 400 species throughout the world. It also contains delicious edible species such as Caesar's mushroom or *Amanita caesarea* (Yang *et al*. 1999). Many *Amanita* species can establish an ECM interaction with a number of plants, including conifers (Pande *et al*. 2004). It is also one of the most important wild fungi in the coniferous and oak forests of northern Iran (Olfati *et al*. 2009). *B. edulis* and its group are the most important fungal groups due to their economic and ecological importance (Sitta & Davoli 2012). They also have a symbiosis with several plant families, including Pinaceae, Fagaceae and Dipterocarpaceae (Dentinger & Suz 2014; Cui *et al*. 2016). In this study, three valuable edible fungi were selected, namely *C. cibarius*, *A. caesarea* and *B. edulis*, that grow naturally in oak and coniferous forests of Northern Iran. There have been a few studies on the symbiosis of *C. cibarius* (Pilz *et al*. 1994, Pachlewski *et al*. 1996, Tjoelker *et al.* 2007, Katanić *et al*. 2019) and *B. edulis* (Tjoelker *et al*. 2007, Katanić *et al*. 2019) with *P. abies*, while there are no previously reported descriptions of *A. caesarea* ectomycorrhizas. Also, the co-inoculation effect of *B. cereus* with any of these fungi was tested, which has not yet been reported in *P. abies*. *B. cereus* is able to fix atmospheric nitrogen and solubilize phosphorus and potassium (Saxena *et al*. 2019). It can also produce siderophore (Zeng *et al.* 2018) and biologically-active metabolites such as Gibberellins, IAA, and organic acids (Khan *et al.* 2020). However, the previous studies have shown the beneficial effects of *B. cereus* in improving mycorrhizal formation of *B. edulis* with *Pinus thunbergii* (Wu *et al*. 2012). Moreover, different strains of this species have been reported in the mycorrhizosphere and the hyphosphere of Norway spruce seedlings ectomycorrhizal with the fungus *Amphinema* byssoides (Geric *et al.* 2000). Due to the poor establishment of *P. abies* seedlings and their slow growth, creating a successful ectomycorrhiza symbiosis can be a promising effort to solve the above problems as well as develop sustainable agriculture. Thus, it is argued that the selected fungi can probably form mycorrhizal with Norway spruce seedlings, *B. cereus* and it may also be able to act as MHB. Therefore, the present study aimed to investigate the effect of valuable ECM fungi symbiosis and MHB (*B. cereus*) on the growth and nutrient uptake of *P. abies.* This study specifically, purports to answer the following questions: (i) Can symbiosis be established between the studied fungi species and *P. abies*? (ii) Which of the fungi can be more effective on mycorrhizal formation as well as improving the seedlings' performance of *P. abies*? (iii) Can co-inoculation of *B. cereus* with the selected ECM species have a beneficial effect on mycorrhizal formation and seedlings performance of *P. abies*?

MATERIALS AND METHODS

Preparation of ECM fungi and seedlings

On October 15, 2017, sporocarps of *B. edulis*, *A. caesarea* and *C. cibarius* (Fig. 1a) were collected from Saravan and Lakan forests, Guilan Province, Iran. The fungi were identified via morphological characterization by the Iranian Research Organization for Science and Technology. The collected fungi were immediately transferred to the laboratory, and fungi caps were sprayed with ethanol 70%. A small piece (2-7 mm) of the inner flesh from the cap was placed in a solid-modified Melin-Norkrans medium (MMN) containing KH_2PO_4 (0.5 g L^{-1}), NaCl (0.025 g L^{-1}), $CaCl_2$ (0.05 g L^{-1}), $(NH_4)_2HPO_4$ (0.25 g L^{-1}), 1% $FeCl_3$ (1.2 mL L^{-1}), $MgSO_4$ (0.15 g L^{-1}), thiamine chloride (100 mg L^{-1}), malt extract (3 g L^{-1}), glucose (10 g L^{-1}), and agar (15 g L^{-1}). The pH was adjusted to 5.6 with 1M

HCl, and then sterilized at 121 °C for 20 min (Lu *et al*. 2016). The cultured samples were incubated at 25 °C in the dark for two weeks until mycelia growth covered the surface of the plates. The subcultures were also conducted as often as necessary (Fig. 1b and c; Kayama & Yamanaka 2016). The seeds of Norway spruce were prepared from Chalus forests, Mazandaran Province, Northern Iran, disinfected with 10% H_2O_2 for 30 min and rinsed 5 times with sterile water. Next, they were sown in pots containing autoclaved plant growth substrate (121°C for 90 min) at 25°C, 60% relative humidity under a 12 h/day photoperiod for germination. Afterward, 60-day-old seedlings were inoculated with ECM fungi and bacteria (Lu *et al*. 2016).

Inoculum preparation

To prepare the inoculum, a solid substrate was prepared with vermiculite/peat/cottonseed (4/1/1, V/V/V), then the substrate was wet with MMN liquid medium (2:1, V/V) in glass containers (0.6 L), each contained approximately 150 mL substrate. The media were autoclaved at 121°C for 90 min. The sterilized substrate was cooled down, and 2-4 pieces of mycelial agar discs (5 mm diameter) were inoculated into the glasses. Inoculated glasses were sealed with Parafilm and placed into an incubator to incubate at 25°C under dark conditions for 10 days until mycelial growth filled the solid substrate in glass containers (Fig. 1d; Wu *et al*. 2012).

Bacterial inoculum

B. cereus ATCC 11778 strain was supplied by the Iranian Research Organization for Science and Technology (IROST). *B. cereus* was activated in nutrient agar (NA) culture media and then inoculated as was already reported by Wu *et al*. (2012) in beef extract liquid medium containing beef extract (2.0 g L^{-1}), peptone (20.0 g L^{-1}), and NaCl (5.0 g L^{-1}). The pH was adjusted to 7 with 1 M HCl and then placed on a shaker at 28°C for 72 h.

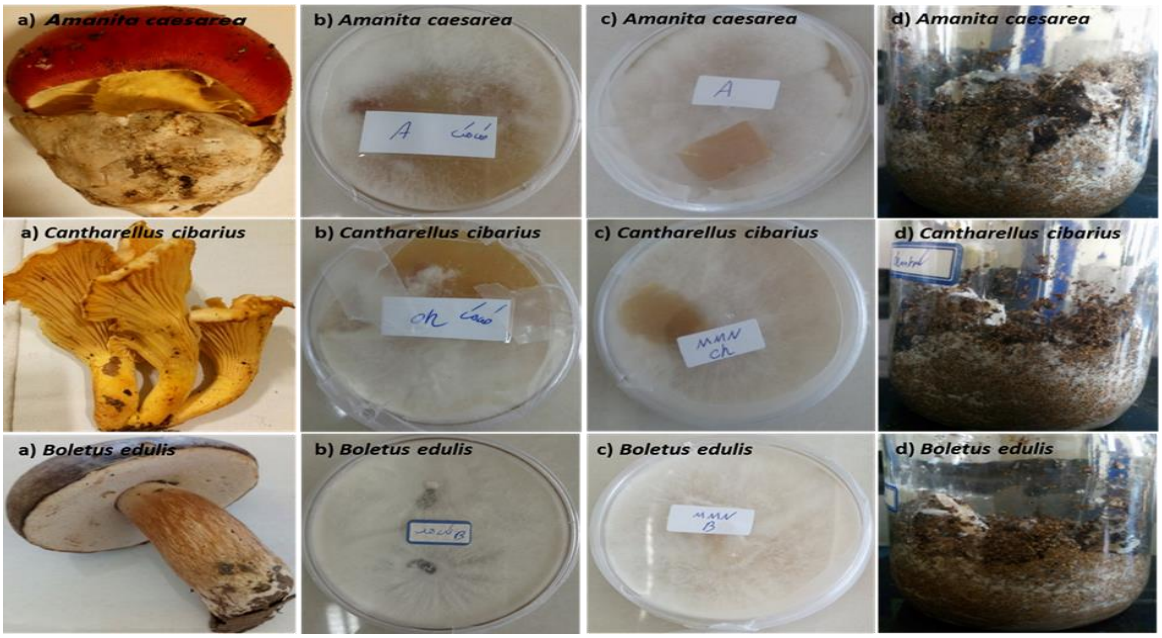

Fig. 1a: Ectomycorrhizal fungi; b: Grown mycelium in MMN medium; c: Subcultures of mycelium in MMN medium; d: Inoculum preparation in glass containers.

Mycorrhizal and bacterial inoculation

The substrate used in the pot experiment consisted of 9:2:1 (V:V:V) mixture of vermiculite, sand, and garden soil and included 7.5 g kg^{-1} total N, 4.2 g kg^{-1} total P, 9.1 g kg^{-1} total K, 0.78% organic carbon, 0.29 dSm^{-1} electrical conductivity and pH 6.85. The substrate was sterilized at 121°C for 90 min to eliminate the native microflora. The roots of 60-day-old seedlings of *P. abies* were covered with mycorrhizal inoculum (10/1, W/W) in the plastic pots (8.5×7×10 cm, top diameter × bottom diameter × height, filled with 350 g substrate as described above) where the seeds were sprouted. For bacterial inoculum, 5 mL bacterial suspensions (5×10^7 CFU g^{-1}) were slowly injected around seedlings (Wu *et al*. 2012). The plants were grown in a growth chamber at 25 °C, 60% relative humidity under a 12 h/day photoperiod for 60 days. When the seedlings were fully established, one plant was kept in each

pot. The pots were then transferred to the greenhouse and remained there until the end of the experiment. The pots were irrigated with distilled water (100 mL) every three days (Lu *et al*. 2016).

Measured parameters

ECM colonization

Evaluation of traits was performed 15 months after plant inoculation. At first, roots were carefully cleaned under tap water and subsequently with distilled water. Then, root samples were cut into 1-cm long, and the percentage of colonization was calculated as described by Wu *et al*. (2012) according to the following formula.

$$\text{ECM colonization (\%)} = \frac{\text{Number of mycorrhizal root pieces}}{\text{Total number of root pieces}} \times 100$$

Chlorophyll

Needle chlorophyll of *P. abies* was measured via spectrophotometer methods as described by Arnon (1956). The chlorophyll was calculated as mg g^{-1} of chlorophyll per plant fresh weight according to the following formula.

$$\text{Ca} = 12.7(\text{A663}) - 2.69(\text{A645}) \times \frac{\text{V}}{1000\,\text{W}}$$

$$\text{Cb} = 20.2(\text{A645}) - 4.68(\text{A663}) \times \frac{\text{V}}{1000\,\text{W}}$$

Ct = Ca + Cb

where Ca: chlorophyll a level; Cb: chlorophyll b level; Ct: Total chlorophyll level; A645: Absorbance at 645 nm; A663: Absorbance at 663 nm; V: volume of the extract; W: tissue weights.

Nutrient uptake

To measure the nutrient uptake of *P. abies*, the aboveground samples were washed and dried in the oven at 60 °C for 72 h. Plant materials were powdered with the grinding mill, then 1 g of samples were burned to ash at 550 °C. After burning, samples were extracted with 10 mL of 15% HCl. The prepared extracts were used to determine all the elements except nitrogen (Aghababaei *et al*. 2011). The phosphorous concentration was measured by a spectrophotometer (Shimadzu-UV-160A, Japan) using a colorimetric method (Olsen & Sammers 1982). Potassium and calcium amount was determined via flame photometer (Jenway-PFP7, UK) respectively as described by Knudsen *et al*. (1982) and Walter & Lanyon (1982). The amount of nitrogen was measured by a Kjeldahl (Gerhardt-VAP40, Germany) after the digestion with sulfuric acid and potassium sulfate (Jackson 1973).

Morphological traits

15 months after inoculation, seedlings were removed from the pots, and morphological traits such as stem height, stem diameter, as well as the root and stem dry weights were measured. Dry weight of root and shoot were taken after drying at 72 °C for 48 h (Kafi *et al*. 2013).

Experimental design and statistical analyses

The experiment was arranged in a completely randomized design with three replications and eight treatments. The treatments included control, bacterial treatment (*B. cereus*), three treatments of fungi (*A. caesarea*, *B. edulis*, *C. cibarius*) and three combined treatments (*A. caesarea* + *B. cereus*, *B. edulis* + *B. cereus*, *C. cibarius* + *B. cereus*). The data were subjected to one-way analysis of variance (ANOVA) using SAS software, version 9.1 (SAS Institute, Cary, NC, USA). Means were compared using least significant difference (LSD) test at 5% probability level.

RESULTS

ECM colonization

The results indicated that *P. abies* seedlings were significantly colonized following the ECM inoculation treatment. ECM colonization was not observed in the control and *B. cereus* treatments (data not shown). ECM colonization rate ranged from 50.33% in *B. edulis* + *B. cereus* to 24.33% in *C. cibarius*. Significant differences were observed between *B. edulis* and *C. cibarius* in both separate and combined inoculations. *B. edulis* and subsequently *A. caesarea* showed the highest colonization rates, while the *C. cibarius* demonstrated the lowest with *P. abies* seedlings. In all three ECM fungi, combined inoculation with bacteria improved mycorrhizal colonization percentage. Thus, in *B. edulis* and *A. caesarea*, a significant difference was observed between the

combined treatments of fungi and bacteria, compared to the separate application of the fungus, while in *C. cibarius*, no significant difference was observed between the separate and combined treatments (Fig. 2).

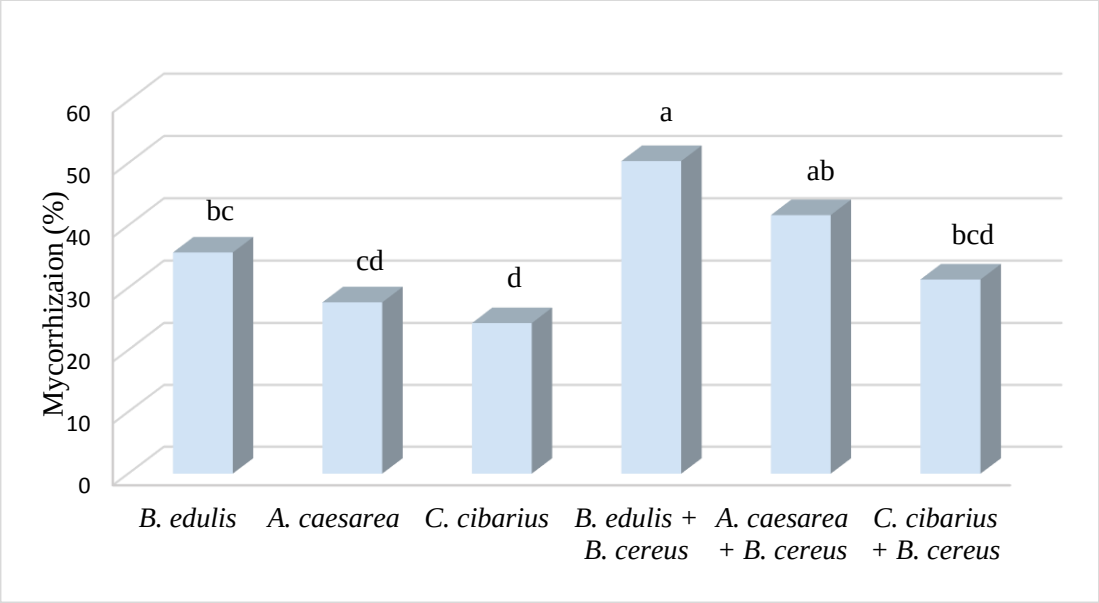

Fig. 2. The effect of *Bacillus cereus* inoculation on mycorrhization percentage of Norway spruce seedlings inoculated with different ectomycorrhizal fungi. The means followed by the same letter are not significantly different by LSD test ($p < 0.05$).

Nutrient uptake

Seedling inoculation with fungi and bacteria exhibited a significant effect on the nutrient uptake of Norway spruce. In all the samples co-inoculated with fungi and bacteria, N and P uptake were significantly increased compared to uninoculated plants. In the combined inoculation with *B. cereus* + *B. edulis*, an increase was found in the K and Ca uptake compared to the control, and also more absorption of all evaluated elements than in bacterial treatments. In addition, it was more effective in increasing the K uptake than *B. edulis* alone. Higher nutrient uptake for all elements was observed in the plants co-inoculated with *B. cereus* + *B. edulis*. The lowest NPK uptake was obtained by the control treatment, while the lowest rate of Ca absorption was related to bacteria. Overall, other treatments also increased nutrient uptake, however, they were not significantly different from the control (Table 1).

Table 1. Result of means comparison of the effect of ectomycorrhizal fungus and *Bacillus cereus* on nutrient uptake in the aboveground of Norway spruce.

Treatments	Nitrogen uptake (%)	Phosphorus uptake (mg g^{-1})	Potassium uptake (mg g^{-1})	Calcium uptake (mg g^{-1})
Control	1.15^{c}	1.56^{c}	6.53^{b}	5.09^{bc}
B. cereus	1.34^{bc}	1.61^{bc}	6.82^{b}	4.92^{c}
B. edulis	1.50^{abc}	1.64^{abc}	7.16^{b}	5.43^{abc}
A. caesarea	1.27^{bc}	1.57^{bc}	6.94^{b}	4.99^{bc}
C. cibarius	1.29^{bc}	1.64^{abc}	6.92^{b}	5.26^{bc}
B. cereus + *B. edulis*	1.85^{a}	1.77^{a}	7.87^{a}	6.07^{a}
B. cereus + *A. caesarea*	1.57^{ab}	1.71^{ab}	7.17^{b}	5.07^{bc}
B. cereus + *C. cibarius*	1.54^{ab}	1.68^{ab}	7.02^{b}	5.6^{ab}
LSD	0.38	0.15	0.65	0.64

In each column, means with the similar letters are not significantly different at the 5% level of probability using the LSD test.

Chlorophyll content

The results indicated that chlorophyll a and total chlorophyll in the needle of Norway spruce were significantly affected by treatments, while there was no observed significant difference in the case of chlorophyll b (Table 2). Plants inoculated with *B. cereus* + *B. edulis* considerably increased the amount of chlorophyll a compared to control. The total chlorophyll contents were also increased following all the combination treatments, compared to the control. The content of chlorophyll a and total chlorophyll in plants inoculated with *B. cereus* + *B. edulis* were

significantly greater than with the bacteria alone, while no significant difference was observed between the combined treatments and the fungus alone. The highest content of chlorophyll a and total chlorophyll was obtained, when plants were co-inoculated with *B. cereus* + *B. edulis*. The lowest value for both chlorophyll a and total chlorophyll was associated with the uninoculated plants (Table 2).

Table 2. Results of means comparison of the effect of ectomycorrhizal fungus and *Bacillus cereus* on chlorophyll in Norway spruce needles**.**

Treatments	Chlorophyll a (mg g^{-1})	Chlorophyll b (mg g^{-1})	Total Chlorophyll (mg g^{-1})
Control	0.607^{b}	0.205^{a}	0.812^{c}
B. cereus	0.636^{b}	0.212^{a}	0.847^{bc}
B. edulis	0.670^{ab}	0.222^{a}	0.891^{abc}
A. caesarea	0.643^{b}	0.212^{a}	0.854^{bc}
C. cibarius	0.636^{b}	0.230^{a}	0.866^{bc}
B. cereus + *B. edulis*	0.738^{a}	0.243^{a}	0.981^{a}
B. cereus + *A. caesarea*	0.681^{ab}	0.251^{a}	0.932^{ab}
B. cereus + *C. cibarius*	0.680^{ab}	0.237^{a}	0.917^{ab}
LSD	0.074	0.067	0.098

In each column, means with the similar letters are not significantly different at the 5% level of probability using the LSD test.

Morphological traits

The results indicated that all morphological traits of Norway spruce except shoot diameters were significantly influenced by the treatments (Table 3). Compared to the controls, the combination treatments (bacterial + fungi) as well as *B. edulis* alone increased plant height, root dry weight and shoot dry weight dramatically. In addition, other treatments exhibited a positive effect on morphological traits and improved them insignificantly. Shoot dry weight of plants co-inoculated with *B. cereus* + *B. edulis* or *B. cereus* + *C. cibarius*, as well as root dry weight of plants co-inoculated with *B. cereus* + *B. edulis* were significantly higher than the occasions in which their inoculations occurred separately. Similarly, the combination of bacteria with each fungus led to a significantly higher shoot height than that with the bacteria alone. The highest and lowest values were obtained by the plants co-inoculated with *B. cereus* + *B. edulis* and uninoculated ones, respectively (Table 3).

Table 3. Results of mean comparison of the effect of ectomycorrhizal fungus and *Bacillus cereus* on morphological traits in Norway spruce**.**

Treatments	Shoot height (cm)	Shoot diameter (mm)	Root dry weight (g)	Shoot dry weight (g)
Control	11.8^{c}	2.25^{a}	0.98^{c}	1.4^{c}
B. cereus	12.45^{c}	2.22^{a}	1.17^{bc}	1.8^{bc}
B. edulis	15.1^{ab}	2.43^{a}	1.4^{b}	2.06^{b}
A. caesarea	13.45^{abc}	2.30^{a}	1.22^{bc}	1.83^{bc}
C. cibarius	12.9b^{c}	2.15^{a}	1.11^{bc}	1.51^{c}
B. cereus + *B. edulis*	15.43^{a}	2.26^{a}	1.93^{a}	2.74^{a}
B. cereus + *A. caesarea*	15.33^{ab}	2.43^{a}	1.32^{b}	2.23^{ab}
B. cereus + *C. cibarius*	15.18^{ab}	2.58^{a}	1.31^{b}	2.26^{ab}
LSD	2.45	0.5	0.32	0.51

In each column, means with the similar letters are not significantly different at the 5% level of probability using the LSD test.

DISCUSSION

Results demonstrated that the ECM fungi of *B. edulis*, *A. caesarea* and *C. cibarius* could successfully colonize *P. abies* seedlings. The Pinaceae family is one of the oldest plants, which had symbiosis with ECM fungi. In previous studies, ECM symbiosis between the genera *Pinus* and *Picea* was suggested (Tedersoo & Brundrett 2017). The *Picea* genus has also been described as a host for the genera of *Amanita*, *Cantharellus* (Solaiman *et al*. 2014) and *Boletus* fungi (Tjoelker *et al*. 2007). In this case, ECM symbioses of *Amanita muscaria* with *P. abies* have been reported in the previous studies (Kottke *et al*. 1987; Schrey *et al*. 2005). In addition, over 100 species of ECM fungi such as *B. edulis* and *C. cibarius* were listed by Tjoelker *et al*. (2007), which could form symbiotic associations with *P. abies*. Moreover, these results are the first to reveal mycorrhization of *P. abies* with *A. caesarea*, so there are no previously reported descriptions in this case. Also, it is shown that mycorrhizal symbiosis was improved by *B. cereus* bacteria and also the dramatic improvement in ECM colonization was observed for *B. edulis* and *A. caesarea*. These results were in agreement with those obtained by Wagner *et al*. (2019), who reported that *B. cereus* enhanced branching of *Tricholoma vaccinum* and increased mycorrhization with *P. abies*. It was

also reported that co-inoculation of *Pinus thunbergii* with *B. cereus* and *B. edulis* stimulated ECM colonization higher than their separate inoculations (Sheng *et al*. 2010; Wu *et al*. 2012). The bacteria involved in enhancing mycorrhiza formation by the plant-fungus symbiosis were defined as MHB (Mediavilla *et al*. 2016). Some MHB species were responsible for multiple helper effects, affecting both plants and mycorrhizal fungi (Bonfante & Anca 2009). In this case, Frey-Klett *et al*. (2007) suggested that some mechanisms may explain the MHB success including the production of growth factors that may promote spore germination, growth of mycelia, improved root branching, greater root colonization, decrease of soil-mediated stress via detoxification of antagonistic substances and deterrence of competitors and antagonists. Results demonstrated that inoculation with all studied ECM fungi, especially in co-inoculated with *B. cereus*, improved all mineral uptake in Norway spruce seedlings. Inoculation of the plants with two or more microorganisms improves the symbiotic relationship as well as the increased nutrient uptake. The results of this study are in line with the studies by Wu *et al*. (2012) in *P. thunbergii* and Ahangar *et al*. (2012) in *Pinus wallichiana* who reported that the mineral nutrients uptake (N, P and K) was successfully increased in plants inoculated with ECM or co-inoculated with HMB. This increase was higher in combined inoculations than in individual inoculations. Most studies have implicated the positive effect of ECM fungi on phosphorus and nitrogen uptake, however, there is no accurate information about other nutrients. Mycorrhizal plants can absorb nutrients from the soil through either plant pathway, which involves direct uptake of nutrients from the soil by root epidermis and root hairs, or with mycorrhizal pathway which involves the uptake of nutrients through the extra-radical mycelium of the fungus and transport to the Hartig net in ECM interactions (Bücking *et al*. 2012). The role of the plant or mycorrhizal pathway to P uptake depends on the plant and fungal species. ECM tree species (e.g. *Pinus*) are considered highly dependent on their fungal symbionts and predominately rely on the mycorrhizal pathway for nutrient absorption (Bücking *et al*. 2012). Accordingly, fungi hyphae uptake nutrients directly from the soil and deliver those nutrients to their host plants (Turk *et al*. 2006). In this case, it is suggested that low-diameter hyphae of fungi are efficiently capable of searching and uptaking the phosphorus in very fine soil pores (Bücking *et al*. 2012). The nutrient uptake, especially P uptake, from the soil through the plant pathway is often limited by the low mobility of nutrients in the soil (Bücking *et al*. 2012). ECM fungi can improve the ability of roots in nutrition absorption (especially P uptake) from the soil by changing the root shape and various biochemical characteristics of the host plant, as well as enlarging the scope of the rhizosphere (Lu *et al*. 2016). Moreover, ECM fungi increase the solubility of unavailable nutrients (Ahangar *et al*. 2012), which can indirectly help to absorb nutrients. For example, phosphatases and organic acids secreted by ECM fungi release phosphorus from organic complexes (Ezawa *et al*. 2005; Alvarez *et al*. 2011). ECM fungi can also synthesize many different hydrolytic enzymes such as protease, chitinase, glucosidase (Agerer 2001; Parrent *et al*. 2009) as well as mobilize and utilize amino acids and amides, such as glutamine, glutamate and alanine, which can represent a considerable N pool, especially in acid-organic soils (Smith & Read 2008). Furthermore, they are able to break down urea to liberate N into a usable form for plants by urease (Nehls *et al*. 2001; Morel *et al*. 2008). ECM fungi also can absorb inorganic N sources as ammonium (NH_4+) or nitrate (NO_3-) from the soil efficiently (Hawkins *et al*. 2000; Jin *et al*. 2005) although they prefer more NH_4^+ than NO_3^- (Bücking *et al*. 2012). According to the findings of this study, inoculated plants exhibited greater chlorophyll content than uninoculated ones. Co-inoculated treatments, especially *B. cereus* + *B. edulis*, were more effective in increasing the chlorophyll a and total chlorophyll. Other authors (Turgeman *et al*. 2011, Zamani *et al*. 2015, Chu *et al*. 2019) have also reported an elevated chlorophyll content in symbiotic plants with ECM fungi, which is in agreement with the results in the present study. The improvement of nutritional and environmental conditions increases the plant ability to produce chlorophyll (Smith & Read 2008). Nitrogen is one of the major components of chlorophyll, and the chlorophyll synthesis requires many elements such as N and P from soils (Li *et al*. 2018). Therefore, in the present study, the elevated chlorophyll content in the inoculated plants may be attributed to the upraised nutrient uptake, especially nitrogen. As mentioned earlier, the nutrients uptake rise was observed in plants inoculated with ECM or a combination of bacteria and ECM. Noteworthy, *B. edulis* alone as well as all combination treatments considerably increased plant height, root dry weight, and shoot dry weight compared to uninoculated plants. ECM fungi can improve root growth through increased production of root meristems and lateral root formation (Bücking *et al*. 2012). Moreover, the beneficial effects of ECM fungi contributing to their plant symbionts are related to the improvement of water and nutrient uptake from the soil (Akhzari *et al*. 2018), carbohydrate distribution (Rincón *et al*. 2007) along with production of phytohormones, including auxins and cytokinins (Bücking *et al*. 2012). Indole acetic acid (IAA) is an important phytohormone, which plays an important role in plant growth by affecting

many functional activities such as cell division, cell elongation, root initiation (Duca *et al*. 2014) and differentiation in the plant and stimulates Hartig' net development (Wagner *et al*. 2019). As mentioned earlier, MHB *Bacillus* in combination with ECM fungi improved colonization, nutrient uptake and growth parameters of Norway spruce seedlings dramatically, which can be in association with the intensification of root colonization by bacteria, the upraised secretion of growth-promoting compounds, and causing a higher abundance of nutrients in the rhizosphere (Bertrand *et al*. 2000; Ahangar *et al*. 2012). So, the role of some bacteria has been illustrated to accelerate the formation of mycorrhizal structures (Mediavilla *et al*. 2016). The effect of MHB is not always limited to mycorrhiza formation and/or functioning (Deveau & Labbé 2016). Some MHB also behaves as the Plant Growth Promoting Rhizobacteria (PGPR), through either direct or indirect effects on plant development and plant health (Martínez *et al*. 2018). So, it is noted that isolates of some bacteria (e.g. *Pseudomonas*, *Rhizobium*, *Serratia*, and *Bacillus*) are capable of producing multiple PGPR activities, such as auxin production, siderophore excretion, P solubilization, and N fixation (Acuña *et al*. 2020). Confirming the above tenet, Rojas-Solis *et al*. (2020) showed that single or co-inoculated tomato plants with two bacterial endophytes, *Bacillus* sp. E25 and *Bacillus* sp. CR71, under saline stress, exhibited an elevation in the root and shoot lengths, chlorophyll content, and biomass parameters, as well as produced IAA, proteases, siderophores, and biofilm. In addition, IAA synthesis has been reported by several *Bacillus* species, including *B. flexus* P4 and *Bacillus* sp. S6, which elevated root length in *Solanum tuberosum* (Ahmed & Hasnain 2010). Moreover, greater chlorophyll content in the inoculated plants suggested more production of photosynthetic products for the growth and development of root systems in the host plant (Lü & Wu 2017).

CONCLUSION

This is an original study on the ectomycorrhizal (ECM) symbiosis of Norway spruce in Iran. Three precious and edible ECM fungi, including *Cantharellus cibarius*, *Amanita caesarea*, *Boletus edulis* and mycorrhiza helper bacteria (*Bacillus cereus*) were used. The results suggested that successful symbiosis was established between the studied fungi species and Norway spruce. The colonization amount of *B. edulis* was higher than *A. caesarea* which in turn was higher than *C. cibarius.* Moreover, these results were the first reports to show the mycorrhization of *P. abies* with *A. caesarea*. Given the implication of this study, it could be concluded that co-inoculation of *B. cereus* with the selected ECM species, especially with *B. edulis* exhibited beneficial effects on mycorrhiza formation and plant performances of Norway spruce. Therefore, the findings of this study could be a promising step for mycorrhizal plant production in the future, whose progress depends on finding the best native microorganism in the plant's natural habitat.

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