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Effect of biocontrol agent *Bacillus subtilis* and mycorrhizal fungus *Glomus mosseae* on growth, mineral nutrition, physiological changes and essential oil quality and quantity of *Thymus daenensis* Celak

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Abstract

In order to study the effects of biocontrol agent (*Bacillus subtilis*) and Arbuscular mycorrhizal (*Glomus mosseae*) on the plant growth, peroxidase (POX) and phenylalanine ammonia-lyase (PAL) activity, nutrient content, essential oil yield and root colonization of *Thymus daenensis* transplants, a randomized complete block design with three replications and four treatments was carried out at the Glasshouse, in 2011-2012. Treatments were included: 1- *Glomus mosseae* 2- *Bacillus subtilis* 3- co-inoculation of *G. mosseae* and *B. subtilis* and 4- no inoculated. Results showed that the Co-inoculation with *G. mosseae* and *B. subtilis* resulted 74% increase in shoot /root dry weight, increased dry herb yield by about 30% and stimulated essential oil yield by 14 % compared to uninoculated controls. The percent of root colonization in the plants inoculated with *G. mosseae* was more than 7 times higher compared to non-inoculated plants, but dual inoculation resulted in a 68 % reduction in root colonization compared to single inoculation with *G. mosseae*. The Zn content significantly increased about three times higher than the control plants after dual inoculation. All microbial inoculation treatments significantly increased the concentration of Thymol and carvacerol in *T. daenensis* plants with respect to the control plants. This study further revealed that the single inoculation with *B. subtilis* significantly enhanced PAL and pox activity compared to the other treatments. Our findings confirm that plants Co-inoculated with *G. mosseae* and *B. subtilis* during the nursery stage, can create a more synergistic effect that supports thyme (*T. deneansis*) quality and quantity yields in a sustainable cultivation

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Introduction

The genus *Thymus* (Lamiaceae), are among the most popular plants throughout the world, commonly used for a long time as herbal teas, flavoring agents (condiment and spice), aromatic, and medicinal plants (Stahl-Biskup & Saez, 2002). Species of *Thymus* are well adapted to the dry climate of the Mediterranean region and widespread in the arid parts of Iran (Amin, 1991). *Thymus daenensis* Celak is an endemic species of *Thymus* that grows in the wild and recently cultivates in different regions of Iran. Within the genus *Thymus,* the influence of the abiotic environmental factors as well as biotic effects on the pattern of essential oil components has been investigated (Pluhar *et al.* 2007; Gouyon *et al.* 1986).

It is well accepted that crop yield and quality are affected by colonization of plant roots with non-pathogenic microorganisms in the rhizosphere (Glick, 1995). Arbuscular mycorrhizal (AM) fungi benefits to their host have been identified include: improved drought resistance (Auge´ *et al.*, 1994), enhanced pathogen resistance (Pozo *et al.*, 2002). Stimulated tolerance of salinity and heavy metals (Mohammad *et al.,* 2003). Increased uptake of macro and micronutrients (Clark and Zeto, 2000; Azaizeh *et al.*, 1995). Other important and beneficial root- microbes are the plant growth promoting rhizobacteria (PGPR) (Perotto and Bonfante, 1997). Their use as a biocontrol agent for sustainable plant protection has been published by some researchers (Suslow, 1982; Glick, 1995). Published results showing that Plants inoculated with the selected strains of non-pathogenic rhizosphere microorganisms can induce systemic resistance to biotic or abiotic stress. (Whipps, 2004, Pozo *et al.* 2005 and Conrath *et al.*, 2006) Limited research has been conducted to investigate the interaction of such microorganisms on the transplant growth, quantitative and qualitative of the secondary metabolites in medicinal plant. The objective of our present research was to evaluate, the effect of inoculation with a mixture of AMF and biocontrol agent on root colonization, plant growth, essential oil yield and quality of *Thymus daenensis* transplants.

Materials and methods

Experimental design

The study was carried out in a randomized complete block design with three replications. Treatments were included: (1- *Glomus mosseae* 2- *Bacillus subtilis* 3- co-inoculation of *G. mosseae and – B. subtilis* 4- no inoculated).

Growth conditions

The experiment was conducted in the Greenhouse condition of Semnan Agriculture and Natural Resources Research station. *T. daenensis* seeds were sown in 12 unsterile micro-plots (4 m^2areas in each plot) on temperatures ranging from 25 ± 3°C day to 15 ± 3°C night, a 16/8 h light/dark photoperiod and a relative humidity of 80–90%. The seeds treated with *B. subtilis* were inoculated with 3 ml of 1 × 10^7cfu/ml of bacterial suspension. For AM treatments 20 g/m^2 of inoculum containing *G. mosseae* colonized root fragments and spores and hyphae was mixed with seeds of *T. daenensis*. Seed rate was kept uniform for all treatments and when seedlings were 15 days old, thinning was done to maintain spacing of 10 cm between and within the rows. Mist irrigation was ensured only when necessary. The plants were allowed to grow and no fertilizer or pesticide was added to the soil during the experiment. Weeding was done at regular intervals. Soil samples were collected before the experiment and air-dried for chemical analysis (Table1).

Plant Growth and Mycorrhizal Colonization

After five months of growth 20 randomly chosen plants were harvested at flowering stage. Dry weights of root and aerial part and shoot/root dry weight was determined. A sub-sample (0.5 g) was taken from each root system to evaluate the percentage of root colonization by AMF. Root samples were cleared with 10% KOH and stained with 0.05% trypan blue in lactophenol as described by Phillips and Hayman (1970), and microscopically examined for AMF colonization by determining the percentage of root segments containing arbuscules, vesicles and hyphae using a grid line intercept method (Giovannetti and Mosse, 1980).

Analysis of Foliar Nutrient Concentrations

Nitrogen was estimated using Kjeltec 2300 after digesting the samples in digestion system (Foss-Tecator) and phosphorus and potassium content of the plants was estimated by vanadomolybdate phosphoric acid and flame photometric method respectively (Jackson, 1973). Magnesium, Zink and manganese were estimated by atomic absorption spectra- photometer (GBC-Avanta PM) using nitrous oxide–acetylene and air–acetylene flame, respectively (Jackson, 1973).

Determination of enzymes special activity

Leaf samples were finely ground in liquid nitrogen and suspended in 100 mm potassium phosphate buffer pH 6.0 at a ratio of 750 µL to 0.1 g tissue. The homogenate was centrifuged at 10,000 rpm for fifteen minutes (4°C), the pellets were discarded. Supernatants were analyzed for their protein content (Bradford, 1976) using bovine serum albumin as a standard protein. Measurement was carried out at 595 nm, and kept at -80°C as a crude extract for further enzymatic assay. Phenylalanine ammonia-lyase activity was assayed according to the method described by D'cuncha (1996). The reaction mixture containing 50 µl crude extract and 2.9 ml of 100 mm L-phenylalanine in 100 mm TrisHCL buffer (pH 8.5), was incubated at 37°C for an hour. The reaction was terminated by the addition of 50 µL of 6N HCl. The reaction mixture without a crude extract was used as a blank for the spectrophotometric determination at 290 nm. The enzyme activity was calculated by a standard curve constructed with trans-cinnamic acid and a unit of phenylalanine ammonia-lyase activity represented the amount of enzyme that yielded 1 µmol of cinnamic acid per hour. Peroxidase activity was assayed spectrophotometrically at 470 nm in 0.28% (v/v) Guayaquil, 0.3% (v/v) hydrogen peroxide (Putter, 1965).

Essential oil analysis

For essential oil analysis, air-dried and ground plant material (100 g) was submitted to water distillation for 2.5 h using an all-glass Clevenger-type apparatus as recommended by the European Pharmacopoeia (Anonymous, 1996). The obtained essential oil was dried over a hydrous sodium sulphate and after filtration, stored in an amber vial at low temperature (4°C) prior to analysis. The oil obtained from aerial parts of *T. daenensis* was analyzed using a Shimadzu DB5-9A gas chromatograph. Phenyl methyl siloxane capillary column (30 m _ 0.25 mm, 0.25 µm film thickness; DB-5) equipped with a FID detector. The oven temperature was maintained at 60°C for 3 min initially, and then raised at the rate of 20°C/min to 240°C. Injector and detector temperatures were set at 280 C° and 300°C, respectively. Helium was used as the carrier gas at a flow rate of 1.5kg/cm^2. The peaks are a percent was used for obtaining quantitative data. GC/MS analysis of the oil was carried out on a Varian -3400 gas chromatograph equipped with a Saturn II, GC/MS detector in Ion trap mode operating under the same conditions as described above. Retention indices were calculated for all components using a homologous series of normal-hydrocarbons (C_7-C_{25}) injected in conditions equal to sample ones. Identification of the components of essential oil was based on retention indices (RI) relative to normal-hydrocarbons and computer matching with the different Libraries, as well as comparisons of the fragmentation pattern of the mass spectra with data published in the literature (Adams, 2001).

Statistical analysis

The data analyses of variance were done by using MSTATC program. Duncan's Multiple Range Test ($P \leq 0.05$) were used to compare means within treatments. Pearson Correlation (2-tailed) between the measured parameters was done by SPSS program.

Results

Root colonization (%)

In the present work *T. daenensis* exhibited excellent mycorrhization. The percent of root colonization of *T. daenensis* inoculated with *G. mosseae* alone (80.67%) or dual inoculated with *B. subtilis* and *G. mosseae* (48%) was higher than in plants treated with *B. subtilis* (7.97%) or control plants (13.04%) (Table 5).

Dry herb yield (g/m^2)

Single inoculation of *B. subtilis* or *G. mosseae* decreased dry herb yield significantly in comparison to the non-inoculated plants. However, dual inoculation significantly enhanced the dry herb yield in comparison to the other treatments (Table 2 & 5).

Table 1. Physical and chemical properties of the soil.

Total Nitrogen (%)	Available Phosphorus (ppm)	Available Potassium (ppm)	EC ($dS.m^{-1}$)|	pH|
0.09	10.08	386.19	4.07	7.79

Dry Shoot/ root

Treatment with *G. mosseae* alone also significantly increased the shoot/root ratio (14.42) but the maximum of it was recorded in dual inoculation (21.02) treatments (Table 2 & 5).

Root dry weight (g. plant^{-1})

Single inoculation with *G. mosseae* significantly enhanced the total root dry weight of *the T. daenensis* plants. Significant decreasing of root dry weight after dual inoculated treatment was recorded (Table 2 & 5).

Table 2. ANOVA results for plant growth and root colonization of *T. daenensis* plants exposed to different bioinoculants.

		Mean of Squares			
Source of Variation	df	Root dry weight	Dry Shoot/root	Dry herb yield	Root AM Colonization
Replication	2	0.0001	1.751 ns	1592.105 ns	14.773 ns
Treatments	3	0.003**	52.836**	48147.42**	3443.583**
Error	6	0.0001	1.371	429.795	13.837
CV%		10.66%	7.84%	6.2%	9.94%

ns: nonsignificant , * and **: Significant at 5% and 1% probability levels, respectively.

Table 3. ANOVA results for quality and quantity of essential oil and enzyme activityof T. daenensis plants exposed to different bioinoculants.

		Mean of Squares					
Source Variation	of df	PAL	POX	Oil content	Oil yield	Thymol	Carvacrol
Replication	2	0.004 ns	0.156 ns	0.036 ns	2.002 ns	19.388 ns	0.652 ns
Treatments	3	0.173**	2.012**	0.028 ns	13.706**	103.192*	3.967**
Error	6	0.018	0.17	0.019	0.78	14.92	0.066
CV%		10.82	10.45	7.93%	15.03%	6.11%	3.68%

ns: Non-significant , * and **: Significant at 5% and 1% probability levels, respectively.

Mineral nutrients

In general, inoculation decreased the N in the leaves. The K concentrations of leaves were significantly lower in dual inoculation than in the control plants. The P concentrations were significantly increased in the leaves after single inoculation with *B. subtilis* (0.99%) and after Dual inoculation (0.89%) compared to control plants (0.56%). The Zn content significantly increased about three times higher than the control plants after dual inoculation (Table 4 &7). The dry herb yield of *T. daenensis* plants showed

positive correlation with the Zn concentration in the leaves (Table8).

PAL special activity

This study further revealed that the Single inoculation with *B. subtilis* significantly enhanced PAL special activity compared to the other treatments (Table 3& 6).

POX special activity

Treatment with *B. subtilis* alone caused a significant increase in POX special activity (3.27U. mg protein^{-1}) in the leaves of plants. The lowest POX activity was observed after single inoculation with *G. mosseae* (1.34U. mg protein^{-1}). Combined treatment with *G. mosseae* and *B. subtilis* also significantly decreased POX activity (2.15U. mg protein^{-1}) compared to the non-inoculated plants (2.69U. mg protein^{-1}) (Table 3& 6).

Table 4. ANOVA results for nutrient uptake by *T. daenensis* plants exposed to different bioinoculants.

		Mean of Squares			
Source of Variation	df	N	K	P	Zn
Replication	2	0.45	0.033	0.001	679.419
Treatments	3	3.629^{ns}	0.335^{ns}	0.124**	1236380**
Error	6	0.213	0.06	0.009	1300.222
CV%		9.69%	9.8%	12.68%	8.19%

ns: Nonsignificant , * and **: Significant at 5% and 1% probability levels, respectively.

Table 5. Effect of bioinoculants on mean plant growth and root colonization (%) in *T. daenensis* plants.

Treatments	Root dry weight (g. plant^{-1})	Dry Shoot/root	Dry herb yield (g/m^2)	Root AM Colonization(%)
Control	0.11 b	12.07 c	370.2 b	13.04 c
B. subtilis	0.1067 c	12.22 bc	178.3 d	7.975 c
G. mossea	0.15 a	14.42 b	307.2 c	80.67 a
G. mossea + B. subtilis	0.08 d	21.02 a	481.9 a	48 b

Means in each column followed by similar letter(s), are not significantly different at 5% probability level, using Duncan's Multiple Range Test.

Essential Oil content (%)

There was no significant difference in essential oil content of inoculated plant and non-inoculated plants (Table 3).

Essential Oil Yield (g/m^2) and quality

Treatment with *B. subtilis* alone decreased the essential oil yield of *T. daenensis* compared to control plants. Combined treatment with *G. mosseae* and *B. subtilis* significantly increased the essential oil yield comparison to the single inoculated treatment. In general, inoculation increased the Thymol (20-22%) and Carvacrol (28-46%) content of essential oil in the *T. daenensis* compared to control plants (Table 3 & 6).

Table 6. Effect of bioinoculants on mean enzyme activity, oil yield (g/m2 and concentration (%) of selected constituents in *T. daenensis* plants.

Treatments	PAL (U. mg protein^{-1})	POX (U. mg protein^{-1})	Oil yield (g/m^2)	Thymol (%)	Carvacrol (%)
Control	1.213 b	2.697 b	7.03 ab	54.4 b	5.39 c
B. subtilis	1.533 a	3.27 a	3.15 c	66.76 a	7.757 a
G. mossea	0.946 b	1.347 d	5.3 b	65.69 a	7.89 a
G. mossea + B. subtilis	1.213 b	2.15 c	8.023 a	65.83 a	6.89 b

Means in each column followed by similar letter(s), are not significantly different at 5% probability level, using Duncan's Multiple Range Test.

Table 7. Effect of bioinoculants on mean nutrient uptake by *T. daenensis* plants.

Treatments	N (%)	K (%)	P (%)	Zn (mg. kg^{-1})
Control	6.3 a	2.623 a	0.5667 b	132.8 b
B. subtilis	3.687 b	2.5 ab	0.9933 a	140.7 b
G. mossea	4.527 b	2.833 a	0.5967 b	85.78 b
G. mossea + *B. subtilis*	4.533 b	2.043 b	0.84 a	403 a

Means in each column followed by similar letter(s), are not significantly different at 5% probability level, using Duncan's Multiple Range Test.

Table 8. Pearson Correlation(2-tailed)between root colonization and dry herb yield with the other measured parameters on *Thymus daenensis* plants.

	Root colonization	Dry herb yield	Root dry weight	N	P	K	Zn	Oil content	Thymol	Carvacerol	PAL	POX
Root colonization	1	0.299	0.472	-0.151	-0.371	0.049	0.170	-0.362	0.341	0.442	-0.780**	-0.857**
Dry herb yield	0.299	1	-0.428	0.482	-0.290	-0.318	0.709**	0.070	-0.165	-0.352	-0.350	-0.364

**Correlation is significant at the 0.01 level (2-tailed).

Discussion

In the present work inoculation of *T. daenensis* with *G. mosseae* or/and *B. subtilis* was investigated for the first time. It was evident from this experiment that *B. subtilis* decreased the root colonized by *G. mosseae*. Increased POX activity in *T. daenensis* plants after *B. subtilis* inoculation could play a role in the root wall reinforcement and probably limiting root colonization. Liszkay *et al* (2004) obtained similar findings in maize roots. The root colonization did not show significant correlation with the total yield production in *T. daenensis* plants (Table 8). This result is in conformity with the earlier findings of Plenchet *et al* (1982) and Baum *et al* (2006), they found that the percent of colonization of host roots by AM fungi was not correlated with benefits to the plants. In the present study, a decreased growth after single inoculation with *B. subtilis* or *G. mosseae* was observed. However, Dual inoculation greatly increased the total dry yield, shoot/ root ratio and P and Zn concentration in *T. daenensis* leaves. This synergistic effect is in conformity with the findings of Gryndler *et al* (2002) in strawberry plant and Medina *et al* (2003) in *Medicago sativa*. Garbay (1994) suggested that *Bacillus* spp, could produce phytohormones and play a helper role in the plant-fungus interaction. In the experiment of Azcon *et al* (2010) a promoted of the plant growth after dual inoculated with AM fungi and *B. cereus* has been shown. Results from our present study showed that in *T.deneansis* plants enhanced total yield was correlated with elevated Zn concentration in the leaves. Such an increased P and Zn uptake due to dual inoculation with AM fungi and PGPRs was reported by lakshmipathy *et al* (2002). Zn content in calcareous soil is less than sufficient level. Under Zn limitation, uptake of it is improved by the AM fungi symbiosis (Chen *et al* 2003). In our study the co-inoculation of *G. mosseae* and *B. subtilis* might be due to facilitate soil acidification of the rhizosphere, improved Zn concentrations in *T.deneansis* plants. This result is in conformity with the findings of Subramanian *et al* (2008; 2009). In the present experiment, plants inoculated with *B. subtilis* displayed a more antioxidant (POX) and defense enzyme (PAL) activity, which may indicate the mechanism of bacterial-elicited induced systemic resistance in *T.deneansis* plants, as observed by Bargabus *et al* (2002) and Saravanakumar *et al* (2007). In this study, single inoculation with *G. mosseae* or dual inoculation decreased POX activity in plants. Similarly, Fries *et al* (1996) showed that, inoculation of *Zea mays* L. with *G. intraradices* decreased POX activity during the full establishment of the AM symbiosis. In this study, dual inoculation with *G. mosseae* and *B. subtilis* stimulated essential

oil yield of *T. deneansis* because of the increase in biomass production. Similar synergistic effect on essential oil yield has been reported after dual inoculation with PGPR and AM fungi by Singh *et al* (2012) and Alam *et al* (2011). The production of metabolites and phytohormones by *Bacillus* spp is one of the mechanisms expected to be positively affected AM functions (Dwivedi *et al*, 2009 and Kohler *et al,* 2007). In the present experiment, the quality of essential oil has improved on all microbial inoculations. This result is in conformity with the finding of Alam *et al* (2011) in geranium plants and Kapoor *et al* (2004) on *Foeniclum vulgar*.

Conclusion

This investigation indicates the pre-inoculated transplants of *T. deneansis* with appropriate combination of biocontrol agents and AM fungi without chemical pesticides or fertilizers, can decrease deleterious effects of single inoculation and create a more synergistic effect that supports thyme (*T. deneansis*) quality and quantity yields in a sustainable cultivation.

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