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Potential effect of *Piriformospora indica* on plant growth and essential oil yield in *Mentha piperita*

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Abstract

Pot culture experiments were used to evaluate the inoculation of peppermint plants (*Mentha piperita*) with *Piriformospora indica.* The effect of inoculation was observed in growth, yield, and composition of the essential oil of peppermint. Hydro distillation was used to extract oil from the dry matter of the shoot. Subsequently, GC/MS was used to determine their composition. The largest plant heights, dry shoot and root weights, and numbers of nodes were observed in pots that were inoculated with *P. indica*. The highest essential oil yield was obtained with *P. indica*-inoculated plants. GC and GC/MS revealed that *P. indica* enhanced menthol levels. T-test analysis showed that differences between treatment with *P. indica* inoculation and control were significant in root length, shoot dry weight, number of nodes and essential oil yield.

Key words – Labiatae – peppermint – symbiosis

Introduction

A member of the Labiatae family, peppermint (*Mentha piperita* L.) is a sterile natural hybrid of *M. aquatica* × *M. spicata* (Tucker 1992). Among the ailments treated by this plant are nausea, stomach cramps, menstrual cramps, indigestion, vomiting, flatulence, and parasitosis (Fonseka-Kruel & Fernandes 2003). Peppermint also possesses stimulant, carminative, antiseptic, antispasmodic, anti-inflammatory, antibacterial and antifungal properties (Guedón & Pasquier 1994, Gershenzon et al. 2000, Inoue et al. 2002, Samarth & Kumar 2003, Ruiz del Castillo et al. 2004, Duarte et al. 2005). *Mentha piperita*, as is the case for other members of the same plant group, produces various metabolites including tannins, flavonoids, terpenes, and phenolic acids (Guedón & Pasquier 1994). Some identified compounds have been discovered as possessing antimicrobial properties including linalool, 1, 8-cineole, limonene, and menthol (Mazzanti et al. 1998, Iscan et al. 2002).

Piriformospora indica, a filamentous fungus, belongs to the order Sebacinales (Verma et al. 1998). The development of axenically cultivable *P. indica* has opened the door for the study of plant-fungi interactions. Cultivated in vitro and applied to plant hosts in controlled experiments, it is now possible to closely analyze fungi influence on plant morphogenesis and secondary

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metabolism. *P. indica* is able to colonize the roots of both mono- and dicotyledonous plants (Pham et al. 2004, Verma & Arya 1998).

P. indica colonization can enhance crop plant yield by increasing the vegetative tissue yield, the number of inflorescences and flowers (Rai et al. 2001, Dolatabadi et al. 2011a), and the average seed weight (Rai et al. 2001, Peskan-Berghofer et al. 2004, Barazani et al. 2005). Chemical analyses revealed increased concentrations of several compounds in *P. indica*-colonized plants such as the antifungal spilanthol in *Spilanthes calva* (Rai et al. 2004), pharmaceutically relevant compounds such as podophyllotoxins from *Linum album* (Baldi et al. 2010), saponin from *Chlorophytum* sp. (Gosal et al. 2010) or asiaticoside from *Centella asiatica* (Satheesan et al. 2012) and amount of the essential oils in *Thymus vulgaris* and *Foeniculum vulgare* (Dolatabadi et al. 2011a, b, Franken 2012). In this study, we investigated the effect of *P. indica* on the performance of *M. piperita* in pot culture experiment.

Materials & Methods

Fungal culture

Kaefer's medium was used to grow *P. indica* in petri dishes (Kaefer 1977). The fungus was used to inoculate the plates and then plates were incubated at 25±1 °C for a week.

In vitro stock plant

Stem node explants excised from the Institute of Medicinal Plants and Natural Products Research Karaj, Iran were used to create stock cultures. These explants were washed in running water. To surface sterilize, explants were immersed for 5 minutes in 70% ethanol then soaked for 10 minutes in sodium hypochlorite (1%) with 0.01% Tween 20. The explants were rinsed four times in sterile distilled water.

Nodes were placed in an MS medium (Murashige & Skoog 1962). Added to this medium were 30 g/L sucrose, 1 mg/L thiamine, and 8 g/L agar. Prior to autoclaving for 20 minutes at 120 °C the pH was adjusted to 5.8. The explants were stored in a growth chamber under cool white fluorescent lamps that retained a constant temperature of 25 °C and a 16:8 light/dark photoperiod regime.

In vitro plant inoculation

For in vitro plant inoculation experiments, 30-day-old micropropagated plants provided the source of apex. A 5 mm diameter mycelial disc of 7-day-old culture of *P. indica* was used to inoculate the transparent culture jars destined for plant-fungus co-culture. Each culture jar contained 50 ml of MS medium. Upon completion of the inoculation, peppermint shoots were removed from just behind the second pair of the upper leaves and then were placed into fungal discs.

Pot experiment

The plant material employed in the experiment for in vivo plant inoculation was micropropagated plants from in vitro cultures.

Following 20 days, micropropagated plants that were inoculated with *P. indica* were transferred to each pot culture and grown in a 2:1:1 sterile mixture of sand, peat, and perlite, with the following chemical properties: EC 0.9 ds/m, pH 7.12, total nitrogen 0.3%, organic carbon 0.7%, available phosphorus 8.2 mg/kg and potassium 285 mg/kg in green house at 24/18 °C day/night temperature. The photoperiod ranged from 16 h of light to 8 h of dark and a 7% formaldehyde solution was used to fumigate the soil.

The experiment was done with three shoots inoculated with *P. indica* and three non-inoculated shoots in a completely randomized design. Plants grown in pots were analyzed after 75 days. Using the methods described by Phillip & Hayman (1970) and Dickson et al. (1998),

segments of the plant were stained. Using a magnification of 10–40×, root pieces were analyzed with a microscope.

Essential oil analysis

Depending on the type of culture, oil was extracted from 15 g of the dry material through hydrodistillation in a Clevenger-like device for up to 2 hours. Anhydrous sodium sulfate was used to dry the product which was then stored in vials at 4°C until their essential oils were analyzed. The essential oils obtained from different treatments were analyzed using gas chromatography (Thermo-UFM Model) equipped with a Ph-5 capillary column (5% dimethylsiloxane phenyl, 10m length, 0.1 mm i.d. and 0.25 µm film thicknesses) and FID detector. The column was programmed as follows: carrier gas, helium 0.5 ml/min; injection temperature 280 °C increased to 200 °C at 5°C/min; injector temperature, 180 °C; detector temperature, 220 °C. The oven temperature was run from 60 °C to 285 °C at the rate of 80 °C /min. For the GC-MS analyses, samples were analyzed using a Varian 3400 gas chromatograph attached to a Saturn II mass spectrometer operating in electron impact ionization mode (70 eV). The compounds were separated on DB-5HT column (30 m length, 0.25 mm i.d., and 0.25 µm film thicknesses). The column temperature was raised from 40 ºC to 250 ºC at a rate of 4 ºC/min; injector temperature, 260 ºC; and MS transfer line, 270 ºC. The components were identified by Kovats indices (KI) which compared relative to C7-C25n-alkanes and mass spectra with authentic standards as well as spectral data from library files and literature (Shibamoto 1987, Adams 1989, Davies 1990).

Statistical analysis

SAS 6.12 was used to statistically calculate the gathered data. Significant differences between treatments were determined using the T test.

Results

A high rate of colonization and the production of many chlamydospores in root cells were observed upon microscopic inspection of roots inoculated with *P. indica* (Fig. 1).

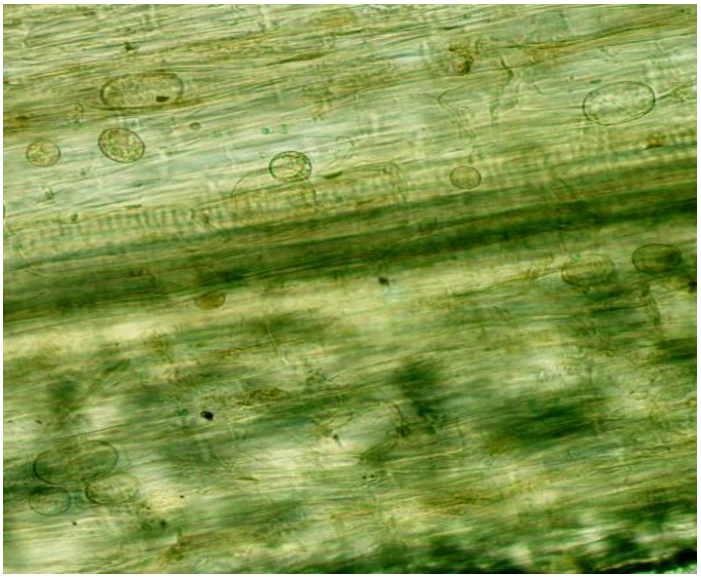

Fig. 1 – Detection of chlamydospores of *Piriformospora indica* in root cells of *Mentha piperita*.

T-test analysis showed that differences between treatment with *P. indica* inoculation and control were significant in root length, shoot dry weight, number of nodes and essential oil (Table 1, $P < 0.05$).

Table 1 Effect of endophytic fungi on the growth characteristics of *Mentha piperita* plants grown under greenhouse condition.

Parameter	*Piriformospora indica* Mean±SD	Control Mean±SD	T-test
Plant height (cm)	51.47 ± 8.62	42.60 ± 4.67	ns
Root length (cm)	31.33 ± 4.62	19.66 ± 4.27	*
Shoot dry weight (g)	7.02 ± 1.20	4.2 ± 0.99	*
Root dry weight (g)	1.41 ± 0.33	0.90 ± 0.17	ns
Number of nodes	252.4 0 ± 17.55	186.73 ± 20.08	*
Essential oil	0.78 ± 0.1115	0.56 ± 0.0473	*

T-test: *P ≤ 0.05; **P ≤ 0.001; ns not significant.

After 75 days, plants that were grown in cultures were analyzed. Increased vigour, extended leaves, thicker stems, and more buds and stolons were the characteristics of plants that were inoculated with *P. indica* during the experiment. *P. indica* inoculated plants were the tallest at 51.47 cm. The control plants were at 42.6 cm.

Similarly, *P. indica* inoculated plants possessed the longest roots (31.33 cm), while control plants possessed the shortest roots (19.66 cm) (Table 1). Likewise, measurements recorded of dry shoot weight showed differences between two treatments. Those plants that were co-cultured with *P. indica* showed the increase of the shoot dry weight as much as 67% compared to control plants. Generally, *P. indica* inoculated plants produce the highest dry root weights while control plants produced the lowest dry root weights.

Additionally, *P. indica* inoculation showed the potential of increasing the total number of nodes. The average number of nodes increased 35% when inoculated with *P. indica* (252.4) compared to the control plants (186.73) (Table 1).

Essential oil concentration also increased in pot cultures that were inoculated with *P. indica*. Essential oil yield, both the highest (0.78% w/w) and the lowest (0.56% w/w), were observed with *P. indica* and the control (Table 1).

The gas chromatographs and essential oil compositions of *M. piperita* are shown in Fig. 2 and Table 2. The major constituents of the essential oil in *P. indica*-inoculated, and control plants were isomenthone (30.62 and 30.94%, respectively), menthol (28.5 and 21.48%, respectively), piperitenone (18.18 and 19.18%, respectively), menthofuran (4.68 and 3.18%, respectively) and p-mentha-3,8-diene (2.86 and 4.53%, respectively) (Table 2).

Discussion

Soil microorganisms can promote plant growth by transferring soil organic matter, producing plant growth regulators (PGRs), mobilizing inorganic nutrients, and acting antagonistically towards pathogens via several mechanisms (Bolton et al. 1993).

According to our results, peppermint plants are cognizant of the fitness benefits that come with association with members of the Sebacinales fungus. *P. indica* inoculation caused a complete plant reaction. Not only does *P. indica* colonize the roots of a wide variety of plant species, but also it promotes their growth and development (Sahay & Varma 1999, Kumari et al. 2003, Peskan-Berghofer et al. 2004). This study demonstrated that those plants treated with *P. indica* developed more efficiently in plant height, root length, plant and root dry weight, and essential oil yield than control plants that were not treated with *P. indica*. *P. indica* inoculation could significantly increase the root length, shoot dry weight, number of nodes and essential oil yield compared to control plants. The more intense proliferation in inoculated plants might be due to synthesized phytohormones (Singh et al. 2000, Varma et al. 2001). *P. indica* produces small amounts of auxins

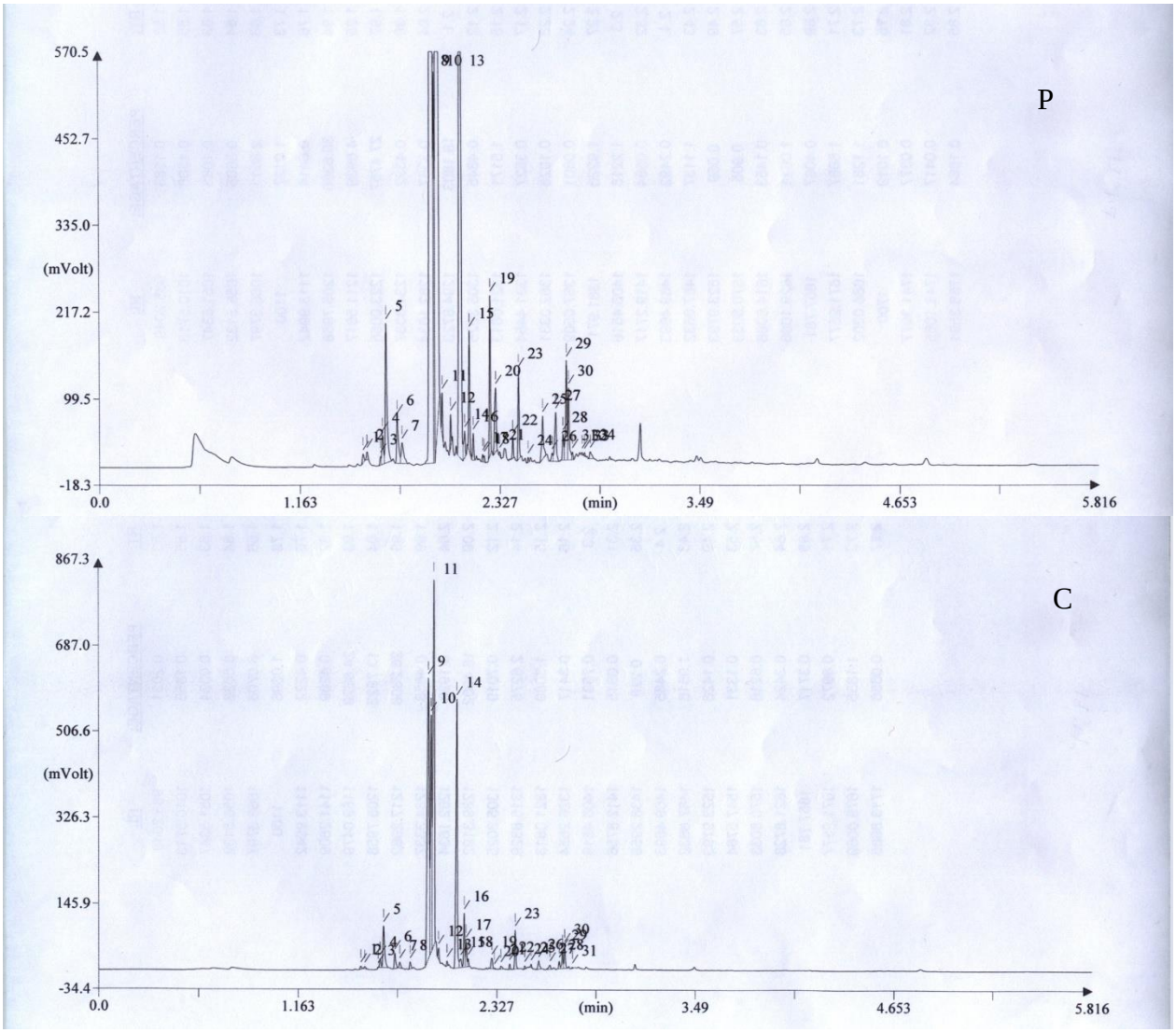

Fig. 2 – Effect of *Piriformospora indica* (P) in chromatogram for peppermint oil (*Mentha piperita*). C: control.

and relatively large amounts of cytokinins. The cytokinins levels are higher in colonized roots relative to the uncolonized roots of the control plants (Vadassery et al. 2008). Root growth and at least some of the effects of *P. indica* on the host plants are derived from the production of auxin (Sirrenberg et al. 2007). Also, differences in growth may have been caused by greater absorption of water and mineral nutrients due to extensive colonization of roots. Varma et al. (1999) reported that plants inoculated with *P. indica* showed the promotion of plant growth and biomass production. Rai et al. (2001) showed that in the presence of *P. indica*, root length, biomass, basal stem, leaf area, overall size, and the production of flowers and seeds of *Spilanthes calva* and *Withania somenifera* increased. The amount of menthol was higher in *P. indica*-inoculated plant. As noted by Mucciarelli et al. (2003), endophytic co-habitation affected the growth of peppermint while decreasing the concentration of menthofuran and increasing the concentration of menthol. In vitro and in vivo cultures of *Thymus vulgaris* showed that the plant height, root length, fresh and dry weight (plant and root), number of shoot nodes, oil yield, and the percentage of thymol were all improved with *P. indica* inoculation (Dolatabadi et al. 2011a). The maximum dry weight of the green tissue and root and plant height, maximum number of umbels and dry weight of 1000 fruits were obtained with *P. indica*. The *P. indica* inoculation significantly increased oil yield as compared to non-inoculated control plants. GC and GC/MS studies revealed that the level of anethole was increased with *P. indica* (Dolatabadi et al. 2011b, Oberwinkler et al. 2013). In the

present study, our observations and data showed that *P. indica* could affect growth and increase essential oil levels of peppermint.

Table 2 Effect of *Piriformospora indica* inoculation on concentration (%) of various constituents in *Mentha piperita.*

Essential oil components	Treatments	
	Piriformospora indica	**Control**
γ - terpinene	0.58	0.46
p-mentha-3,8-diene	2.86	4.53
Linalool	1.04	3.14
Pulegol	0.62	0.56
Isomenthone	30.62	30.94
menthofuran	4.68	3.18
menthol	28.50	21.48
pulegone	0.43	3.23
piperitenone	18.18	19.18
isomenthyl acetate	0.52	0.50
neoiso-isopulegol acetate	1.49	1.38
piperitenone oxide	0	1.18
geranyl acetate	2.88	0
longifolene	1.18	1.36
germacrene D	1.14	1.02
β- bisabolol	1.56	1.87
longiborneol	1.08	4.54

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