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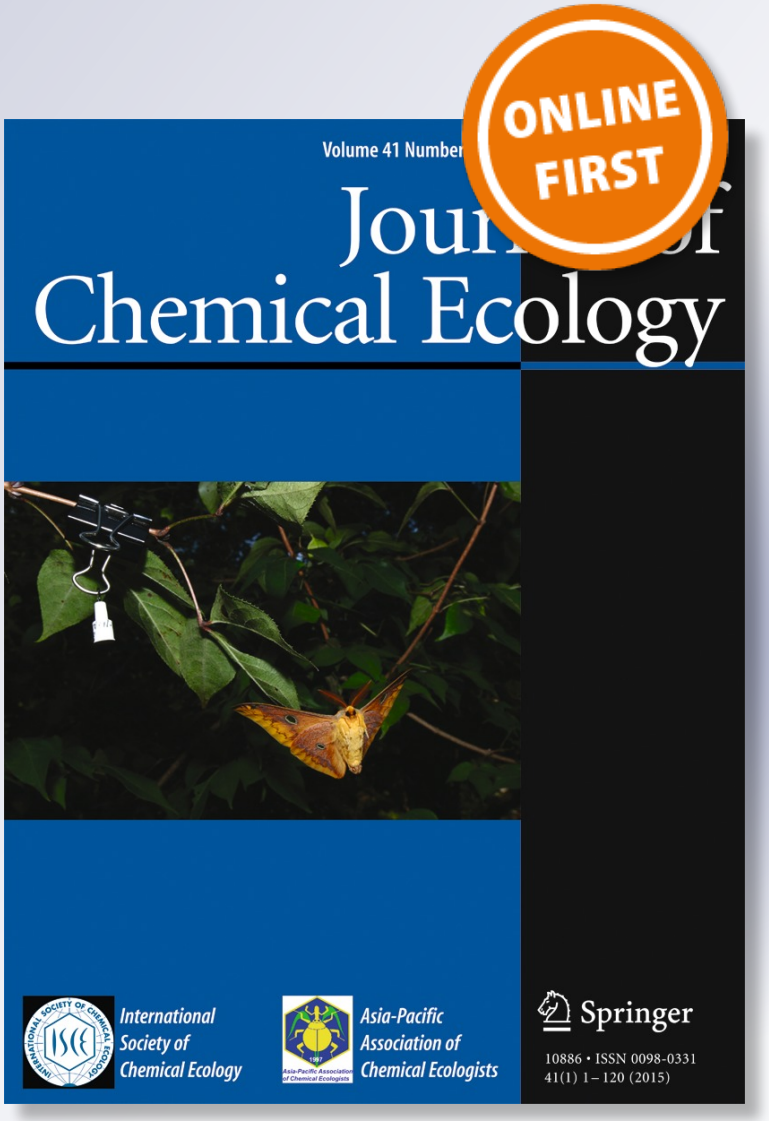

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Anatomical, Morphological, and Phytochemical Effects of Inoculation with Plant Growth- Promoting Rhizobacteria on Peppermint (*Mentha piperita*)

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Abstract Plant growth-promoting rhizobacteria (PGPR) generally exert their effects through enhancement of plant nutrient status and/or phytohormone production. The effects of PGPR on aromatic plant species are poorly known. We measured plant growth parameters, chlorophyll content, trichome density, stomatal density, and levels of secondary metabolites in peppermint (*Mentha piperita*) seedlings inoculated with PGPR strains *Bacillus subtilis* GB03, *Pseudomonas fluorescens* WCS417r, *P. putida* SJ04, or a combination of WCS417r+ SJ04. The treated plants, in comparison with controls, showed increases in shoot biomass, root biomass, leaf area, node number, trichome density, and stomatal density, and marked qualitative and quantitative changes in monoterpene content. Improved knowledge of the factors that control or affect biosynthesis of secondary metabolites and monoterpene accumulation will lead to strategies for improved cultivation and productivity of aromatic plants and other agricultural crops without the use of chemical fertilizers or pesticides.

Keywords Glandular trichome · Mint · Secondary metabolites · Stomatal density · Lamiaceae sustainable agriculture

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Introduction

Peppermint (*Mentha*×*piperita*) is cultivated worldwide for production of essential oils (EOs) and fresh or dried herbs, and is one of the most important EO crops (Lawrence 2007).

Fresh and dried peppermint herbs are used for teas and for flavoring of foods and beverages. Peppermint EOs are used extensively as aromatic agents (*e.g.*, in chewing gum, candy, toothpaste, mouthwash, and aromatherapy), pharmaceuticals, antimicrobial agents, and ecofriendly pesticides (MIRC 2010). Herbs, extracts, and EOs of peppermint and spearmint have a long history of medicinal usage for therapy or symptomatic treatment of numerous human diseases and disorders (Zheljazkov *et al*. 2010).

Aromatic plants belonging to the family Lamiaceae are economically important because of their EO production. The EOs are produced exclusively by glandular trichomes (McCaskill and Croteau 1995), which are epidermal structures covering the aerial portion of the plant.

There are two types of glandular trichomes, termed peltate and capitate, located on both the upper and lower leaf surfaces (Werker 2000). Peppermint (*M. piperita*) has both types. Only the peltate glandular trichomes accumulate monoterpenes. These trichomes contain secretory cells that are responsible for oil synthesis. Nascent EO is secreted into an emerging cavity formed by the separation of a preformed layer of cuticular material (Rios-Estepa *et al*. 2010). The density of glandular trichomes on leaves in the genus *Origanum* (oregano, marjoram) has been positively correlated with plant EO content (Bosabalidis 2002). Intensive farming practices for the purpose of high crop yield and quality traditionally involve the extensive use of chemical fertilizers, which are expensive and have negative environmental impacts. During the past few decades, there has been steadily increasing study and

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utilization of environmentally safe, sustainable, and organic agricultural food and feed production strategies that reduce negative environmental effects (Lind *et al*. 2004). "Organic agriculture" is a production system that avoids or minimizes the use of synthetic fertilizers, pesticides, and growth regulators, relying instead on biofertilization, crop rotation, crop residues, mechanical cultivation, and biological pest control to maintain soil productivity. Reduced yield is a major problem and concern in organic production systems (Lind *et al*. 2004). For the numerous medicinal and aromatic plant species that are consumed without further processing, it is important that no synthetic compounds be present in the harvested crop.

Many species of bacteria, most of which are found in the rhizosphere (the narrow region of soil associated with the roots of plants), have beneficial effects on plant growth and on crop yield and quality. Such bacteria, collectively termed "plant growth-promoting rhizobacteria" (PGPR), promote plant growth through both direct and indirect mechanisms (Kloepper 1993; Niranjan *et al*. 2006; Van Loon 2007). Direct mechanisms of PGPR include production of stimulatory bacterial volatile organic compounds (VOCs) (Wenke *et al*. 2010) and phytohormones, reduction of plant ethylene level, and enhancement of plant nutrient status (release of phosphates and micronutrients from insoluble sources; nonsymbiotic nitrogen fixation) (Van Loon 2007). Indirect effects of PGPR include functioning as biocontrol agents to reduce diseases, based on enhancement of disease-resistance mechanisms (induced systemic resistance) (Bakker *et al*. 2007), promotion of other beneficial symbioses, and protection of plants by degrading xenobiotics in contaminated soils (Figueiredo *et al*. 2010). Depending on the PGPR species, two or more of the above growth-promoting mechanisms may be present (Vessey 2003).

Enhancement of plant growth parameters and secondary metabolic responses in aromatic plants often result from inoculation of beneficial microbes. The effects of PGPR inoculation on sweet marjoram, Italian oregano, sweet basil, and wild marigold vary depending on the inoculated strain. PGPR-inoculated plant species display host response specificity (Banchio *et al*. 2009, 2010; Cappellari *et al*. 2013). In most studies, the growth parameters evaluated were increased significantly by *P. fluorescens* inoculation. Essential oil yield was increased to varying degrees. Studies of plant interactions involving arbuscular mycorrhizal (AM) fungi in aromatic plants haveshown increases of plant growth and EO production (Copetta *et al*. 2006; Khaosaad *et al*. 2006; Zeng *et al*. 2013). Mycorrhization allows the root system to exploit a greater volume of soil by extending the root zone, reaching small soil pores not accessible by root hairs, and acquiring organic phosphates through production of extracellular acid phosphatases (Bouwmeester *et al*. 2007).

Another strategy for exploiting microbial potential is to combine the attributes of different microbe strains to achieve an outcome that encompasses numerous or complementary beneficial effects (Lucy *et al*. 2004). Microorganisms display varying degrees of cooperation/ mutualism whereby they benefit themselves or other organisms, and positive outcomes are enhanced (Vessey 2003). Recent studies have addressed the factors that promote or regulate EO production (Chalchat *et al*. 1997; Hudaib *et al*. 2002), and the inhibitory effects of various abiotic stresses on EO production (Karousou *et al*. 1998; Panou-Filotheou *et al*. 2001). Many experiments have addressed the role of PGPR inoculation in aromatic plants (Banchio *et al*. 2009; Cappellari *et al*. 2013; Santoro *et al*. 2011). However, there have been no comparative studies on the role of inoculation and co-inoculation with native strains on plant growth at the morphological or anatomical level, or on production of secondary metabolites (particularly monoterpenes) in commercially important aromatic plants. It has been suggested that indigenous isolates are preferred in selection of bacteria for inoculation of crop plants, because they are already adapted to the environment and are intrinsically more competitive than are non-indigenous bacteria (Bhattarai and Hess 1993). In general, our knowledge of morphological and physiological features of aromatic plants associated with rhizobacteria remains limited and fragmentary.

We performed a comparative analysis of the effects of *M. piperita* root inoculation in three PGPR strains (indigenous and non-indigenous; singly and in combination) on plant morphological, anatomical (glandular trichome and stomatal density), and phytochemical features.

Methods and Materials

Bacterial Strains, Culture Conditions, Media, and Treatments

Three bacterial strains previously reported as PGPRs were studied: (i) *Pseudomonas fluorescens* WCS417r; (ii) *Pseudomonas putida* SJ04 (a native fluorescent strain isolated from rhizospheric soil collected with a commercial crop of *Mentha*×*piperita* (San José) in Villa Dolores, Córdoba, Argentina and tested for plant growth-promoting activity (GenBank KF312464.1); (iii) *Bacillus subtilis* GB03 (Banchio *et al*. 2009). Each strain was grown on LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) for routine use and maintained in nutrient broth with 15 % glycerol at −80 °C for long-term storage.

Each bacterial culture was grown overnight at 30 °C with rotation at 120 rpm until reaching exponential phase, washed twice in 0.9 % NaCl by Eppendorf centrifugation (4300×*g*, 10 min, 4 °C), resuspended in sterile water, and adjusted to a final concentration of ~10^6 colony forming unit (CFU)/ ml for use as inoculum. Plants were grown in plastic pots (diam 12 cm, depth 22 cm) containing 250 g sterilized vermiculite. *Mentha piperita* seedlings were planted (one per pot) in

vermiculite and inoculated with 1000 μl bacterial suspension. The treatments were: (1) sterile water (control); (2) GB03; (3) WCS417r; (4) SJ04; (5) WCS417r plus SJ04.

For experiments, single colonies of bacteria grown on nutrient agar were transferred to 100-ml flasks containing the appropriate culture medium and grown aerobically on a rotating shaker (150 rpm) for 48 hr at 28 °C. The bacterial suspensions were diluted in sterile water to a final concentration of 10^6 CFU/ml as determined by optical density measurement. Plants were treated with 1 ml of the resulting suspension for treatments (2), (3), and (4), or with 1 mL each of WCS417r and SJ04 for treatment (5). Ten plants were used for each treatment.

Greenhouse Experiments

Young shoots from *M. piperita* plants grown in Traslasierra Valley (Córdoba province, Argentina) were surface disinfected by soaking for 1 min in 17 % sodium hypochlorite solution, and rinsed 3X in sterile distilled water. Disinfected shoots were cultured in 100 mL MS culture medium containing 0.7 % (*w*/*v*) agar and 1.5 % (*w*/*v*) sucrose (Murashige and Skoog 1962). All culture media contained 30 g/L sucrose and 7.5 g/L agar. Stage I (initial shoot-tip culture): After shoot culture for 30 day as above, apical meristems with foliar primordia, not showing contamination, were aseptically removed from terminal buds. Explants were cultured in test tubes in 10 ml MS medium with 0.53 μM naphthaleneacetic acid (NAA) and 0.26 μM benzyladenine (BA) (Santoro *et al*. 2011).

Stage II (growth and multiplication): Plantlets obtained from tips were multiplied by single node culture in MS medium with 0.53 μM NAA and 0.28 μM BA. pH was adjusted to 5.8, and growth regulators were added prior to autoclaving (20 min, 121 °C). Temperature was maintained at 22 °C. Photoperiod was 16 hr/day with ~2000 Lux light radiation from cool white fluorescent tubes.

Stage III (rooting and acclimatization): At d 7 of culture, rooting plantlets were obtained at the *in vitro* multiplication stage, transplanted directly into vermiculite in a greenhouse, and watered by a micro-irrigation system.

Plants were grown in a growth chamber with controlled conditions of light (16/8 hr L/D cycle), temperature (22± 2 °C), and relative humidity (~70 %). Bacterial suspensions as described above were applied to experimental seedlings, and sterile water was applied to control seedlings. All plants received Hoagland's nutrient medium (20 ml/pot) once per week (Banchio *et al*. 2008). The experiments were performed under non-sterile conditions.

Experiments were replicated three times (10 pots per treatment; 1 plant per pot). Pots were arranged randomly in the growth chamber. Thirty days after inoculation, plants were removed from pots, roots were washed to remove vermiculite, and standard growth parameters (shoot length, leaf number, node number, shoot fresh weight, root dry weight) were measured. Total leaf areas were calculated based on digitized images of leaves using ImageJ (open source software program developed by the National Institutes of Health, Bethesda, MD, USA) as described previously (Turner *et al*. 2000).

Total Chlorophyll Determination

The amount of total chlorophyll in plants was determined as described by Arnon (1949). In brief, leaves (~0.1 g fresh weight) were placed in a mortar, 80 % acetone added, and ground to a fine pulp. The resulting extract was transferred to a Buchner funnel containing a pad of Whatman filter paper. Grinding of the leaf pulp was repeated during filtration of the extract to adjust the final volume of the filtrate to 10 ml. The optical density of the chlorophyll extract was measured by spectrophotometry at wavelengths 650 and 665 nm. The amount of total chlorophyll present in the extract was calculated on the basis of mg chlorophyll per g fresh leaf tissue, according to the following equation (Mc Kinney 1938):

$$\text{Total chlorophyll} = 6.45(\text{absorbance at } 665\text{nm}) + 17.72(\text{absorbance at } 650\text{nm})$$

Trichome Density and Stomatal Density

A layer of acrylic (synthetic nail coating) was brushed onto the adaxial and abaxial sides of the leaf, dried for a few seconds, then carefully extracted and mounted for microscopy with glycerol/ distilled water 1:10 (D'Ambrogio de Argüeso A 1986). Six leaf blades were processed for each treatment. Trichome density and stomatal density (number per mm^2) were calculated from three microscope fields chosen at random for each leaf epidermis. Histological preparations of trichomes and stomata were assessed using a standard Zeiss model 16 microscope. Photomicrographs were taken with a Zeiss Axiophot microscope equipped with image capture and digitization (AxioVision 4.3, with camera AxioCam HRc 200× magnification). Trichomes and stomata were counted using the ImageJ program for image analysis, and their frequency (n/mm^2) was calculated as described previously (Barbieri *et al*. 2012; Mucciarelli *et al*. 2003). Density was expressed as the ratio between mean number of trichomes or stomata per leaf pair and the corresponding leaf area.

Extraction of EOs

Shoot samples were weighed individually and subjected to hydrodistillation in a Clevenger-like apparatus for 40 min. The volatile fraction was collected in dichloromethane, and β-pinene (1 μl in 50 μl ethanol) was added as an internal standard.

Mentha piperita plants contain ~3 % volatile oils, consisting of >50 different compounds. The major EO components, which comprise ~60 % of total oil volume, are 1,8-cineole, linalool, (−) menthone, (−) menthol, and (+) pulegone. These compounds were quantified in relation to the standard β-pinene added during the distillation procedure as above. Flame ionization detector (FID) response factors for each compound generated essentially equivalent areas (differences <5 %).

Chemical analyses were performed using a Perkin-Elmer Q-700 gas chromatograph (GC) equipped with a CBP-1 capillary column (30 m×0.25 mm, film thickness 0.25 μm) and a mass selective detector. Analytical conditions: injector temperature 250 °C, detector temperature 270 °C; oven temperature programmed from 60 °C (3 min) to 240 °C at 4 °/min; carrier gas = helium at a constant flow rate of 0.9 mL/min; source 70 eV. Oil components were identified based on mass spectral and retention time data and confirmed by direct comparison with commercial standard compounds (Banchio *et al*. 2005).

GC analysis was performed using a Shimadzu GC-RIA gas chromatograph fitted with a 30 m×0.25 mm fused silica capillary column coated with Supelcowax 10 (film thickness 0.25 μm). GC operating conditions: injector and detector temperatures 250 °C; oven temperature programmed from 60 °C (3 min) to 240 °C at 4 °/min; detector = FID; carrier gas = nitrogen at a constant flow rate of 0.9 ml/min.

Statistical Analyses

Data were pooled and subjected to analysis of variance (ANOVA) followed by comparison of multiple treatment levels with controls using Fisher's *post hoc* LSD (least significant difference) test. Differences between means were considered significant for P values<0.05. The Infostat software program, version 2008 (Group Infostat, Universidad Nacional de Córdoba, Argentina) was used for all statistical analyses.

Results

Plant Morphology

The effects of PGPR inoculation on *M. piperita* growth and development varied depending on the experimental treatment (inoculation with GB03, WCS417r, SJ04, or WCS417r+SJ04) (Fig. 1; Table 1). Each of the growth parameters evaluated was significantly (P<0.05) altered by each of the four treatments (Table 1). Root dry weights were ~40 % higher for plants in each of the treatment groups than in the control (noninoculated) group (P<0.05). Root length was altered only by WCS417r treatment. Shoot fresh weight for each of the treatment groups was ~380 mg higher than in the control group

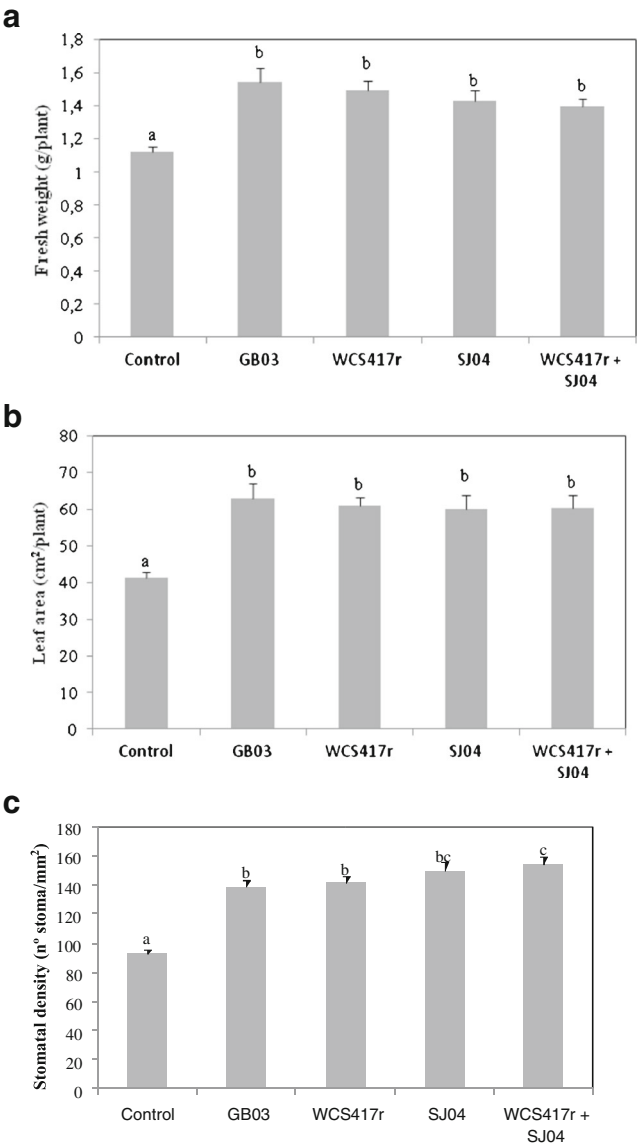

Fig. 1 Growth and development parameters of 30-day old *Mentha piperita* plants in a control group and four treatment groups (inoculated or co-inoculated with PGPR) as described in M&M. **a** fresh weight. **b** leaf area. **c** stomatal density. Different letters above bars within a panel indicate significant differences according to Fisher's LSD test (P<0.05)

(Fig. 1a). This increase was due to a combination of increased leaf number, shoot length (Table 1), and leaf area (25 cm^2 higher) (Fig. 1b).

Leaf chlorophyll a, chlorophyll b, and total chlorophyll concentrations were significantly higher for each of the treatment groups than the control group. All three chlorophyll values were higher for the GB03 group than the other treatment groups (Table 2). Chlorophyll a and b values were higher for the SJ04 group than the WCS417r group. For all three chlorophyll values, the WCS417r+SJ04 co-inoculated group was intermediate between the SJ04 and WCS417r singly-inoculated groups.

Table 1 Morphological parameters (mean±SE) of *Mentha. piperita* plants in a control group and four treatment groups (inoculated or co-inoculated with PGPR) as described in M&M

Plant growth parameters			Plant morphogenesis parameters			
	Root length (cm)	Root dry weight (mg)	Shoot length (cm)	Node number	Leaf number	Ramification number
Control	29.96±0.59*a*	97.94±4.66*a*	17.96±0.52 *a*	18.96±0.32 *a*	40.4±0.82 *a*	5.34±0.21*a*
GB03	32.67±1.02 *ab*	138.13±10.60 *b*	20.46±0.76 *b*	22.22±0.94 *bc*	45.83±2.02 *bc*	6.79±0.45 *b*
WCS417r	33.52±0.99 *b*	141.70±8.07 *b*	20.59±0.86*b*	23.08±0.66 *c*	46.31±1.18 *bc*	5.89±0.29 *a*
SJ04	32.37±1.05 *ab*	111.32±6.47 *a*	20.92±0.82 *b*	22.79±0.84 *bc*	47.24±1.55 *c*	6.19±0.30 *ab*
WCS417r + SJ04	32.35±1.01*ab*	139.94±11.95 *b*	19.24±0.75 *ab*	20.63±0.70 *ab*	42.81±1.08 *ab*	5.48±0.27 *a*

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test. ($P<0.05$)

Plant Anatomy

Single non-glandular capitate trichomes and peltate glandular trichomes were present on the adaxial and abaxial leaf surfaces. A small capitate glandular trichome consisted of a globose unicellular head attached to the leaf by a two- or three-celled uniseriate stalk. Peltate glandular trichomes consisted of a large eight-celled head, with an enlarged secretory cavity, attached to a one-celled short stalk. Average numbers of trichomes per cm^2 for the control group and the four treatment groups are shown in Table 3. Trichomes were significantly more abundant on the abaxial than the adaxial surface. Differences in numbers of capitate trichomes were not significant. Peltate trichomes were present on both surfaces for the treatment groups, but their density was lower on the adaxial surface. The density of peltate trichomes on both surfaces was higher for the treatment groups than the control group (Table 3). Differences in peltate trichome density among the four treatment groups were not significant. Stomatal density was higher for the abaxial than the adaxial surface for all treatments (data not shown). Overall stomatal density increased from 93 stoma/mm^2 in the control group to ~140 stoma/mm^2 in the treatment groups. The value was higher for the WCS417r+SJ04 group (154 stoma/mm^2) than for the other three treatment groups (Fig. 1c).

Table 2 Chlorophyll content (μg/ g fresh weight; mean±SE) in *Mentha. piperita* control and treatment groups

	Chlorophyll a	Chlorophyll b	Total chlorophyll
Control	76.66±3.55 *a*	39.08±2.35 *a*	119.37±5.92 *a*
GB03	106.49±2.73 *c*	58.21±1.66 *c*	169.92±3.02 *c*
WCS417r	88.89±1.98*b*	51.14±1.80*b*	136.93±1.40 *b*
SJ04	104.25±3.54 *c*	55.10±1.71 *bc*	164.30±5.13 *c*
WCS417r + SJ04	97.77±4.00 *c*	51.28±1.57 *b*	155.30±7.49 *c*

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test ($P<0.05$)

Secondary Metabolites

GC analysis of EOs revealed striking differences between the treatment and control groups (Fig. 2). These differences were due mainly to variations in major EO components (Fig. 3) and total EO yield.

Total EO concentrations were in the range of 0.5 % (for control plants) and 2 % (for treated plants) fresh weight, similar to findings in other aromatic species (Werker 2000).

Monoterpene accumulation values (μg per g fresh weight) were 4.69 for the control group, 15–20 for the singly-inoculated groups, and 29.2 for the WCS417r+SJ04 group (~6-fold higher than the control value; $P=0.001$) (Fig. 2). Yields of the major EO components ((+) pulegone, (−) menthone, (−) menthol, 1,8-cineole, and linalool) were higher in all four treatment groups than in the control group (Fig. 3). Menthol yield increased to 2.09 μg/g fresh weight ($P<0.05$) in the WCS417r+SJ04 group in comparison with 0.22 μg/g fresh weight in the control group and 1.1–1.2 μg/g fresh weight in the other three treatment groups. Similar trends were observed for menthone, 1,8–cineole, linalool, and pulegone; yield was always higher for the WCS417r+SJ04 group than the other three treatment groups. Yields for each of the major

Table 3 Density (per mm^2; mean±SE) of peltate and capitate glandular trichomes in *Mentha. piperita* control and treatment groups

Abaxial face		Adaxial face		
	Peltate	Capitate	Peltate	Capitate
Control	3.89±0.37 *a*	10.00±1.25 *ab*	2.33±0.13 *a*	3.33±0.65 *a*
GB03	5.71±0.34 *b*	12.16±3.40 *b*	4.05±0.28 *b*	3.05±0.74 *a*
WCS417r	5.36±0.41*b*	6.90±1.21 *a*	3.44±0.21 *b*	3.75±0.72 *a*
SJ04	5.44±0.34 *b*	7.03±0.98 *a*	3.84±0.26 *b*	3.61±0.48 *a*
WCS417r + SJ04	6.07±0.24 *b*	7.50±0.94 *ab*	3.67±0.18 *b*	2.50±0.43 *a*

Means followed by the same letter within a column are not significantly different according to Fisher's LSD test ($P<0.05$)

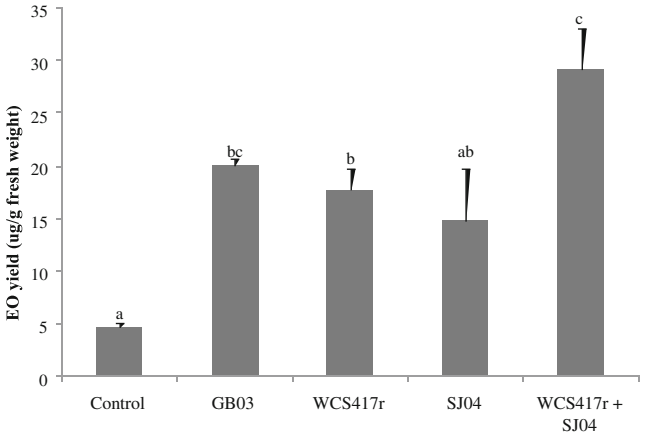

Fig. 2 Essential Oil (*EO*) yields in the *Mentha piperita* control and treatment groups. Different letters above bars indicate significant differences according to Fisher's LSD test ($P<0.05$)

EO components were higher for the GB03 group than the WCS417r or SJ04 groups, but the differences between these three singly-inoculated groups were generally not significant.

Discussion

Plant Morphology

Enhanced growth and development following inoculation with PGPR has been reported for many plant species (Gray and Smith 2005; Van Loon 2007; Vessey 2003). The causes of the enhancement vary depending on the species, and may include both direct and indirect mechanisms (Glick 1995; Gupta *et al*. 2002). In the present study, the magnitude of the effects of PGPR on *M. piperita* growth and development varied depending on the inoculated strain (GB03, WCS417r, SJ04, or WCS417r+SJ04). Single inoculation with the three PGPR strains evaluated resulted in significant increases in almost all plant growth and morphogenesis parameters measured. The only exception was the lack of effect of SJ04 on root development. Shoot fresh weight was enhanced significantly (~40 %) in each of the treatment groups compared with the control group. This effect appeared to be due primarily to increased leaf area in the WCS417r+SJ04 group, and in part to increased leaf and node number and shoot length in the GB03, WCS417r, and SJ04 groups. These findings rule out the possibility that the enhanced growth resulted simply from increased plant hydration. Root dry weight was higher in the GB03, WCS417r, and WCS417r+SJ04 groups than in the control group. This increase was due primarily to increased number of lateral roots (data not shown). Root length was affected only by WCS417r treatment. Vessey (2003) described various effects of PGPR on root morphology. Zhang *et al*. (2007) reported that enhanced lateral root formation led to increased root surface area and nutrient uptake potential.

We previously obtained similar results for *Origanum majoricum*, *O. majorana*, and *Tagete minuta* inoculated with WCS417r (Banchio *et al*. 2010; Cappellari *et al*. 2013). GB03 enhanced growth parameters of *O. majoricum* and *O. basilicum* (basil) (similar to its effects on *M. piperita* in the present study), but had no effect on development parameters of *O. majorana*. *In vitro* studies showed that VOCs emitted by WCS417r and GB03 produced effects similar to those observed here in *M. piperita*, and were more striking in plants affected by GB03 (Santoro *et al*. 2011). In the present study, developmental parameters in the WCS417r+SJ04 group were not significantly different from those in the singly-inoculated or control groups.

In this study, plants received Hoagland's nutrient solution, in which nitrogen and other nutrients are readily available. The growth stimulatory effects observed were therefore not due to phosphate solubilization, sulfate oxidation, or increased nitrate availability (Kloepper 1993). Rather, the enhancement of growth following PGPR inoculation was presumably due to increased production and emission of growth hormones and/or VOCs by the bacteria (Ryu *et al*. 2005; Santoro *et al*. 2011; Van Loon 2007). Such components, collectively termed "plant growth regulators", are organic substances that affect plant

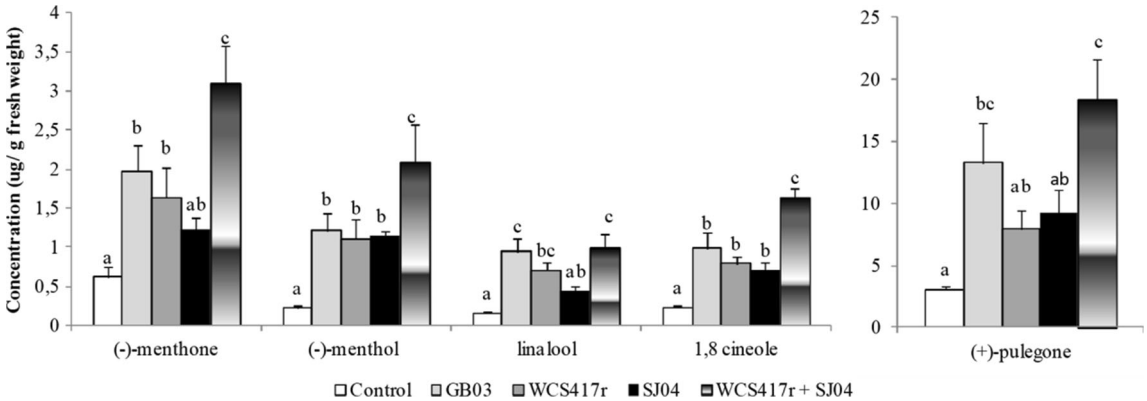

Fig. 3 Concentrations of major Essential Oil (*EO*) components in *Mentha piperita* control and treatment groups. Different letters above bars indicate significant differences according to Fisher's LSD test ($P<0.05$)

physiological processes at extremely low concentrations and play regulatory roles in plant growth and development (Dobbelaere *et al*. 2003).

Cultures of various PGPR have been shown to produce phytohormones in substantial amounts (Zahir *et al*. 2004) that promote plant development. Numerous bacterial strains produce auxins and/or ethylene, and some strains have been reported to produce cytokinins and gibberellins (Van Loon 2007). Studies during the past few decades have shown that rhizobacteria are capable of releasing functional VOCs (Vespermann *et al*. 2007). Zhang *et al*. (2008) showed that exposure to *B. subtilis* VOCs enhanced photosynthetic efficiency, chlorophyll content, and cell expansion. We observed increased fresh weight and root dry weight in *M. piperita* exposed to GB03 and WCS417r VOCs (Santoro *et al*. 2011).

Plant Anatomy

Trichome distribution is not uniform over the leaf surface; there are distinct differences between the abaxial and adaxial epidermis. Consistent with the findings of Turner *et al*. (2000), we observed approximately twice as many trichomes on the abaxial as on the adaxial surface. After 30 days, plants in the four treatment groups had greater numbers of peltate trichomes on both the adaxial and abaxial surfaces. Glandular trichome density is controlled by numerous environmental and hormonal factors. Several studies indicate that jasmonates in particular play a role (Ament *et al*. 2004; Boughton *et al*. 2005; Lange and Ahkami 2013; Li *et al*. 2004). Stomata are involved in photosynthesis and transpiration, two essential processes in plants. Increased stomatal density, as observed in our treatment groups, theoretically could enable plants under well-hydrated conditions to increase conductance for gas exchange at the leaf surface and thereby avoid photosynthetic limitation due to suboptimal CO_2 supply (Schlüter *et al*. 2003). The more stomata per unit area, the more CO_2 can be taken up, and the more water can be released (Berry *et al*. 2010).

Chlorophyll concentration is an important parameter in evaluation of plant photosynthetic efficiency and response to environmental stress (Zhu *et al*. 2012). We observed higher concentrations of chlorophyll a, chlorophyll b, and total chlorophyll in the PGPR-treated groups than in the control group, in agreement with previous findings (Colla *et al*. 2008). A likely explanation is that PGPR-inoculated plants can absorb more water and nutrients than can non-inoculated plants (Subramanian and Charest 1997) and are, therefore, less susceptible to oxidative stresses that damage the photosynthetic apparatus (Evelin *et al*. 2009).

Secondary Metabolites

Our previous studies showed that PGPR inoculation increased monoterpene production in various aromatic plant species (Banchio *et al*. 2009, 2010; Cappellari *et al*. 2013). In the present study, total EO yield in the four treatment groups was 4- to 6-fold higher ($P<0.001$) than in the control group. In our previous studies, *P. fluorescens* inoculation increased total EO yield (relative to controls) 2.5-fold in *O. majoricum*, 24-fold in *O. majorana*, and by 50 % in *T. minuta* (Banchio *et al*. 2010; Cappellari *et al*. 2013), and bacterial VOCs increased 2-fold in *M. piperita* (Santoro *et al*. 2011). The present study showed differential effects of GB03 on monoterpene accumulation. We found previously that exposure to *B. subtilis* VOCs significantly increased EO yield in *O. basilicum* (Banchio *et al*. 2009), but had no effect on total monoterpene level or EO yield in *O. majoricum* or *O. majorana* seedlings, or in *M. piperita* (Banchio *et al*. 2009; Santoro *et al*. 2011). The effects of rhizobacteria and their VOCs on these secondary metabolites varied depending on the strain, suggesting that the rhizobacteria are recognized by the host plant in a strain-specific manner.

Levels of the major EO components analyzed (1,8-cineole, linalool, (−) menthone, (−) menthol, (+) pulegone) were markedly different in the four treatment groups from the control group. (+) Pulegone was by far the predominant component, accounting for ~50 % of total EOs. Our findings suggest an increase in terpene biosynthesis in the inoculated plants, although we did not directly measure this process. Similar results have been obtained for various aromatic plant species treated with arbuscular mycorrhizal fungi. Gupta *et al*. (2002) inoculated *Mentha arvensis* cultivars with the fungus *Glomus fasciculatum* and observed increases in plant height, shoot growth, and oil content. Khaosaad *et al*. (2006) reported altered EO concentration in *Origanum sp*. Copetta *et al*. (2006) observed altered glandular trichome abundance and EO yield in *O. basilicum* exposed to mycorrhizal fungi.

Expression of the gene controlling terpenoid biosynthesis in *Mentha aquatica* increases in response to herbivore feeding; the majority of terpene production is diverted to synthesis of (+)-menthofuran, which has been shown to repel herbivorous mint leaf beetles in bioassay tests (Lamiri *et al*. 2001). Monoterpene synthesis is promoted by herbivore feeding in *Minthostachys mollis* (Banchio *et al*. 2005) and in other plant species, most likely thus protecting damaged leaves from further attack (Harrewijn *et al*. 2001; Hummelbrunner and Isman 2001).

Essential oil concentrations and composition in plants play several key roles in plant-environment interactions and plant-plant communication. Terpenoids are crucial components in plant defensive responses to abiotic and biotic stresses (Unsicker *et al*. 2009; Vickers *et al*. 2009), signaling among plant organs (Heil and Silva Bueno 2007), and plant-plant communication (Baldwin *et al*. 2006; Lange and Ahkami 2013).

The increases in EO synthesis observed in the present study presumably represent defensive responses to colonization by microorganisms. Several EO compounds in *M. piperita* exert insecticidal, antifungal, and/or antibacterial effects (Sangwan

et al. 2001). Induction of systemic resistance, which can be elicited not only by pathogens and herbivores but also by beneficial microorganisms and certain synthetic compounds, provides plants with an enhanced capacity for rapid and effective activation of cellular defensive responses against pathogen or insect attack (Conrath 2011). Induction of systemic resistance against herbivores also involves "priming" of jasmonate-dependent responses and other ye tunknown mechanisms (Pineda *et al*. 2010; van Oosten *et al*. 2008). Priming of plant defenses by beneficial microorganisms has been proposed to be a consequence of the modulation of plant immune systems associated with establishment of the symbiosis, and related changes in defense-related signaling (Pozo and Azcón-Aguilar 2007; Zamioudis and Pieterse 2012). The increased monoterpene concentrations we observed in inoculated plants may result from growth-promoting substances, secreted by PGPR, that affect plant metabolic processes. Increases in stomatal density and chlorophyll content in inoculated plants may be reflected in a higher rate of carbon assimilation and result in increased terpenoid biosynthesis, which requires carbon fixation through photosynthesis (Ghirardo *et al*. 2011). Terpenoid biosynthesis depends on primary metabolism (photosynthesis) and oxidative pathways for carbon and energy supplies (Singh *et al*. 1991). Giri et al. (2003) found that net photosynthesis of PGPR-hosting plants is correlated with nutritional status. Factors that increase dry matter production affect the interrelationship between primary and secondary metabolism, leading to increased biosynthesis of secondary products (Shukla *et al*. 1992). In the present study, trichome density was higher in the treatment groups than in the control group. Oil yield is strongly correlated with the total number and developmental distribution patterns of glandular trichomes, the biosynthetic machinery that quickly and efficiently converts imported carbohydrates into EOs (Lange *et al*. 2011; Lange and Turner 2012). Rios-Estepa et al. (2010) proposed a mechanism whereby increased expression of a biosynthetic gene induces production of glandular trichomes, facilitating the production and storage of EOs.

In this study, single inoculation with *Pseudomonas* (WCS417r or SJO4) or *B. subtilis* GB03 affected plant growth and development, trichome density, and chlorophyll content to similar degrees. Co-inoculation with WCS417r and SJO4 produced the greatest increases in stomatal density and levels of secondary metabolites, indicating a synergistic effect on monoterpene biosynthesis. This finding may reflect a higher number of CFU per seedling; the plants were inoculated with 106 CFU/mL from each strain. On the other hand, previous studies on co-inoculation with various PGPR strains indicated that most of the strains did not have negative effects on each other (Marimuthu *et al*. 2002; Vestberg and Cassells 2009). Compositional studies of various microbial communities have shown that *Pseudomonas* species generally are able to adapt to a wide range of environments and to increase population size (Van Loon 2007; Vessey 2003) because of their ability to metabolize a wide range of carbonaceous substances excreted by plant roots, the versatility of their metabolism, and their rapid growth rates (Figueiredo *et al*. 2010; Lucy *et al*. 2004; Vestberg and Cassells 2009). Other reports indicate that co-inoculation with certain combinations of PGPR strains has greater or comparable effects on plant protection and plant growth promotion in comparison with separate inoculation (Schisler *et al*. 1997; Schmidt *et al*. 2004). Native strains may be preferred in such cases in selection of bacteria for inoculation of crop plants, because they are already adapted to the environment and are more competitive than are non-indigenous strains. In summary, we describe a novel approach to evaluate the effects of specific PGPR strains on *M. piperita*. Bacterial inoculants are an effective biotechnological tool for stimulating secondary metabolism in plants. Studies of the activities of these inoculants will help clarify certain adaptive processes that are poorly understood at present. There is steadily increasing demand for environmentally safe, sustainable, organic agricultural practices that reduce negative environmental effects associated with food and feed production (Lind *et al*. 2004). A worldwide shift is underway from traditional inorganic farming methods toward ecofriendly, organic farming methods. For the numerous medicinal and aromatic plant species that are consumed without further processing, it is important that no synthetic compounds be present in the harvested crop. Such crop plants are ideal candidates for development of growth-promoting strategies involving PGPR and biofertilizers.

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