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RESEARCH PAPER

Plant growth-promoting effects of native *Pseudomonas* strains on *Mentha piperita* (peppermint): an *in vitro* study

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) affect growth of host plants through various direct and indirect mechanisms. Three native PGPR (*Pseudomonas putida*) strains isolated from rhizospheric soil of a *Mentha piperita* (peppermint) crop field near Córdoba, Argentina, were characterised and screened *in vitro* for plant growth-promoting characteristics, such as indole-3-acetic acid (IAA) production, phosphate solubilisation and siderophore production, effects of direct inoculation on plant growth parameters (shoot fresh weight, root dry weight, leaf number, node number) and accumulation and composition of essential oils. Each of the three native strains was capable of phosphate solubilisation and IAA production. Only strain SJ04 produced siderophores. Plants directly inoculated with the native PGPR strains showed increased shoot fresh weight, glandular trichome number, ramification number and root dry weight in comparison with controls. The inoculated plants had increased essential oil yield (without alteration of essential oil composition) and biosynthesis of major essential oil components. Native strains of *P. putida* and other PGPR have clear potential as bio-inoculants for improving productivity of aromatic crop plants. There have been no comparative studies on the role of inoculation with native strains on plant growth and secondary metabolite production (specially monoterpenes). Native bacterial isolates are generally preferable for inoculation of crop plants because they are already adapted to the environment and have a competitive advantage over non-native strains.

INTRODUCTION

Mentha (mint) is a major genus in the family Lamiaceae, with ~16 species and a worldwide distribution (Guntert *et al.* 2001; McKay & Bumberg 2006). Mint is grown commercially in many countries. The essential oils accumulated in leaves are commonly used in the cosmetic, pharmaceutical, food, confectionary and liquor industries. The aerial parts are widely used for treatment of colds, bronchitis and other respiratory diseases (McKay & Bumberg 2006). In regard to plant extracts, essential oil components in general are being used increasingly in the food, cosmetic and pharmaceutical industries for reasons of relative safety and popularity with consumers. The antioxidant properties of many plant extracts have beneficial effects in human health (Newman & Cragg 2007). Essential oils from certain *Mentha* species have been shown to function as free radical scavengers and as primary antioxidants that react with free radicals and inhibit the destructive effects of reactive oxygen species (ROS) in biological systems and foods (Nickavar *et al.* 2008).

Lamiaceae species are characterised by non-photosynthetic glandular trichomes on both upper and lower leaf surfaces (Werker 2000). Peppermint (*M. piperita*) has peltate and capitate glandular trichomes. Only the peltate trichomes, which contain secretory cells responsible for essential oil synthesis, accumulate monoterpenes. Essential oils are secreted into an emerging cavity formed by the separation of a preformed layer of cuticular material (Rios-Estepa *et al.* 2010).

We have previously investigated inoculation of various aromatic plant species with plant growth-promoting rhizobacteria (PGPR) as a strategy to improve yield (Banchio *et al.* 2009; Santoro *et al.* 2011; Cappellari *et al.* 2013). Many studies of bacterial populations living in the root environment (rhizosphere) of plants have demonstrated the predominance and ecological importance of the genus *Pseudomonas* (fluorescent pseudomonads; Patten & Glick 2002; Naik *et al.* 2008; Loaces *et al.* 2011; Parejko *et al.* 2012; Rameshkumar *et al.* 2012). Taxonomy of the genus is based on cell morphology, presence of flagella and Gram-negative type of cell wall. The key phenotypic feature of *Pseudomonas* is the production of pigments, particularly pyocyanin, pyoverdine and other diffusible fluorescent pigments (Palleroni 2005). The bacteria typically live on the root surface or nearby, play crucial roles in soil health and plant development (Kloepper 1993) and affect plant growth either directly or indirectly (Van Loon 2007). Indirect promotion of plant growth occurs through prevention of the harmful effects of phytopathogenic organisms, primarily by production of phenazines and other antibiotics, phenolics (Keel *et al.* 1996), pyrrole-type compounds, polyketides and peptides (Babalola 2010). Direct promotion of plant growth occurs through providing the plant with a useful compound synthesised by the bacteria, or facilitating plant uptake of specific nutrients from the environment. *Pseudomonas* species produce siderophores

(Mavrodi *et al.* 2001) and participate in phosphate solubilisation (Anzuay *et al.* 2013), thereby making soil iron and phosphorus accessible to the plant. Fluorescent pseudomonads also produce the phytohormone indole-3-acetic acid (IAA; Patten & Glick 2002) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which sequesters the ethylene precursor (Van Loon 2007).

Intensive farming practices aimed at high crop yield and quality traditionally require the extensive use of chemical fertilisers, which are expensive and have negative environmental impacts. There is increasing interest in environmentally safe, sustainable and organic agricultural practices that reduce negative environmental effects associated with food and feed production. 'Organic agriculture' is a production system that avoids or minimises the use of synthetic fertilisers, pesticides and growth regulators, relying instead on biofertilisation, crop rotation, crop residues, mechanical cultivation and biological pest control to promote or maintain soil productivity. Reduced yield is a major problem and concern in organic production systems (Lind *et al.* 2004). In the case of the numerous medicinal and aromatic plant species that are consumed without further processing following harvest, it is important that no synthetic compounds be present at harvest.

In view of the above considerations, *Pseudomonas* species have great potential as inoculants of crop plants. In selecting bacterial strains as inoculants, native (indigenous) isolates are typically preferable because they are already adapted to the local environment and therefore more competitive than non-native strains (Bhattarai & Hess 1993). Few studies to date have focused on native PGPR strains as inoculants for improving growth of aromatic and medicinal plants. We evaluated the effects of direct inoculation with native fluorescent *Pseudomonas* strains isolated from the *M. piperita* rhizosphere on plant growth parameters and on qualitative and quantitative essential oil composition.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Three native pseudomonad strains (*Pseudomonas putida* SJ04, *P. putida* SJ25, *P. putida* SJ48) isolated from rhizosphere soil of *M. piperita* plants in a commercial agricultural field in San José, Villa Dolores, Córdoba province, Argentina, and a reference strain (*P. fluorescens* WCS417r; Van Peer *et al.* 1991) were studied. Each strain was grown on Luria-Bertani (LB) medium (10 $g \cdot l^{-1}$ tryptone, 5 $g \cdot l^{-1}$ yeast extract, 10 $g \cdot l^{-1}$ NaCl) for routine use, and maintained in nutrient broth with 15% glycerol at −80 °C for long-term storage.

Indole-3-acetic acid production

Indole-3-acetic acid (IAA) level was qualitatively assayed as described in Brick *et al.* (1991). Bacteria were inoculated as a dot on LBTD4 agar plates (LB medium supplemented with L-tryptophan 5 mM, 0.06% sodium dodecyl sulphate, 1% glycerol). The plates were overlaid with a nitrocellulose membrane disk (diameter 45 mm; Nytran, Amersham, UK) and incubated until colonies reached ~2-mm diameter. The membrane was removed from the plate and treated with Salkowsky reagent (50 ml perchloric acid, 1.0 ml 0.5 M ferric chloride). Bacteria that produced a characteristic red halo within the membrane surrounding the colony were considered to be positive for IAA production. Some plates were inoculated with *Azospirillum brasilense* strain Sp7 as a positive control. Qualitative results were obtained based on comparison of colours developed by native fluorescent strains *versus* the positive control. The assay was replicated twice.

Siderophore production

Siderophore production was qualitatively assayed using the chrome azurol S (CAS) method with some modification. Petri dishes were coated with 15 ml M9 medium. After the medium solidified, one half was cut off and replaced by 7.5 ml CAS-blue agar (Schwyn & Neilands 1987). Bacteria were inoculated in the boundary between the two media. A CAS-agar plate was inoculated with *Sinorhizobium meliloti* strain Rm1021 as negative control (Schwyn & Neilands 1987). Plates were incubated for 48 h at 30 °C. CAS reaction was determined by colour change from blue to orange. The lack of siderophore production by the negative control was evidenced as absence of orange colour around the colonies. The technique was validated using colour development by colonies of the native fluorescent strains. The assay was replicated twice.

Phosphate solubilisation ability

Phosphate solubilisation ability was qualitatively assayed by inoculating bacteria as a single point on Petri dishes coated with Pikovskaya medium (Pikovskaya 1948). Plates were incubated for 48 h at 30 °C. Formation of a clear zone around bacterial colonies was considered to be a positive reaction. *S. meliloti* RM1021 was used as a negative control. The lack of phosphate solubilisation in the negative control was evidenced as absence of a clear zone around the colonies. The technique was validated as formation of clear zones around colonies of the native fluorescent strains. The assay was replicated twice.

Plant micropropagation

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 three times in sterile distilled water. The disinfected shoots were cultured in 100 ml MS culture medium containing 0.7% (w/v) agar and 1.5% (w/v) sucrose (Murashige & Skoog 1962).

Stage I (initial shoot tip culture)
Apical meristems with foliar primordia, not showing contamination, were aseptically removed from terminal buds after 30 days. Explants were cultured in test tubes with 40 ml MS medium containing 0.66 $mg \cdot l^{-1}$ indole-butyric acid.

Stage II (growth and in vitro multiplication)
Plantlets obtained from tips were multiplied by single node culture, pH of MS medium was adjusted to 5.6–5.8, and cultures were autoclaved for 20 min at 121 °C. Explants were grown in a growth chamber under controlled conditions of light (light/dark cycle 16/8 h), temperature (22 ± 2 °C) and relative humidity (70%).

Direct inoculation

Petri dishes coated with MS semisolid medium (0.5% agar) were inoculated with bacteria at concentration 10^6 CFU dish^{-1}. After solidification of the medium, one node from an aseptically cultured plant was placed in the centre of the dish. Reference strain *P. fluorescens* WCS417r was used as a positive control and distilled water as a negative control. Dishes were sealed with Parafilm, arranged in a completely randomised design and placed in a growth chamber under controlled conditions as above. Plants were harvested after 30 days. Experiments were replicated three times (ten dishes per treatment, one plant per dish).

Evaluation of plant growth-promoting effects

Effects of bacterial inoculation on plant growth were evaluated as described previously (Santoro *et al.* 2011). After harvesting of plants from Petri dishes, roots were rinsed with water to remove medium, and standard growth parameters (shoot length, root length, node number, leaf number, shoot ramification number, shoot fresh weight, root dry weight) for each plant were evaluated.

Trichome density

Third node leaves were immersed for 48 h in clarifying solution (5% sodium hypochlorite, 2% potassium hydroxide, 1:1 v/v), rinsed five times in sterile distilled water, coloured with safranin for 20 s, rinsed again, and mounted on microscope slides in glycerol/distilled water 1:10 (D'Ambrogio de Argüeso 1986). Three leaf blades were processed for each treatment. Trichome density was calculated from three randomly chosen microscope fields for each adaxial leaf epidermis. Histological preparations were evaluated using a standard Zeiss model 16 microscope. Photomicrographs were taken at 10× magnification with a Zeiss Axiophot microscope equipped with image capture and digitisation (software program AxioVision 4.3 (Carl Zeiss MicroImaging GmbH, Jena, Germany), with AxioCam HRc camera). Trichomes were counted using the Adobe Photoshop software for image analysis, and their frequency expressed as number mm^{-2} (Barbieri *et al.* 2012).

Essential oil extraction and analysis

Individual shoot samples were weighed, subjected to hydrodistillation in a micro Clevenger-like apparatus for 30 min, and volatile fraction was collected in dichloromethane. An internal standard (0.1 µl dodecalactone in 50 µl ethanol) was added.

Chemical analyses were performed using a gas chromatograph (GC) (model Clarus 600; PerkinElmer, Waltham, MA, USA) equipped with a CBP-1 capillary column (30 m × 0.25 mm, film thickness 0.25 mm) and mass-selective detector. Analytical conditions: injector and detector temperatures 250 and 270 °C; oven temperature programmed from 60 °C (3 min) to 240 °C at 4° min^{-1}; carrier gas ¼ helium at constant flow rate 0.9 ml·min^{-1}; source 70 eV. Essential oil components were identified based on mass spectra and retention times, in comparison with standards (Banchio *et al.* 2009). GC analysis was performed using a PerkinElmer Clarus 500 GC, fitted with a 30 m × 0.25 mm fused silica capillary column coated with Supelcowax 10 (film thickness 0.25 mm). GC operating conditions: oven temperature programmed from 60 °C (3 min) to 240 °C at 4° min^{-1}, injector and detector temperature 250 °C; detector FID; carrier gas ¼ nitrogen at constant flow rate 0.9 ml·min^{-1}.

Statistical analysis

Data were subjected to (i) ANOVA followed by comparison of multiple treatment levels with control using *post-hoc* Fisher's LSD (least significant difference) test, and (ii) principal components analysis (PCA). The purpose of PCA was to extract and display relationships among factors in the multivariate data set (four bacterial strains, essential oil content after inoculation, IAA production parameters). Statistical analyses were performed using Infostat software version 2.0 (Group Infostat, Universidad Nacional de Córdoba, Argentina). Differences between means were considered to be significant for $P < 0.05$.

RESULTS

Plant growth-promoting activity of native bacterial strains

The *P. putida* strains evaluated were isolated from roots in the *M. piperita* rhizosphere as described in Material and Methods. Biochemical characteristics of each strain (phosphate solubilisation, siderophore production, IAA production) are summarised in Table 1.

Phosphate solubilisation
Each of the tested strains grown on synthetic medium displayed phosphate solubilisation, as indicated by formation of a clear zone around the colony (Table 1).

Siderophore production
The ability of bacterial strains to sequester iron confers a competitive advantage. Of the three tested strains (SJ04, SJ25, SJ48), only SJ04 displayed siderophore production, as indicated by formation of a distinct yellow halo on CAS plates after 24 h incubation (Table 1).

Table 1. Biochemical characteristics of native fluorescent *Pseudomonas* strains isolated from rhizosphere soil of *Mentha piperita* plants from an agricultural field in Córdoba, Argentina, and a reference strain (WCS417r).

strain	species	access number	phosphate solubilisation	siderophore production	IAA production
SJ04	*P. putida*	KF312467.1	+	+	+
SJ25	*P. putida*	KF312473.1	+	–	+
SJ48	*P. putida*	KF312479.1	+	–	±
WCS417r	*P. fluorescens*		+	+	–

Table 2. Effect of direct inoculation of native and reference strains as in Table 1, and a control, on plant growth parameters of *Mentha piperita*.

	length (cm)	dry weight (mg)	length (cm)	node number	ramification number	leaf number
control	2.05 ± 0.36	0.73 ± 0.27 a	5.15 ± 0.50	8.25 ± 0.75	0.25 ± 0.05 a	18.00 ± 2.83 a
SJ04	1.98 ± 0.11	0.95 ± 0.37 ab	4.16 ± 0.22	7.25 ± 0.25	0.75 ± 0.25 b	19.13 ± 1.59 a
SJ25	1.87 ± 0.39	1.11 ± 0.20 b	4.69 ± 0.51	7.43 ± 0.57	0.57 ± 0.20 b	19.14 ± 1.87 a
SJ48	1.74 ± 0.24	1.27 ± 0.18 b	5.40 ± 0.44	7.90 ± 0.28	0.50 ± 0.17 b	21.30 ± 1.84 b
WCS417r	2.24 ± 0.36	2.74 ± 0.47 c	5.34 ± 0.52	7.78 ± 0.46	1.11 ± 0.31 c	26.22 ± 2.09 b

Data shown are mean ± SE. Values within a column followed by the same letter are not significantly different according to Fisher's LSD test ($P > 0.05$). a, Root; b, Shoot.

Indole-3-acetic acid production

IAA is the major auxin in plants, and controls many important physiological processes, including cell enlargement, cell division, tissue differentiation and responses to light (Patten & Glick 2002). The time course of auxin production was evaluated, and maximum production was found to occur usually in the stationary phase. The amount of produced IAA was high for strains SJ04 and SJ25, and variable for SJ48 (Table 1).

Biomass

Direct inoculation tests were performed in culture medium of micropropagated plants. PGPR strains (isolates SJ04, SJ25, and SJ48, reference WCS417r) were inoculated at a concentration of 10^6 CFU plate^{-1} and incubated for 30 days. Direct inoculation did not result in significant differences in parameters such as root length, shoot length and node number (Table 2). Root dry weight increased in all directly inoculated plants; most strikingly, a 2 mg increase in WCS417r. Shoot ramification number increased significantly ($P < 0.05$) for treatment with all strains, and the increase was two- to three-fold higher for native strains than for controls. Leaf number increased significantly (20–40% higher than in controls; $P < 0.05$) after treatment with WCS417r or SJ48 (Table 2).

Shoot fresh weight was increased significantly after treatment with each of the strains (Fig. 1), and was ~140 and 190 mg higher for SJ48 and WCS417r, respectively, than for controls ($P < 0.05$).

Trichome number increased significantly after treatment with each of the strains (Fig. 2); the number was ~2.5-fold higher for SJ25 and ~60% higher for the other strains in comparison with controls.

Secondary metabolites

Total essential oil content in plants inoculated with three of the tested bacterial strains was significantly ($P < 0.05$) higher than in controls (Fig. 3). The increase was ~two-fold for native strain SJ25, and ~60% for reference WSC417r and native SJ04. Essential oil content in native SJ48 was not significantly different from that in controls.

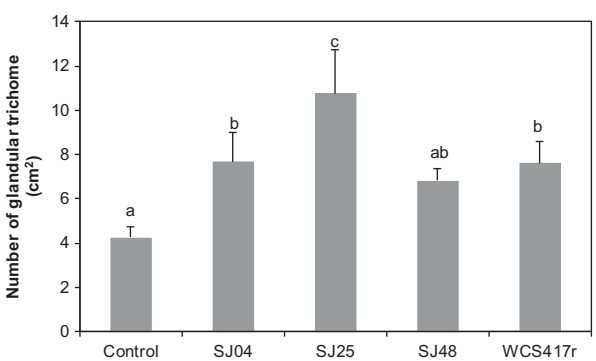

Fig. 2. Number of glandular trichomes per mm^2, at day 30, in *M. piperita* plants inoculated with five PGPR strains as in Fig. 1. Letters above bars indicate significant differences according to Fisher's LSD test ($P < 0.05$).

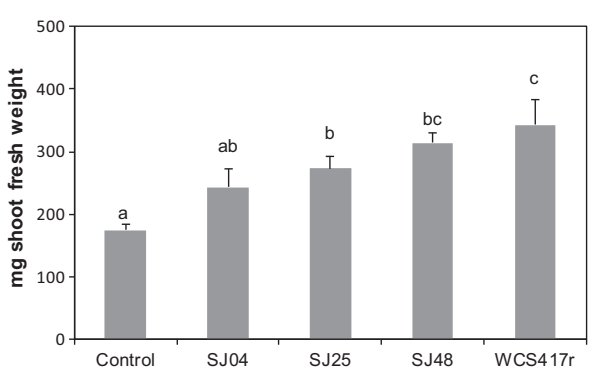

Fig. 1. Shoot fresh weight (mg) of *M. piperita* inoculated with native and reference *Pseudomonas* strains and a control. Letters above bars indicate significant differences according to Fisher's LSD test ($P < 0.05$).

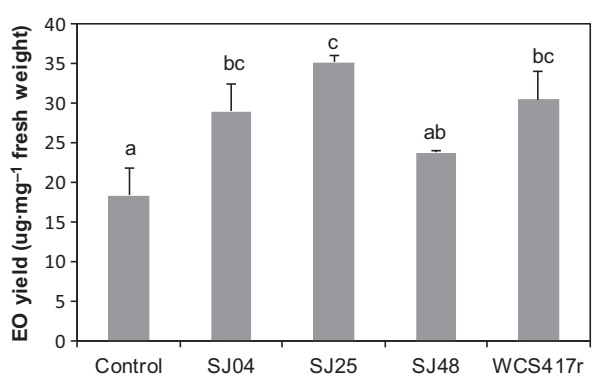

Fig. 3. Total essential oil yield in *M. piperita* plants inoculated with five PGPR strains as in Fig. 1. Letters above bars indicate significant differences according to Fisher's LSD test ($P < 0.05$).

Table 3. Variation in content (μg·mg^{-1} fresh weight) of major essential oils of *M. piperita* inoculated with bacterial strains as in Table 2.

EO	limonene (μg·mg^{-1} FW)	terpineol (μg·mg^{-1} FW)	menthone (μg·mg^{-1} FW)	menthofuran (μg·mg^{-1} FW)	menthol (μg·mg^{-1} FW)	pulegone (μg·mg^{-1} FW)
control	0.20 ± 0.03	0.13 ± 0.01 a	0.13 ± 0.05 a	0.20 ± 0.09	0.55 ± 0.08 a	17.13 ± 3.15
SJ04	0.17 ± 0.02	0.23 ± 0.03 b	0.23 ± 0.03 b	0.20 ± 0.04	4.20 ± 0.45 c	15.73 ± 4.91
SJ25	0.26 ± 0.08	0.63 ± 0.09 c	0.18 ± 0.04 b	0.17 ± 0.05	3.55 ± 0.67 bc	17.21 ± 3.46
SJ48	0.26 ± 0.09	0.40 ± 0.10 b	0.19 ± 0.05 b	0.16 ± 0.02	2.68 ± 0.06 b	19.46 ± 0.72
WCS417r	0.20 ± 0.01	0.33 ± 0.07 b	0.22 ± 0.06 b	0.37 ± 0.11	0.70 ± 0.12 a	27.18 ± 3.43

Data shown are mean ± SE. Values within a column followed by the same letter are not significantly different according to Fisher's LSD test ($P > 0.05$).

Effects of direct bacterial inoculation on the major monoterpenes present in *M. piperita* essential oils were variable (Table 3). Terpineol content increased significantly for treatment with all four strains: up to four-fold for SJ25. The increase of menthone was similar (~50%) for the four strains. Menthol showed the largest increases for treatment with SJ04 (~seven-fold) and SJ25 (~six-fold). Levels of limonene, menthofuran and pulegone were not significantly altered ($P > 0.05$) for any of the strains.

Principal components analysis

The PCA was performed to correlate effects of inoculation with the four PGPR strains with production of IAA and monoterpenes (Fig. 4). This type of analysis provides a graph that facilitates visualisation and interpretation of the data set and the variables. For PCA, the observations (cases) were the inoculated strains and their phylogenetic affiliations, and the variables were IAA production, total essential oil content and content of major monoterpenes after treatment.

A plot defined by the first two principal components was sufficient for our purpose because it explained most (86%) of the variation in the data and gave a cophenetic correlation coefficient of 0.98. In the two-dimensional coordinate system based on the first two principal components (Fig. 4) it was possible to differentiate the four strains in spite of their phylogenetic affiliations. Strain SJ25 was located in proximity to the variables total essential oil (TEO), trichome number (NT), limonene content (LIM) and terpineol content (TERP). WCS417r was located in proximity to the variables menthofuran content (MF) and pulegone content (PUL). SJ04 and SJ48 were fairly close to each other and located between the variables menthol content (MOL) and menthone content (MNE).

In regard to associations among variables, we observed a strong positive correlation (acute angle in Fig. 4) between the variables IAA production and menthol content. There were also positive correlations among total essential oil content, trichome number, limonene content and terpineol content, and among menthofuran, pulegone and menthone contents. Surprisingly, no associations (*i.e.* right or obtuse angles in Fig. 4) were observed between menthone and menthol content, or among total essential oils and the major essential oil components (>60% of total) pulegone and menthofuran.

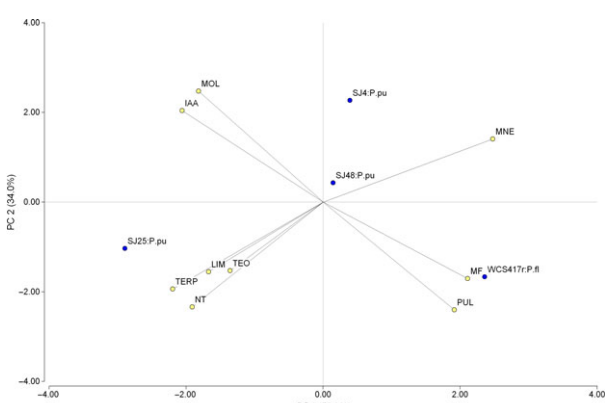

Fig. 4. Plot of first two principal components (PC1 and PC2) from PCA, illustrating relationships among strains, phylogenetic affiliations, major essential oil components of *M. piperita* after PGPR inoculation and bacterial IAA production. Diamonds indicate combinations of biotypes and affiliations. Circles indicate biological variables: IAA production, LIM (limonene content), TERP (terpineol content), MNE (menthone content), PUL (pulegone content), MF (menthofuran content), MOL (menthol content), TEO (total essential oils) and NT (trichome number).

DISCUSSION

Plant growth-promoting rhizobacteria (PGPR) are a heterogeneous, beneficial group of microorganisms living in the rhizosphere or on root surfaces of the host plant (Kloepper 1993). We inoculated *P. putida* strains isolated from *M. piperita* roots and rhizosphere soils on micropropagated plants *in vitro*. Fluorescent *Pseudomonas* strains have been isolated from rhizosphere soils of numerous crop plants, including cotton, rice (Loaces *et al.* 2011), banana (Naik *et al.* 2008), rape (Patten & Glick 2002), sugar cane (Rameshkumar *et al.* 2012), wheat and barley (Mavrodi *et al.* 2001; Parejko *et al.* 2012). Most research to date has focused on the genus *Pseudomonas* because of its wide distribution in a variety of environments and its ease of culture under laboratory conditions (Palleroni 2005). Few studies have focused on PGPR isolated from rhizospheres of *Mentha* (mint) or other aromatic crop plants, perhaps because of a presumption that essential oils might be released in root exudates and exert antimicrobial effects (Chen *et al.* 2004).

The PGPR can affect plant growth indirectly or directly through siderophore production (Babalola 2010). Secretion of siderophores by rhizobacteria can stimulate plant growth either by improving iron nutrition to the plant or by reducing the availability of iron to phytopathogens, thus protecting plant health (Glick 2012). In the present study, siderophore production was observed only for native strain SJ04. However, high numbers of siderophore-producing *Pseudomonas* strains have been isolated from other crop species, including tomato, rice and soybean (Naik *et al.* 2008; Babalola 2010). Phosphate-solubilising bacteria are common in the rhizosphere (Anzuay

et al. 2013). Solubilisation of phosphate in the rhizosphere appears to be the most common mechanism whereby PGPR increase nutrient availability to the host plant (Kloepper 1993; Glick 2012). Each of the native strains tested in the present study was capable of phosphate solubilisation.

Phytohormone production is another important process whereby PGPR affect plant growth. Most commonly, IAA secreted by PGPR increases root number and root length, thereby increasing root surface area and the capacity of the plant to access soil nutrients. Each of our native *P. putida* strains produced IAA in the presence of the precursor L-tryptophan. The native strains tested displayed strong plant growth-promoting properties, including concurrent IAA production, siderophore production and phosphate solubilisation. Strain SJ04 was positive for all tested PGPR activities. Bacterial isolates in many studies have been found to display multiple PGPR traits, which may promote plant growth directly, indirectly or synergistically.

In vitro inoculation with the four tested strains (SJ04, SJ25, SJ48, WCS417r) had varying growth-promoting and developmental effects on *M. piperita*. Shoot fresh weight was significantly higher (~40–100%) in the treatment groups than in controls. This effect appeared to be due primarily to increased leaf area (data not shown), and in part to increased leaf number and ramification number. These findings rule out the possibility that the enhanced growth resulted simply from increased plant hydration. Root dry weight was higher for all tested strains than in controls. This increase was due primarily to increased number of lateral roots. Single inoculation with the tested native strains resulted in significant increases in all the measured growth parameters except shoot length and root length. Native SJ48 and reference WCS417r produced significant increases in all measured growth parameters (Table 2) and on plant biomass. Similar results have been reported in several plant species. In studies of wheat, lettuce, radish and pine, Vessey (2003) observed various effects of PGPR on root morphology mediated by phytohormone production. Zhang *et al.* (2007) found that enhanced lateral root formation led to increased root surface area and nutrient uptake potential in *Arabidopsis thaliana*. Similar findings have been obtained in inoculation trials in other aromatic plants, *e.g.* our studies of *Origanum majoricum, O. majorana* and *Tagetes minuta* grown in vermiculite pots and inoculated with WCS417r (Banchio *et al.* 2010; Cappellari *et al.* 2013).

In the present *in vitro* study, plants were grown in semi-solid MS medium in which nitrogen and other nutrients were readily available. The observed plant growth-promoting effects were therefore not due to phosphate solubilisation or siderophore production (Kloepper 1993). Rather, the growth enhancement following PGPR inoculation was presumably due to increased bacterial production and emission of growth hormones and/or volatile organic compounds (VOCs; Glick 2012; Ryu *et al.* 2004). Such components, collectively termed 'plant growth regulators', are organic substances that affect plant physiological processes at extremely low concentrations and play regulatory roles in plant growth and development (Dobbelaere *et al.* 2003). The five well-known classes of plant growth regulators are auxins, gibberellins, cytokinins, ethylene and abscisic acid (ABA; Dobbelaere *et al.* 2003). Considerable research attention has been paid to the role of auxin, a phytohormone. IAA is a naturally occurring, major auxin in plants and controls many important physiological processes, including cell enlargement, cell division, tissue differentiation and responses to light and gravity (Leveau & Lindow 2005). In the present study, each of the native strains was capable of producing IAA, and some of the observed effects were presumably due to the ability to utilise root exudates and synthesise plant growth regulators. Bharucha *et al.* (2013) observed similar capacity in *P. putida* isolates from *Medicago sativa*.

On the other hand, the observed plant growth-promoting effects might also be related to functional VOCs released by the rhizobacteria (Santoro *et al.* 2011). Studies in recent decades have demonstrated the ability of rhizobacteria to synthesise and release VOCs that travel short or long distances through the soil (Wenke *et al.* 2010). Zhang *et al.* (2007) showed that exposure to *B. subtilis* VOCs enhanced photosynthetic efficiency, chlorophyll content and cell expansion. We have previously observed increases comparable to those in the present *in vitro* study in shoot fresh weight and root dry weight in *M. piperita* plants inoculated with *B. subtilis* GB03 and WCS417r VOCs (Santoro *et al.* 2011).

To analyse essential oil production, we counted numbers of peltate trichomes on day 30. Peltate trichome numbers on the adaxial surface were higher in the four treatment groups, particularly SJ04 and SJ25, than in controls. Glandular trichome density is controlled by numerous environmental and hormonal factors; jasmonates in particular play an important role (Li *et al.* 2004; Lange & Ahkami 2013).

Levels of the major essential oil components analysed (limonene, terpineol, menthone, menthol, pulegone, collectively comprising ~90% of total essential oils) were markedly different in the four treatment groups than in controls. Pulegone was by far the predominant component, accounting for ~75% of total essential oils; menthone and menthol are the predominant monoterpenes of peppermint plants grown in soil (Guntert *et al.* 2001). Our previous study (Santoro *et al.* 2013) indicated that the predominance of pulegone in these particular assays is a consequence of the *in vitro* culture conditions (Santoro *et al.* 2013). Menthone level increased in all tested strains, but only SJ04- and SJ25-treated plants displayed increased levels of menthol, the following compound in the biosynthetic pathway and the monoterpene of major commercial interest in peppermint. SJ04 and SJ25 were also associated with the highest trichome number and total essential oil content. Enhanced essential oil content was not due to increased biomass because it was not associated with the highest values of shoot fresh weight. These findings suggest that rhizobacteria induce biosynthesis of secondary metabolites, whose levels were calculated per gram fresh weight.

Terpene biosynthesis in inoculated plants appeared to be increased in our study, although we did not directly measure this process. Similar results have been obtained for other 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 essential oil concentration in *Origanum* sp.; and Copetta *et al.* (2006) observed increases of glandular hair abundance and essential oil yield in *O. basilicum* exposed to mycorrhizal fungi. Our previous studies have demonstrated increased monoterpene production in several aromatic plant species inoculated with PGPR (Banchio *et al.* 2009, 2010;

Cappellari *et al.* 2013). In the present study, total essential oil yield in the four treatment groups was 60% to 100% higher ($P < 0.001$) than in controls. In our previous studies, *P. fluorescens* inoculation increased total essential oil yield 2.5-fold in *O. majoricum*, 24-fold in *O. majorana* and 50% in *T. minuta* (Banchio *et al.* 2010; Cappellari *et al.* 2013), and increased bacterial VOCs two-fold in *M. piperita* (Santoro *et al.* 2011). The effects of rhizobacteria and their VOCs on secondary metabolites clearly vary depending on the bacterial strain. All the studies mentioned above were performed with *Pseudomonas* species, and various strains of *P. putida* in the present study produced different responses, suggesting that rhizobacteria are recognised by the host plant in a strain-specific manner.

The observed increase in monoterpene levels in *M. piperita* may result from induction of systemic resistance, a phenomenon that occurs upon stimulation of a plant's defence mechanisms. Various non-pathogenic PGPR strains have the ability to induce systemic disease resistance in plants against broad-spectrum phytopathogens (Kloepper 1993; Glick 2012). Induction of systemic resistance has been shown to increase plant cell wall strength and alter plant physiology and metabolic responses, leading to enhanced synthesis of defence chemicals upon challenge by pathogens and/or abiotic stress factors (Wittstock & Gershenzon 2002). Essential oil concentrations and composition in plants play several key roles in plant–environment interactions and plant–plant communication. Several monoterpenes are synthesised *de novo* in aromatic and other types of plant in response to herbivory, apparently to prevent damage from further attacks (Harrewijn *et al.* 2001; Banchio *et al.* 2005). Increased synthesis of essential oils is a defence response to colonisation by microorganisms, since several essential oils have antimicrobial properties (Zheljazkov *et al.* 2010). Increases in essential oil synthesis observed in the present study presumably represent defence responses to colonisation by microorganisms. Several essential oil compounds in *M. piperita* exert insecticidal, antifungal and/or antibacterial effects (Sangwan *et al.* 2001).

Induction of systemic resistance can be elicited not only by pathogens and herbivores but also beneficial microorganisms and certain synthetic compounds. It provides plants with an enhanced capacity for rapid and effective activation of cellular defence responses against pathogen or insect attack (Pineda *et al.* 2013). The phenomenon typically involves up-regulated expression of defence-related genes only after the plant is attacked, and is therefore less costly than expression of constitutive or (to a lesser extent) induced defences (Van Hulten *et al.* 2006). Induction of systemic resistance against herbivores also involves 'priming' of jasmonate-dependent responses (Van Oosten *et al.* 2008; Pineda *et al.* 2013) and other yet-unknown mechanisms (Valenzuela-Soto *et al.* 2010). Priming of plant defences by beneficial microorganisms has been proposed to be a consequence of the modulation of plant immune systems associated with establishment of symbiosis and related changes in defence-related signalling (Pozo & Azcón-Aguilar 2007; Zamioudis & Pieterse 2012).

Expression of the gene controlling terpenoid biosynthesis in *Mentha aquatica* increased in response to herbivore feeding (Lamiri *et al.* 2001). Monoterpene synthesis was similarly promoted by herbivory in *Minthostachys mollis* (Banchio *et al.* 2005) and other plant species (Harrewijn *et al.* 2001; Hummelbrunner & Isman 2001). The increased monoterpene content observed in inoculated plants in the present study may result from growth-promoting substances secreted by PGPR that affect plant metabolic processes. Terpenoid biosynthesis depends on primary metabolism and oxidative pathways for carbon and energy supply (Singh *et al.* 1991). Giri *et al.* (2003) found that 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 treated groups than in controls. Essential oil yield is strongly correlated with total number and developmental distribution patterns of glandular trichomes, part of the biosynthetic machinery that rapidly and efficiently converts imported carbohydrates into essential oils (Lange *et al.* 2011; Lange & Turner 2013).

Single inoculation with *Pseudomonas* in the present study affected plant growth, plant development and trichome density. SJ04 and SJ25 treatment produced the largest increases in secondary metabolite levels and glandular trichome number, whereas SJ48 and WCS417r produced the largest increases in biomass. Because the tested native strains all belonged to the same species, these findings indicate that responses to single inoculation are strain-specific. The effects of particular strains could result from either single or combined action of bacterial metabolites and regulators on host plant roots (Glick 2005; Ambrosini *et al.* 2012).

Multivariate PCA in the present study helped clarify the relationships among the cases and variables, and facilitated visualisation and interpretation of the data set. The distribution of the strains in Fig. 4 reflects the complexity of interactions and the combination of responses of each strain to each evaluated variable. However, the positions are functions of the variables representative of each strain. For example, the position of SJ25 reflects enhancement of the compounds involved in the early steps of monoterpene biosynthesis (Mahmoud & Croteau 2002) as a representation of its inoculation effects, despite the striking increase of total essential oil content and the number of trichome variables. On the other hand, the position of WCS417r reflects the induction of menthofuran and pulegone production (Table 3). The type of compounds that undergo enhancement is consistent with the biocontrol PGPR activity of the reference strain, in view of the strong antimicrobial effect of these monoterpenes (Van Peer *et al.* 1991). The behaviour of the group consisting of SJ04 and SJ48 is explained by the combination of plant responses to inoculation on menthol and menthone content.

The PCA also provides insight regarding relationships among the variables. A positive correlation is evident in Fig. 4 between trichome number and total essential oil content extracted from treated plants. Thus, increased essential oil content was a consequence of higher trichome number rather than higher monoterpene accumulation in these epidermal structures. The variables of pulegone, menthofuran and menthone content are positively correlated with each other, consistent with the monoterpene biosynthetic pathway; pulegone is the precursor of menthofuran and menthone. On the other hand, the strong correlation between menthol content and IAA production indicates that production of a bacterial auxin promotes maturation of the monoterpene of highest commercial interest extracted from peppermint.

These findings improve our knowledge of the role of native PGPR (*Pseudomonas*) strains in enhancing physiological processes of host plants, and help elucidate the mechanism of essential oil production in peppermint. This is a first step and a basis for future studies aimed at improved agricultural practices for aromatic plants in general.

CONCLUSIONS

The findings present an option for transition from traditional inorganic farming methods toward eco-friendly organic farming methods. For the numerous medicinal and aromatic plant species that are consumed without further processing, it is important to avoid the presence of any synthetic compounds in the harvested crop, or any adverse environmental impacts. Inoculation of *M. piperita* (peppermint) with PGPR significantly enhances biomass and essential oil production, allowing for reduced use of fertilisers, sustainable agricultural production and reduced environmental impacts.

In the present study, we observed differences in responses between the native and non-native evaluated strains, illustrating the strain specificity of the microorganism–plant interactions. Native bacterial isolates are generally preferable for inoculation of crop plants because they are already adapted to the environment and have a competitive advantage over non-native strains in enhancing the quality of essential oils, particularly menthone and menthol content. Certain native PGPR strains, such as those evaluated in the present study, are promising candidates for bio-inoculant formulation. Commercial development of *P. putida* and other PGPR for growth promotion of *M. piperita* and other aromatic crops will require carefully designed field trials.

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