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Increase of secondary metabolite content in marigold by inoculation with plant growth-promoting rhizobacteria

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ABSTRACT

The effects of single inoculation and co-inoculation of two plant growth-promoting rhizobacteria (PGPR) (*Pseudomonas fluorescens*, *Azospirillum brasilense*) on growth and essential oil (EO) composition and phenolic content were evaluated in marigold (*Tagetes minuta*). Plant growth parameters (shoot fresh weight, root dry weight, leaf number, node number) were measured. EO yield increased 70% in *P. fluorescens*-inoculated and co-inoculated plants in comparison with control (non-inoculated) plants, without altering EO composition. The biosynthesis of the major EO components was increased in the inoculated plants. Shoot fresh weight and EO yield were significantly higher in *P. fluorescens*-inoculated and in co-inoculated plants than in control plants. The total phenolic content was 2-fold higher in singly-inoculated or co-inoculated treatments than in controls. In view of the economic importance of monoterpenes and phenolic compounds for a variety of applications in the food and cosmetic industries, *P. fluorescens* and other PGPR have clear potential for improving the productivity of cultivated aromatic plants. Better understanding of the processes that affect the accumulation of secondary metabolites will lead to increased yields of these commercially valuable natural products.

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1. Introduction

Wild marigold (*Tagetes minuta*) is an annual plant species native to southern South America that has become widespread throughout the world. The essential oil (EO) obtained from this species, known as "Tagetes oil" to both retailers and end users, is a commercially valuable product (Singh and Singh, 2003) that is used primarily in the preparation of high-grade perfumes (Kaul et al., 2000). Because of the high demand for Tagetes oil, there has been increasing cultivation of *T. minuta* for commercial production (Ghera and Leon, 1999).

Intensive farming practices aimed at high crop yield and quality traditionally require the extensive use of chemical fertilizers, which are expensive and have negative environmental impacts. There has been increasing interest in environmentally "friendly", sustainable, and organic agricultural practices (Zahir et al., 2004). In many cases, the use of bio-fertilizers that contain beneficial microorganisms rather than synthetic chemicals has been found to enhance plant growth through improved nutrient supply and to promote environmental health and soil productivity (Van Loon, 2007).

Numerous species of bacteria, most of which are associated with the plant rhizosphere, have been shown to have beneficial effects on plant growth and on crop yield and quality. Such bacteria are collectively called "plant growth-promoting rhizobacteria" (PGPR). They promote plant growth indirectly by reducing or eliminating the deleterious effects of pathogenic organisms through various mechanisms that include the induction of host resistance to the pathogen (Van Loon and Glick, 2004; Van Loon, 2007). PGPR promote plant growth directly by providing the host plant with synthesized compounds, facilitating nutrient uptake, fixing atmospheric nitrogen, solubilizing phosphorus and other minerals, producing siderophores that solubilize and sequester iron, synthesizing phytohormones (e.g., auxins, cytokinins, gibberellins) that enhance various stages of plant growth, and synthesizing enzymes that modulate plant growth and development (Lucy et al., 2004; Gray and Smith, 2005). Numerous studies have demonstrated improvement in plant growth and development following seed or root inoculation with microbial strains that are capable of producing plant growth regulators (Zahir et al., 2004).

Certain aspects of PGPR interactions have been well studied; e.g., growth effects, nutritional exchange, biocontrol of plant pathogens, and tolerance of water stress and other adverse environmental conditions. However, our knowledge regarding the potential of PGPR to

Abbreviations: CFU, colony-forming unit(s); EO, essential oil; IAA, indole acetic acid; PGPR, plant growth-promoting rhizobacteria.

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affect the secondary metabolic pathways of aromatic and medicinal plants remains fragmentary.

We report here the results of a comparative analysis of the effects of *T. minuta* root inoculation with two PGPR strains on plant development and on the qualitative and quantitative synthesis of EOs and phenols.

2. Material and methods

2.1. Bacterial strains, culture conditions, media, and treatments

Two bacterial strains previously reported as PGPRs, *Pseudomonas fluorescens* WCS417r and *Azospirillum brasilense* Sp7 (Van Loon, 2007) were 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.

Bacterial cultures were grown overnight at 30 °C with rotation at 120 rpm until reaching the exponential phase. Each culture was then washed twice in 0.9% NaCl by centrifugation (4300 × g, 10 min, 4 °C) in an Eppendorf centrifuge, resuspended in sterile water, and adjusted to a final concentration of $\sim 10^9$ colony-forming units (CFU)/mL for use as an inoculum. Plants were grown in plastic pots (diameter 12 cm, depth 22 cm) containing 250 g sterilized vermiculite. *T. minuta* seeds were surface sterilized in 70% ethanol for 5 min, rinsed 5 times with sterile water, dipped in 1% NaCl for 1 min, rinsed 5 times with sterile water, planted in vermiculite (one seed per pot), and inoculated with 1 mL of bacterial suspension. The treatments were: (1) WCS417r; (2) Sp7; (3) WCS417r plus Sp7; (4) sterile water (control).

2.2. Greenhouse experiments

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

The experiments were replicated (10 pots per treatment; 1 plant per pot), and the pots were arranged randomly in the growth chamber. Ninety days after inoculation, the plants were removed from the pots, the roots were washed to remove vermiculite, and standard growth parameters (shoot length, leaf number, node number, shoot fresh weight, root dry weight) were measured.

2.3. Extraction of EOs

The shoot samples were individually weighed and subjected to hydrodistillation in a Clevenger-like apparatus for 40 min, and the volatile fraction was collected in dichloromethane. Delta dodecalactone (0.1 μL in 50 μL ethanol) was added as an internal standard.

T. minuta contains up to 1% volatile oils, which consist of 15 or more different compounds depending on the chemotype (e.g., Zygadlo et al., 1990; Gil et al., 2000; Tomova et al., 2005). We identified the major EOs, accounting for ~75% of the total EO volume, as limonene, (Z)-β-ocimene, dihydrotagetone, (E)-tagetone, (Z)-tagetone, linalool, humulene, (E)-ocimenone, (Z)-ocimenone. EO components were identified based on mass spectral and retention time data in comparison with those of standard compounds. Each of these EOs consisted mainly (>70%) of monoterpenes. These compounds were quantified relative to the delta dodecalactone standard added during the distillation procedure. Flame ionization detector (FID) response factors for each compound generated equivalent areas with negligible differences (<5%).

Chemical analyses were performed using a PerkinElmer Q-700 gas chromatograph 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 of 0.9 mL/min; source 70 eV. 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 of 0.9 mL/min.

2.4. Determination of total phenols

Total phenols were determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Each plant extract (0.5 mL) or gallic acid (standard phenolic compound) was mixed with Folin-Ciocalteu reagent (0.5 mL, diluted with 8 mL distilled water) and aqueous Na_2CO_3 (1 mL, 1 M). After 1 h, the level of total phenols was determined by colorimetry at a wavelength of 760 nm. Total phenol values were expressed in terms of mg gallic acid (a common reference compound) equivalent per g plant dry weight (Lan et al., 2007).

2.5. Estimation of numbers of rhizobacterial CFU

In vitro trials were performed to estimate the presence and the co-existence of the two inoculated bacterial strains. Single colonies of the 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 h at 28 °C. The bacterial suspensions were diluted in sterile water to a final concentration of 10^5 CFU/mL as determined by optical density measurement. Plants were treated with 1 mL of one of the resulting suspensions for treatments (1) WCS417r and (2) Sp7 or with 1 mL each of the two suspensions for treatment (3) WCS417r plus Sp7. Sterilized *T. minuta* seeds were cultivated in 100 mL of basal medium (BM) consisting of Murashige and Skoog (1962) salts plus 0.7% (w/v) agar and 1.5% (w/v) sucrose (Santoro et al., 2011). All culture media contained 30 g/L sucrose and 7.5 g/L agar. When the seeds had germinated and the first leaves appeared, the plants were inoculated or co-inoculated with 1 mL of the bacterial suspensions.

The plants were removed from the pots 7 or 14 days after inoculation. Ten plants were used for each treatment. Roots were resuspended in 100 mL sterile saline solution and ground in a sterilized mortar. Various dilutions of the ground material were placed on LB agar and supplemented with antibiotics according to the natural resistance of the particular strains for each co-inoculation. For counting *P. fluorescens* WCS417r and *A. brasilense* Sp7, the antibiotic kanamycin (Km) and the antibacterial agent Cm (chloramphenicol), respectively, were added to the medium at a final concentration of 50 μg/mL. The plates were incubated at 28 °C, and the number of viable cells was determined by plating diluted samples. The number of CFU was determined after 24 and 48 h incubation.

2.6. Statistical analyses

The 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 in means were considered to be significant for p values < 0.05. The Infostat software program, version 2008 (Group

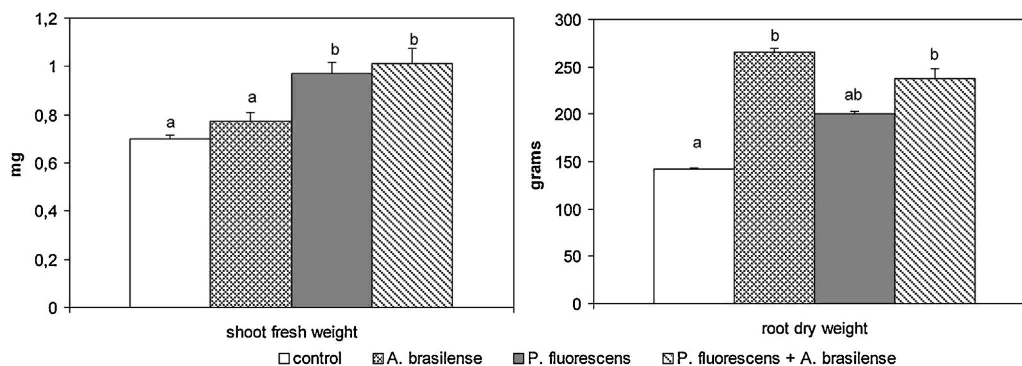

Fig. 1. Shoot fresh weight and root dry weight of *T. minuta* plants singly inoculated or co-inoculated with two PGPR strains. Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

Infostat, Universidad Nacional de Córdoba, Argentina) was used for all statistical analyses.

3. Results and discussion

3.1. Plant growth parameters

Increases in growth and development parameters following inoculation with PGPR have been reported for many plant species (Banchio et al., 2005, 2009; Gray and Smith, 2005; Van Loon, 2007; Vessey, 2003; Zahir et al., 2004). In the present study, the effects of PGPR inoculation on *T. minuta* growth and development varied depending on the inoculated strain (*P. fluorescens* WCS417r, *A. brasilense* Sp7, or their combination) (Fig. 1; Table 1). Almost all of the growth parameters evaluated were significantly ($p < 0.05$) modified by each of the three inoculation treatments (Table 1). The only exception was stem length.

Inoculation with *A. brasilense* caused significant increases in leaf number, root length, and root dry weight but not in shoot fresh weight (Fig. 1; Table 1). The root dry weight was 80% higher than that of control (non-inoculated) plants. In our previous study of peppermint (*Mentha piperita*), the volatile compounds emitted by *A. brasilense* did not cause a significant change in stem fresh weight (Santoro et al., 2011). In contrast, the inoculation of Italian oregano (*Origanum x majoricum*) with *A. brasilense* resulted in a 30% increase in fresh weight (Banchio et al., 2010). The increase of *T. minuta* root weight in the present study was due primarily to an increased number of lateral roots (data not shown). Various effects of PGPR on root morphology have been reported (Vessey, 2003). Enhancement of lateral root formation leads to increased root surface area and nutrient uptake potential (Zhang et al., 2007). *Azospirillum* exerted a clear phytostimulatory effect on the root system of *T. minuta.* The production of auxin has been shown to stimulate root growth and to promote plant growth (Lucy et al., 2004; Babalola, 2010).

Individual inoculation of *P. fluorescens* and its co-inoculation with *A. brasilense* significantly increased (by ~50%) the shoot fresh weight (Fig. 1). Similar trends were observed for the leaf number and node number (Table 1). The leaf number was 33% higher in *P. fluorescens*-inoculated and co-inoculated plants than in controls, as reflected by the increased shoot fresh weight (Fig. 1). The root dry weight in these treated plants was significantly (~35%) increased, partly because of an increase in the root length (Table 1). Similar increases in our study of marjoram (*Origanum majorana*) were observed for shoot length, shoot weight, leaf number, node number, and root dry weight (Banchio et al., 2008a,b).

Fluorescent pseudomonads are a group of PGPR that have been reported to improve the overall growth of various crops (Dey et al., 2004; Vikram, 2007). Inoculation of *P. fluorescens* improved plant growth through the production of growth-promoting substances such as indole acetic acid (IAA) and cytokinins (De Salamone et al., 2001; Vikram, 2007). The roles of auxins and cytokinins in enhancing plant cell division and root development have been well documented (Arshad and Frankenberger, 1998). IAA is involved in root initiation, cell division, and cell enlargement (Gray and Smith, 2005). Cytokinins promote cell division, cell enlargement, and tissue expansion in certain plant parts (Dey et al., 2004; Gray and Smith, 2005).

In the present study, plants were grown in vermiculite under an artificial growing system. We chose this inert substrate because its use facilitates the removal of the plant without damaging the roots and because the porosity of vermiculite facilitates the movement of volatile compounds emitted by rhizobacteria (Santoro et al., 2011). All plants received Hoagland's nutrient solution in which nitrogen and other nutrients were readily available. The growth-stimulatory effects shown in Fig. 1 and Table 1 were therefore not due to the solubilization of phosphates, oxidation of sulfates, increased nitrate availability, extracellular production of antibiotics, or induction of plant systemic resistance (Kloepper, 1993). Rather, the enhanced growth of *T. minuta* observed following bacterial inoculation was presumably due to increased production of growth hormones and/or volatile compounds emitted by the PGPR (Banchio et al., 2009; Santoro et al., 2011).

3.2. Secondary metabolites

Phenolic compounds are a major class of plant secondary metabolites and one of the most common and widespread types of plant components in general. They are essential for the growth and reproduction of plants. Some are produced constitutively, while others are induced as a plant defensive response. In contrast to basic metabolism, which refers to the anabolic and catabolic processes that are required for cell maintenance and proliferation, secondary metabolism refers to the compounds present in specialized cells that are not directly essential for basic photosynthetic or respiratory metabolism but are considered to be necessary for plant survival in the external environment (Lattanzio et al., 2006). It has been well established that secondary metabolites are not simply waste products of primary metabolism that accumulate in the plant cell because of the absence of an efficient excretory system (Kliebenstein, 2004; Theis and Lerdau, 2003; Whiting, 2001). Secondary metabolites act as defensive compounds (against herbivores, microbes, viruses, or competing plants) and as signaling compounds (to attract pollinating or seed-dispersing animals) and also protect the plant from ultraviolet radiation and oxidants (Kutchan, 2001).

In the present study, the total phenolic content was 2-fold higher ($p < 0.005$) in singly-inoculated or co-inoculated plants than in controls (Fig. 2). Although the Folin-Ciocalteu method is commonly

Table 1
Effects of single inoculation and co-inoculation with two PGPR strains on the growth and morphogenesis of *T. minuta* plants. The values shown are mean ± standard error (SE). Means followed by the same letter in a given column are not significantly different according to Fisher's LSD test (criterion of significance: $p < 0.05$).

	Plant growth		Plant Morphogenesis	
	Root length (cm)	Shoot length (cm)	Node number	Leaf number
Control	27.0 3 ± 0.20 *a*	11.81 ± 0.35 *a*	9.76 ± 0.32 *a*	12.44 ± 0.40 *a*
A. brasilense	38.18 ± 2.39 *b*	11.49 ± 0.49 *a*	9.50 ± 0.36 *a*	14.94 ± 0.45 *b*
P. fluorescens	37.59 ± 0.75 *b*	12.44 ± 0.36 *a*	10.71 ± 0.26 *b*	16.18 ± 0.54 *c*
A. brasilense + *P. fluorescens*	33.93 ± 1.81 *b*	12.34 ± 0.54 *a*	10.38 ± 0.27 *b*	16.68 ± 0.52 *c*

used for the determination of total phenolic content in botanical and biological samples (Ignat et al., 2011), it has limitations. Other reducing agents may also react with the Folin-Ciocalteu reagent and contribute to the total absorbance, resulting in overestimation of the total phenolic content. The differences from control levels obtained using this method was particularly significant.

Similar results were obtained in *Piper belte* L. inoculated with *Serratia marcescens* (Lavania et al., 2006). Singh et al. (2003) showed that the inoculation of chickpea seeds with *P. fluorescens* and *P. aeruginosa*, singly or in combination, induced the synthesis of specific phenolic acids (gallic, ferulic, chlorogenic) and increased total phenol content at various stages of plant growth.

There has been recent interest in phenolic acids because of their potential protective role, through the ingestion of fruits and vegetables, against oxidative damage diseases (coronary heart disease, stroke, cancers). Recent studies have clearly demonstrated the important antioxidant activities of phenolic compounds and the advantages of their use as natural antioxidants in processed foods (Lattanzio et al., 2006).

In terms of EO composition, we showed previously that inoculation with rhizobacteria increased the production of monoterpenes (Banchio et al., 2009), which are biosynthesized in specialized anatomical structures termed glandular trichomes on the leaf surface (Gershenzon et al., 2000; McConkey et al., 2000). In the present study, *P. fluorescens*-inoculated or co-inoculated plants showed a 50% increase ($p = 0.02$) in total EO yield (Fig. 3). In our previous studies, *P. fluorescens* increased the total EO yield in *O. x majoricum* (2.5-fold) and in *O. majorana* (24-fold) relative to controls (Banchio et al., 2008a,b, 2010), and bacterial volatile compounds increased the EO yield 2-fold in *M. piperita* (Santoro et al., 2011). Inoculation with *A. brasilense* alone did not significantly affect the total monoterpene content in *T. minuta*, in contrast with the 2.5-fold increase observed in *O. x majoricum* (Banchio et al., 2010).

The components of interest, isolated by column chromatography and identified by GC-MS were: limonene, (Z)-*β*-ocimene, dihydrotagetone, (E)-tagetone, (Z)-tagetone, linalool, humulene, (E)-ocimenone, (Z)-ocimenone (Fig. 4). Levels of the major EO components analyzed i.e., (E)-tagetone, (Z)-(E)-ocimenone, (Z)-β-ocimene, and limonene (which accounted for ~60% of total EOs), were different in inoculated plants than in controls in most cases (Fig. 5), only humulene and linalool did not differ significantly ($p > 0.005$). (E)-ocimenone was by far the predominant component, representing ~50% of total EOs. *A. brasilense* inoculation increased the levels of (E)-ocimenone and (E)-tagetone by 71% and 66%, respectively ($p < 0.005$) (Fig. 5). *P. fluorescens* inoculation caused an increase in all of the EO components except (*Z*)-β-ocimene, humulen and linalool; and the larger increases were observed in co-inoculated plants (Fig. 5). These findings suggested an increase in terpene biosynthesis, although we did not perform direct measurement of this process. In a study of water mint (*Mentha aquatica*), increased expression of the gene controlling terpenoid biosynthesis was observed as a response to herbivore feeding; most of the terpene production was diverted to the synthesis of (+)-menthofuran, which was found to repel herbivorous mint leaf beetles (*Chrysolina herbacea*) in bioassay tests (Zebelo et al., 2011). Monoterpene synthesis was also induced by herbivore feeding in *Minthostachys mollis* (Banchio et al., 2005) and other plant species, apparently to protect damaged leaves from further attack (Harrewijn et al., 2001).

The concentrations and compositions of EOs in plants play important ecological roles. The increases in EO synthesis observed in the present study presumably represent defensive responses to colonization by microorganisms. Several EO compounds in *T. minuta* (limonene, (Z)-β-ocimene, (E)-ocimenone, (E)-tagetone) have been shown to display insecticidal, antifungal, and/or antibacterial effects (Sangwan et al., 2001; Nikkon et al., 2009).

Few studies have attempted to elucidate the relative quantitative and qualitative contributions of rhizobacteria to the formation of secondary compounds in plants. Results similar to ours were obtained for arbuscular mycorrhizal (AM) fungi in various aromatic plants. Gupta et al. (2002) inoculated the AM *Glomus fasciculatum* fungus in cultivars of *Mentha arvensis*, and observed increases in plant height, shoot growth, and oil content. Khaosaad et al. (2006) reported the alteration of EO concentration (but not EO composition) by mycorrhization of *Origanum* sp. Copetta et al. (2006) observed changes in glandular hair abundance and EO yield in *Ocimum basilicum*. Increased oil yield was associated with a significantly larger number of peltate glandular trichomes (the main site of EO synthesis). AM fungi increase plant growth and EO production because mycorrhization allows the root system to exploit a greater volume of soil by: (1) extension of the root zone; (2) reaching smaller soil pores that are not accessible by root hairs; (3) acquisition of organic phosphates by production of extracellular acid phosphatases (Bouwmeester et al., 2007).

Our results show that individual inoculation with *A. brasilense* or *P. fluorescens* affected plant growth and development, with *P. fluorescens* being more effective. However, co-inoculation with these two PGPR produced the greatest increases in the evaluated parameters (plant growth and secondary metabolites), indicating a synergistic effect of the two strains when applied together. To elucidate this synergistic effect, we performed an *in vitro* experiment

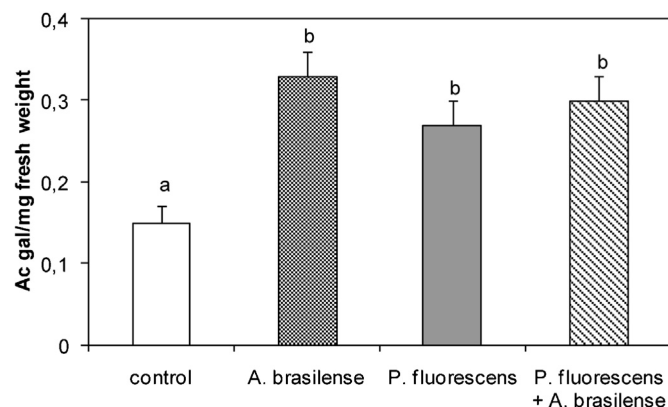

Fig. 2. Total phenol content in *T. minuta* singly inoculated or co-inoculated with two PGPR strains. Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

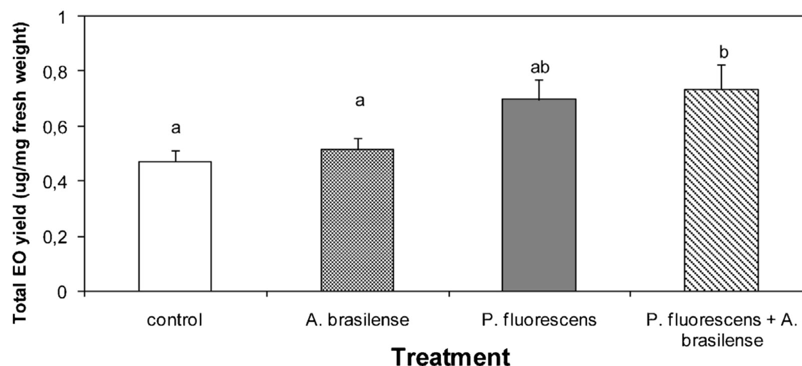

Fig. 3. Shoot EO concentration in *T. minuta* plants singly inoculated or co-inoculated with two PGPR strains. Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

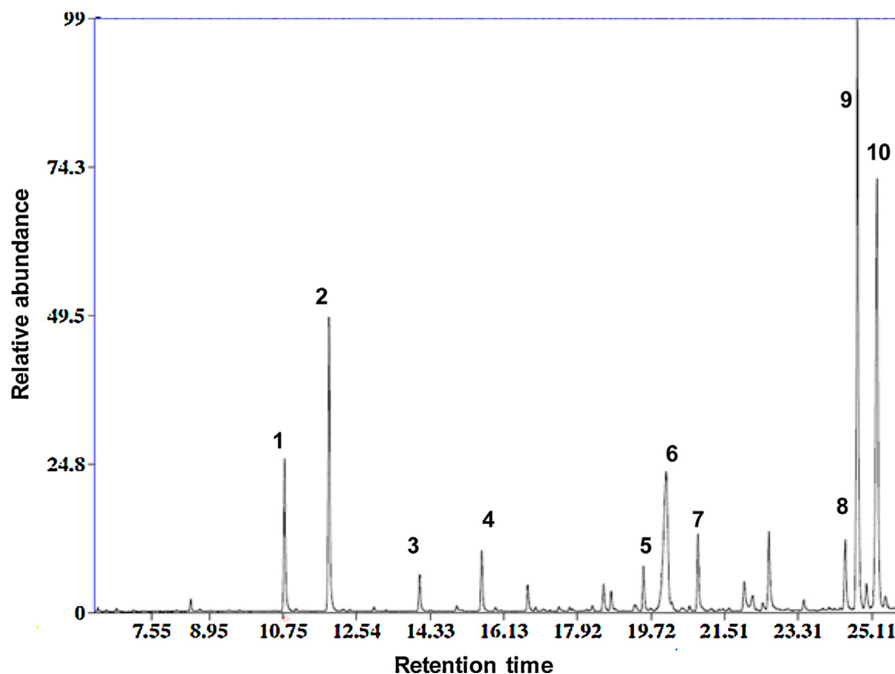

Fig. 4. GC/MS chromatogram of the essential oil from *T. minuta*. The main components were identified as: limonene (1), β-ocimene (2), dihydrotagetone (3), α pinene oxide (4), (Z) tagetone (5), (E) tagetone (6), linalool (7), α humulene (8), (E) ocimenone (9) and (Z) ocimenone (10).

to determine the presence and coexistence of the two rhizobacteria in *T. minuta* roots. Chemicals secreted into the soil by roots, broadly referred to as root exudates, are known to influence the activity of microbial communities in the rhizosphere. Root border cells thus have the potential to promote the growth of beneficial microorganisms and to inhibit the growth of (or to actively repel) pathogenic organisms (Humphris et al., 2005). The quantity and quality of the exudates produced by border cells depend on the plant genotype. The microbial communities present in the rhizosphere are clearly affected by the ability of the microorganisms to respond to and utilize particular compounds released from border cells (Hawes et al., 2003). We observed in our experiments that the *P. fluorescens*

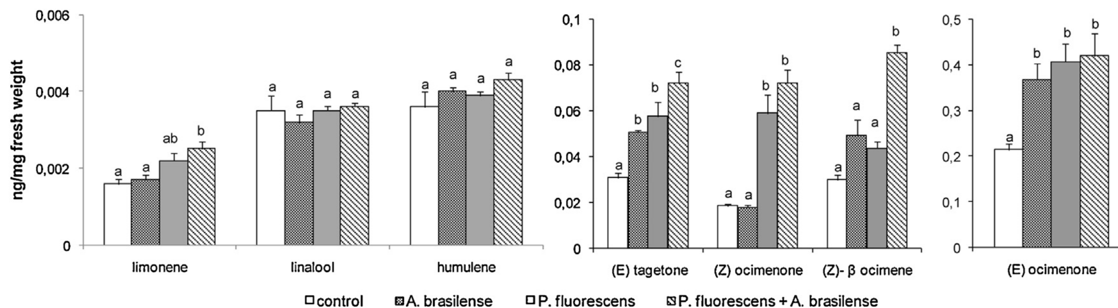

Fig. 5. Concentrations of major EO components in shoots of *T. minuta* plants singly inoculated or co-inoculated with two PGPR strains. Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

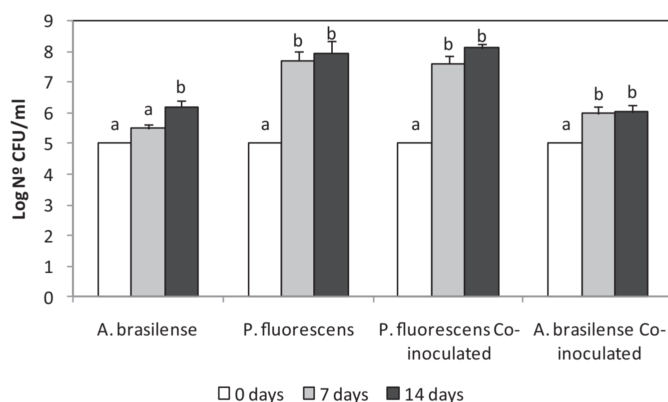

Fig. 6. Rhizobacterial populations in *T. minuta* roots.

population increased from 10^5 CFU/mL at day 0–10^8 CFU/mL at day 7 of inoculation and then remained roughly constant ($p > 0.05$ for the comparison between days 7 and 14) (Fig. 6). The *A. brasilense* population increased during this time from 10^5 to 10^6 CFU/mL ($p < 0.05$ for the comparison between days 7 and 14). The co-presence of the two strains was observed in the co-inoculation experiments, confirming that they were co-present in time and place. *P. fluorescens* showed the same behavior when co-inoculated as when alone (10^8 CFU/mL; $p > 0.05$), whereas *A. brasilense* when co-inoculated increased its population during the first 7 days and then maintained the population (10^6 CFU/mL) (Fig. 6). In all experiments, the plants were inoculated with 10^5 CFU/mL and *P. fluorescens* was better able than *A. brasilense* to adapt and increase its population. This finding may be due to the fact that *Pseudomonas* have a more rapid growth rate than do *Azospirillum*. Composition studies of various microbial communities showed that *Pseudomonas* is generally dominant in relation to *Azospirillum*, *Bacillus*, and *Streptomyces* (Soroa-Bell et al., 2009). *Pseudomonas* bacteria are abundant microorganisms in the rhizosphere because of their ability to metabolize a wide range of carbonaceous substances exuded from plant roots, the versatility of their metabolism, and their rapid growth rate (Hernandez et al., 2008). The population size of *Azospirillum* was estimated as 1–10% of the total population of the rhizosphere and reached an average of 10^3–10^6 CFU/g in wheat (Bashan et al., 2004); these findings are consistent with ours. Previous studies on co-inoculation with combinations of PGPR strains indicated that most of the strains used in the mixtures did not interfere negatively with each other (Vestberg and Cassells, 2009). On the other hand, many reports indicate that certain mixtures of microbial strains show synergistic (or at least comparable) effects on plant protection and plant growth promotion in comparison with separate inoculation (Barea et al., 2005).

Bacterial inoculants are an efficient biotechnological tool for stimulating secondary metabolism in plants, and studies of their activities will increase our understanding of certain adaptive processes that are poorly understood at present. We found that the inoculation of certain PGPR caused the systemic induction of monoterpene and phenolic compound pathways in *T. minuta*. Our results suggest that co-inoculation with *P. fluorescens* and *A. brasilense* strains may significantly increase plant growth and reduce the amount of fertilizers required for economically viable *T. minuta* crop production.

Future studies will clarify the role of other ecological factors such as root colonization sites and competition between PGPR strains and indigenous soil microflora. Carefully controlled field trials are needed before *P. fluorescens*, *A. brasilense*, and other PGPR strains can be commercially applied for improving the growth of *T. minuta* and other aromatic crops.

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