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ARTICLE

Systemic Induction of Monoterpene Biosynthesis in *Origanum* × *majoricum* by Soil Bacteria

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Italian oregano (*Origanum* × *majoricum*) was subjected to root system inoculation with three species of plant growth-promoting rhizobacteria (PGPRs) (*Pseudomonas fluorescens*, *Bacillus subtilis, Azospirillum brasilense*), and essential oil (EO) content and plant growth were measured. Composition of monoterpenes, a major EO component, was analyzed qualitative and quantitatively by gas chromatography. Total EO yield for plants inoculated with *P. fluorescens* or *A. brasilense* was 3.57 and 3.41 μg/mg fresh weight, respectively, ~2.5-fold higher than controls, without change of quantitative oil composition. The major EO compounds, *cis*- and *trans*-sabinene hydrate, γ-terpinene, carvacrol, and thymol, showed increased biosynthesis. Carvacrol was the only terpene showing significant increase of *R*% in plants inoculated with *A. brasilense*. Plant growth parameters (shoot and root fresh and dry weights, numbers of leaves and nodes) were evaluated. Shoot fresh weight was significantly increased by all three PGPR species, but only *P. fluorescens* and *A. brasilense* increased root dry weight. These two species have clear commercial potential for economic cultivation of O. × *majoricum*. Knowledge of the factors affecting yield and accumulation of monoterpenes is essential for improving production of these economically important plant compounds.

KEYWORDS: Aromatic plants; essential oil; monoterpene; *Origanum* × *majoricum*; plant growth promoting rhizobacteria

INTRODUCTION

A large variety of bacterial and fungal species have evolved functional, mutualistic relationships with plants. The group of bacteria termed plant growth-promoting rhizobacteria (PGPRs) colonize the rhizosphere and are able to increase plant growth and/or protect plants against pathogens (*1*). PGPRs are involved in such key ecological processes as nutrient cycling, seedling establishment, and soil enrichment. PGPRs applied as inoculants can improve plant growth and health through nitrogen fixation, nitrate reduction, phytohormone production, promotion of biological control activity (*2*).

Widespread use of nitrogen fertilizers has increased crop yield, but also has deleterious effects on ecosystems, e.g., pollution of ground and surface waters by nitrates (NO_3), soil acidification, and production of nitrous oxide (N_2O), a "greenhouse gas", through denitrification (*3*). New biotechnological methods for less aggressive crop protection are based on the use of beneficial microorganisms applied as biofertilizers. "Organic agriculture" is a production system which 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 in organic production systems is a major problem (*4*). In the case of many aromatic and medicinal plants, which are typically consumed after harvest without further processing, it is important that synthetic compounds not be present.

Plants of importance in modern agriculture and trade, in addition to the traditional food, forage, and fiber crops, increasingly include species with secondary metabolites having desired aromatic or therapeutic qualities, or providing source material for the perfume and chemical industries. Lipid (oil) constituents of certain plants are used as chiral auxiliaries in synthetic organic chemistry, and in microbial transformation of common structures to give highly functionalized substances of economic value (*5*).

Oregano, a member of the family Lamiaceae, is heavily used in the food industry because of its aromatic and antioxidant properties (*6*). One economically important species is *Origanum* × *majoricum* Cambess. (Italian oregano), a hybrid *of O. majorana* L. × *O. vulgare* L. ssp. virens (Hoffm. et Link) Ietswaart (*7*). The abundant essential oils (EOs) located in leaf trichomes of this species are lipophilic volatiles, consisting mostly of monoterpenes, sesquiterpenes, and phenylpropanoid metabolites, widely used as flavoring in foods and beverages, as fragrances, and as fungicides or insecticides in pharmaceutical and industrial products (*3*).

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Inoculation of plants with PGPRs can substantially enhance growth, but the impact of PGPRs on production of secondary metabolites is poorly known. Changes of environmental conditions and agricultural practices can significantly alter overall yield as well as EO composition in oregano (*8*). All plants produce and store volatile hydrocarbons to some degree, but increased synthesis and accumulation of EOs in oregano, as well as other commercial spices that are harvested specifically for robust aromas, have direct economic benefits. In the present study, we examined the effect of direct bacterial root inoculation with PGPRs on EO production and yield in Italian oregano.

MATERIALS AND METHODS

Micropropagation. Young shoots of field plants of *Origanum* × *majoricum* cultivated in Traslasierra Valley (Córdoba, Argentina) were surface disinfected by soaking for 1 h in water, 1 min in 50% alcohol, 15 min in 1.5% sodium hypochlorite with two drops of Tween 20, and rinsed three times in sterile water. Disinfected shoots were cultured in 10 mL of MS culture medium containing 0.8% (w/v) agar and 1.5% (w/v) sucrose (*9*), in 18 mm × 15 cm test tubes. All culture media contained 30 g/L sucrose and 8 g/L agar.

Stage I. Initial Shoot-Tip Culture. After 20 days, apical meristems with foliar primordia not showing contamination were aseptically removed from terminal buds of shoots obtained in the previous step. Explants were cultured in test tubes, in 10 mL MS medium with 0.53 μM naphthalenacetic acid (NAA) and 0.26 μM benzyladenine (BA).

Stage II. Growth and in Vitro Multiplication. Plantlets obtained from tips were multiplied by single node culture, and the MS medium was 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 h/day with ~2000 Lux light radiation from cool white fluorescent tubes.

Stage III. Rooting and Acclimatization. At 15 days of culture rooting plantlets were obtained at *in vitro* multiplication stage, transplanted directly into vermiculite in a greenhouse, and watered by microirrigation system.

Bacterial Strains, Culture Conditions, Media, and Treatments. Three bacterial strains previously reported as PGPRs (*10*, *11*), *Pseudomonas fluorescens* WCS417r, *Azospirillum brasilense* SP7, were grown on LB and *Bacillus subtilis* GB03, on TSA medium, for routine use, and maintained in nutrient broth with 15% glycerol at −80 °C for long-term storage. For experiments, bacteria were grown on nutrient agar, single colonies were transferred to 100 mL flasks containing respective culture medium, and grown aerobically on a rotating shaker (150 rpm) for 48 h at 28 °C. The bacterial suspension was diluted in sterile distilled water to a final concentration of 10^9 CFU/mL, determined by optical density measurement, and each plant was treated with 1 mL of the resulting suspension. Ten plants were used for each treatment.

Greenhouse Experiments. Plants were grown in a greenhouse with controlled conditions of light (16/8 h light/dark cycle), temperature (22 ± 2 °C), and relative humidity (~70%). Bacterial suspension (1 mL) was added directly to vermiculite containing the plantlets around the shoot; controls were inoculated with sterilized water. Plants were watered with Hoagland's nutrient medium once per week, 20 mL per pot (*12*). This study was conducted under nonsterile conditions.

Experiments were replicated (10 pots per treatment; 1 plant per 200 cm^3 pot), with pots arranged randomly in the greenhouse, 90 days after inoculation, during the growing period, plants were removed from pots, roots were washed to remove vermiculite, and growth promoting effects of bacterial treatments were evaluated: shoot length, leaf number, node number, shoot fresh weight, and root dry weight.

Extraction of Essential Oils (EOs). Each shoot sample was weighed and subjected to hydrodistillation in a micro Clevenger-like apparatus for 40 min, and the volatile fraction was collected in dichloromethane. Internal standard added was 0.1 μg of dodecalactone in 50 μL of ethanol.

O. × *majoricum* contains up to 3% volatile oil, comprising more than 35 different compounds (*13*). Major EOs, accounting for about 55% of total volume, were *cis*- and *trans*-sabinene hydrate, γ-terpinene, carvacrol, and thymol. These compounds were quantified with respect to the standard dodecalactone added during distillation procedure. The relative percentage (R%) of major compounds of essential oils was calculated based on total oil yield. The oil components were initially identified based on mass spectral and retention time data and confirmed by direct comparisons with commercial standards from Sigma-Aldrich Co. FID response factors for each compound generated equivalent areas with negligible difference (< 5%). For comparison of the same compound under different treatments, response factors for individual compounds were assumed to be equal (*14*).

Chemical analyses were performed using a Perkin-Elmer 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 and detector temperatures 250 and 270 °C, respectively; 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; mass spectra were recorded in the scan mode at 70 eV. Oil components were identified based on mass spectral and retention time data, in comparison to standard compounds (*15*). 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: oven temperature programmed from 60 °C (3 min) to 240 °C at 4°/min, injector and detector temperatures 250 °C; detector FID; carrier gas = nitrogen at a constant flow of 0.9 mL/min.

EO Quantification. Areas corresponding to all compounds in EOs were added, and total weight of EOs from each sample was calculated in relation to area of the internal standard (corresponding to 0.1 μg of dodecalactone). The shoot was weighed and yield was represented as μg/mg fresh weight.

Statistical Analyses. Data were pooled and subjected to analysis of variance (ANOVA) followed by comparison of multiple treatment levels with control, using post hoc Fisher's LSD (least significant difference) test. Infostat software version 2.0 (Group Infostat, Universidad Nacional de Córdoba, Argentina) was used for all statistical analyses.

RESULTS

Effects of PGPR inoculation on *O.* × *majoricum* development differed for the three rhizobacterial species (*B. subtilis, P. fluorescens, A. brasilense*) (**Figure 1**; **Table 1**). Numbers of leaves and nodes did not differ significantly ($P > 0.05$), but certain differences among the treatments could be detected even after 90 days' growth. Shoot length and root fresh weight were 30 and 70% higher, respectively, in plants inoculated with *P. fluorescens* than in control (noninoculated) plants ($P < 0.05$).

Shoot fresh weight was ~50% higher than control in all inoculated plants (**Figure 1a**). The increase in shoot fresh weight was due to a combination of increased leaf size and elongation of internodes. Root dry weight was 2-fold higher ($P < 0.05$) than controls in plants treated with *P. fluorescens* or *A. brasilense* (**Figure 1b**).

Regarding EO yield, inoculation with PGPRs increased production of certain terpenes (**Figure 2**). Total EO yield for plants inoculated with *P. fluorescens* or *A. brasilense* was 3.57 and 3.41 μg/mg fresh weight, respectively, ~2.5-fold higher than controls ($P = 0.001$).

The major EOs analyzed γ-terpinene, *trans*-sabinene hydrate, *cis*-sabinene hydrate, thymol were altered in PGPR-inoculated plants relative to controls in most cases (**Figure 3**). Only carvacrol was significantly increased (9-fold) by inoculation with *B. subtilis* ($P < 0.05$).

Inoculation led to changes of relative percentage (R%), as well as yield, of EOs (**Table 2**). R% for *trans*-sabinene hydrate, the main EO component, increased from 26.7% in controls to 36.9% in *P. fluorescens*-inoculated plants; R% for γ-terpinene and thymol also increased in this case. R% for *cis*-sabinene hydrate was lower in plants inoculated with *B. subtilis* or *A. brasilense* (6.6% and 10.7% respectively) than in controls (13.3%). Carvacrol was the only terpene showing significant increase of R% in

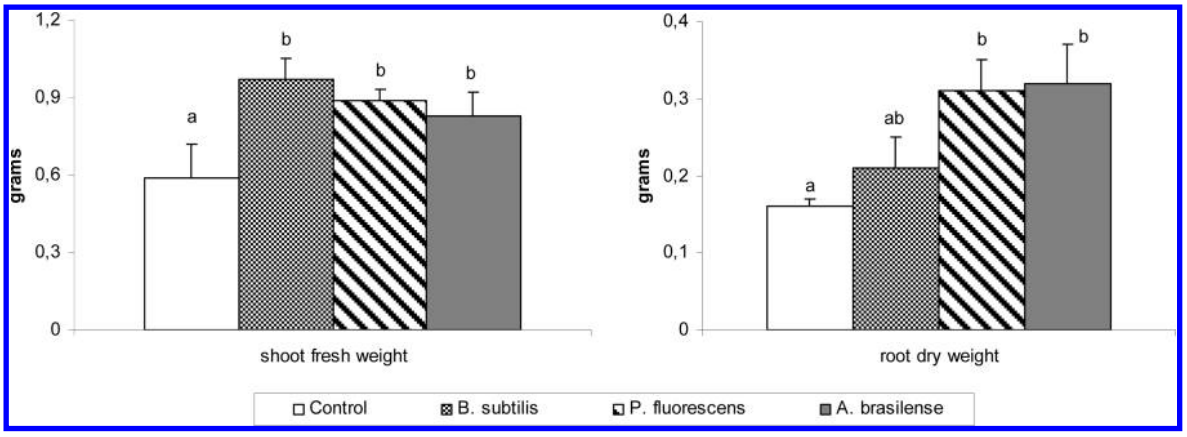

Figure 1. (**a**) Shoot fresh weight and (**b**) root dry weight of *O.* × *majoricum* inoculated with three PGPR strains. Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

Table 1. Effect of *O.* × *majoricum* Inoculation with Different PGPR Strains on Plant Growth Parameters and Essential Oil Yield (Mean ± SE)[a]

treatment	leaves (no.)	shoot length (cm)	node (no.)	root fresh wt (g)
control	19.77 ± 0.60 (7)	10.79 ± 1.43(7) ab	11.90 ± 0.48(8)	0.39 ± 0.07 (7) a
B. subtilis	22.70 ± 2.10(7)	10.50 ± 0.94(7) a	13.60 ± 1.38(7)	0.49 ± 0.08(7) ab
P. fluorescens	19.61 ± 0.59(8)	14.28 ± 1.61(8) b	12.12 ± 0.74(7)	0.67 ± 0.09(8) b
A. brasilense	18.33 ± 1.52(8)	11.38 ± 1.08(8) ab	11.88 ± 0.54(8)	0.55 ± 0.10(8) ab

[a] Values followed by different letter in a column are significantly different according to Fisher's LSD test ($p < 0.05$).

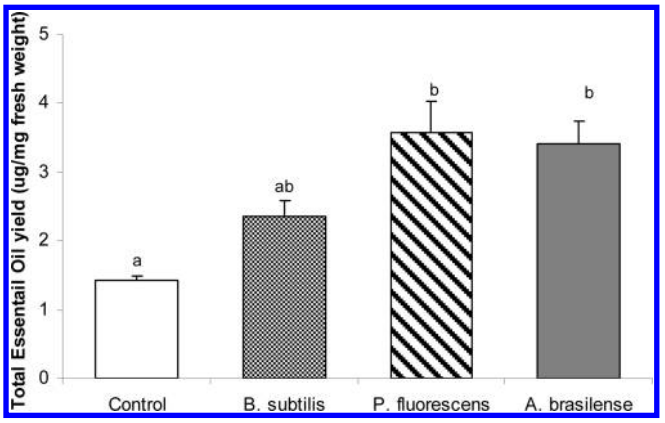

Figure 2. Shoot essential oil concentration in *O.* × *majoricum* inoculated with three PGPR strains. Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

plants inoculated with *A. brasilense* (11.3% compared to 2.6% in controls).

DISCUSSION

Enhanced growth and development following inoculation with PGPRs has been reported for a number of plant species (*11*, *16*, *17*). Possible causes are increases in growth hormone production, phosphate solubilization, sulfur oxidation, nitrate availability, extracellular production of antibiotics, lytic enzymes, hydrocyanic acid, root permeability, competition for available nutrients or root sites, and/or induction of plant systemic resistance (*18*). PGPRs typically alter root morphology (more specifically, increase root surface area) and thereby enhance soil nutrient uptake potential (*16*).

All three PGPR strains we evaluated produced a significant increase in shoot fresh weight, but only *P. fluorescens* enhanced shoot length and root fresh weight, and only *A. brasilense* and *P. fluorescens* increased root dry weight. A similar result was observed in *O. majoricum* inoculated with *P. fluorescens* (*12*). Consistent with our findings, fluorescent pseudomonads were reported to improve overall growth of various crops (*19*). *P. fluorescens* enhanced plant growth through production of growth-promoting substances such as indole acetic acid (IAA) and cytokinins (*19*, *20*). The role of auxins and cytokinins in enhancing plant cell division and root development is well-known (*21*). IAA is involved in root initiation, cell division, and cell enlargement (*17*), and increases root surface area and consequent access to soil nutrients. Cytokinins promote cell division, cell enlargement, and tissue expansion in certain plant parts (*17*). *A. brasilense*, in addition to its nitrogen fixing ability, secretes phytohormones such as auxins, cytokinins, and gibberellins. Auxins are quantitatively the most abundant phytohormones secreted by *Azospirillum*. Auxin production, rather than nitrogen fixation, is considered the major factor responsible for stimulation of rooting and enhancement of plant growth (*22*).We did not observe increase of shoot/root biomass in plants inoculated with *B. subtilis*, which has been reported as a PGPR in many plant species (*10*). In sweet basil (*Ocimum basilicum*), shoot and root biomass (on both fresh and dry weight basis) increased 2-fold following direct PGPR inoculation or exposure to bacterial volatile emissions (*23*).

Concentration and composition of oils in plants serve important ecological roles. Increased synthesis of EOs provides a defensive response to colonization by microorganisms, since several EOs have antimicrobial properties (*5*). Analogously, monoterpene synthesis is induced by herbivore feeding in *Minthostachys mollis* (*15*) and other plant species, apparently to protect damaged leaves from further attack (*3*).

There have been few attempts to elucidate relative quantitative and qualitative contributions of rhizobacteria to formation of plant secondary compounds. In the present study, inoculation with *P. fluorescens* and *A. brasilense* increased EO yield in *O.* × *majoricum*. This increase was not due to increased biomass, and therefore may result from enhanced biosynthesis of terpenes. Thirty-eight constituents, accounting for >95% of total oil content, were identified. The EO composition of *O.* × *majoricum* we found was very similar to that reported by Tabanca (*13*),

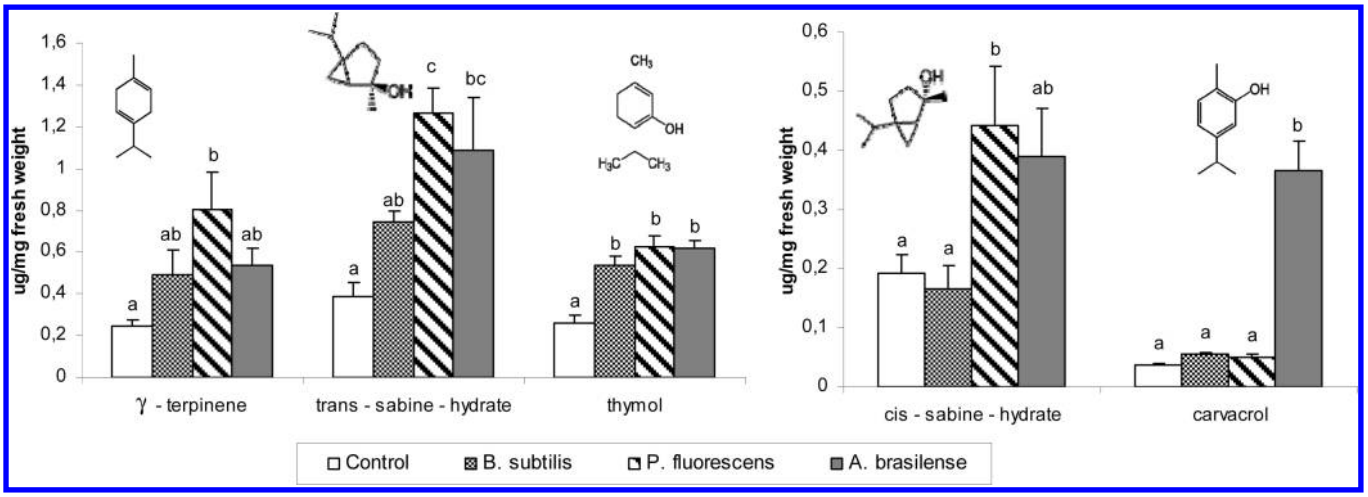

Figure 3. Concentration of main shoot essential oil components of *O.* × *majoricum* inoculated with three PGPR strains. Letters above bars indicate significant differences according to Fisher's LSD test ($p < 0.05$).

Table 2. Variation in Relative Percentage (*R*%) of the Main Shoot Oil Constituents of *O.* × *majoricum* Inoculated with Three PGPR Strains (Mean ± SE)[a]

treatment	γ- terpinene	*cis*-sabinene hydrate	*trans*-sabinene hydrate	thymol	carvacrol
control	17.98 ± 2.15 *a*	13.30 ± 2.07	26.72 ± 3.79	18.27 ± 2.79	2.63 ± 0.16 *a*
B. subtilis	19.58 ± 2.94 *a*	6.63 ± 1.06	33.46 ± 3.26	23.41 ± 1.48	2.43 ± 0.21 *a*
P. fluorescens	20.08 ± 2.80 *a*	12.10 ± 3.04	36.95 ± 2.50	20.41 ± 4.68	1.57 ± 0.21 *a*
A. brasilense	17.16 ± 3.18 *a*	10.74 ± 1.83	30.18 ± 4.47	18.56 ± 1.25	11.35 ± 2.08 *b*

[a] Values followed by different letter in a column are significantly different according to Fisher's LSD test ($p < 0.05$).

although percentages of the main components showed slight differences. Quality, quantity, and composition of extraction products can vary depending on climate, soil composition, plant organ, age, and vegetative cycle stage (*8*).

In this study, inoculation with *A. brasilense* or *P. fluorescens* caused a 2-fold increase of EO accumulation. This finding suggests increased terpene biosynthesis, although direct measurements were not made. The systemic induction of monoterpenes in *O.* × *majoricum* is consistent with previous reports in other aromatic plant species (*12*, *23*). Yield and accumulation of the major EOs (α-terpineol, terpinen-4-ol, *trans*-sabinene hydrate, *cis*-sabinene hydrate) in *Origanum majorana* were increased by inoculation with *P. fluorescens*, but not with *B. subtilis*. Treatment of *Ocimum basilicum* with *B. subtilis* increased total EO yield and content of eugenol and terpineol (*23*).

Induction of secondary metabolite responses has been reported in other beneficial microbe-plant interactions involving arbuscular mycorrhizal (AM) fungi. Gupta et al. (*24*) inoculated the AM fungus *Glomus fasciculatum* in cultivars of wild mint (*Mentha arvensis*), and observed increased plant height, shoot growth, and oil content. Khaosaad et al. (*25*) observed changes of EO concentration (but not composition) following mycorrhizal inoculation of *Oreganum sp*. Copetta et al. (*26*) found increased abundance of glandular hairs, and EO yield, in inoculated *Ocimum basilicum*. The increased oil yield was associated with a 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 (a) extending the root zone; (b) reaching smaller soil pores not accessible by root hairs; (c) acquiring organic phosphates through production of extracellular acid phosphatases (*27*).

Terpene compounds help the plant's photosynthetic apparatus recover from brief episodes of high temperature. Isoprene may physically stabilize the thylakoid membranes at high temperature, or quench reactive oxygen species, such as ozone, that cause membrane damage (*28*). Improved biosynthesis of secondary metabolites can be triggered by certain stress factors (*29*). Nonpathogenic rhizobacteria have been shown to stimulate secondary metabolism of plants through a mechanism termed ISR (induced systemic resistance) (*30*). Biological agents can act as effective elicitors of key enzymes involved in secondary metabolism (*31*), which are clearly related to plants' defense mechanisms against pathogens despite being induced by nonpathogenic bacteria (*18*). The major EO components of *O.* × *majoricum* EO (*cis*-sabinene hydrate, *trans*-sabinene hydrate, γ-terpinene, carvacrol, thymol), which are significantly increased by inoculation with soil bacteria, display antibacterial and antifungal properties (*3*). Stimulation of secondary metabolism by production of siderophores, cell wall polysaccharides, and/or salicylic acid (*29*) has been well documented in Gram-negative *Pseudomonas* bacteria. Siderophores produced by *Bacillus* sp. also stimulated the systemic response involved in induction of secondary pathways, although the mechanism is not known (*32*).

Biosynthesis of terpenoids depends on primary metabolism, e.g., photosynthesis, and oxidative pathways for carbon and energy supply (*33*). Giri (*34*) found that net photosynthesis of PGPR-hosting plants increases as a result of improved nutritional status. Factors that increase dry matter production may influence the interrelationship between primary and secondary metabolism, leading to increased biosynthesis of secondary products (*35*). Increased plant biomass appears to result in greater availability of substrate for monoterpene biosynthesis (*3*). The increased concentration of monoterpenes in inoculated plants may be attributed to growth-promoting substances, produced by the inoculated microorganism, that affect plant metabolic processes. Since plants in the present study were grown in enriched medium containing nitrogen and other nutrients, bacterial metabolites are the most likely growth-promoting substance.

Knowledge of the adaptative mechanisms of plants is of interest from an ecophysiological point of view, but these mechanisms also constitute an important starting point—probably the key—to improving plant production, including optimization of secondary metabolite production. The use of fungal and

bacterial inoculants is an efficient biotechnological alternative for stimulating secondary metabolism in plants, and may also lead to relevant information on certain adaptive processes which are poorly understood. We found that inoculation of certain PGPRs causes systemic induction of monoterpene pathways in *Origanum,* suggesting that inoculation with *P. fluorescens* and/or *A. brasilense* can significantly increase productivity and reduce the amount of fertilizer required for economically viable *O.* × *majoricum* crops. Further studies will clarify which PGPRs are most effective to enhance EO production. Carefully designed and controlled field trials are essential before the ability of *P. fluorescens* and *A. brasilense* to promote growth of *O.* × *majoricum* or other aromatic crops can be commercially exploited.

ABBREVIATIONS USED

EO, essential oil; IAA, indole acetic acid; PGPR, plant growth-promoting rhizobacteria.

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