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Research Paper

Inducing phenolic production and volatile organic compounds emission by inoculating *Mentha piperita* with plant growth-promoting rhizobacteria

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) work primarily by improving the nutrient status of plants and increasing plants' phytohormone production. Although this response has been demonstrated in many plant species, it is not well understood in aromatic plant species. To examine the effect of PGPR strains on the emission of volatile organic compounds (VOCs), total phenolic production and phenylalanine ammonia-lyase (PAL) activity, shoots of peppermint (*Mentha piperita*) plants were inoculated or co-inoculated with strains of beneficial rhizobacteria.VOC emissions for the inoculated groups were ~3-fold higher than for controls. Co-inoculated plants produced the greatest increase in VOC emission in comparison to singly inoculated and control plants. The synthesis of phenolic compounds in leaves of all the treated plants was enhanced in comparison with controls; and higher activity of the PAL enzyme was observed in inoculated plants. In view of the economic importance of VOCs and phenolic compounds for a variety of applications in the food and cosmetic industries, *P. fluorescens, B. subtilis* and *P. putida* SJ04 have the potential to improve the productivity of cultivated aromatic plants. Better understanding of the processes that affect secondary metabolites accumulation will lead to increased yields of these commercially valuable natural products.

1. Introduction

Plants are sessile organisms that must discriminate among a variety of challenges posed by the surrounding biotic and abiotic environment, and respond to them. Appropriate responses allow them to allocate their resources in an optimal manner for growth, reproduction, and defense. Over time, plants have evolved many physical and chemical defense systems to combat stress (Kessler and Heil, 2011). The huge diversity of existing phytochemicals and the evolution of secondary metabolism have resulted in increasingly complex interactions with biotic stresses (e.g., herbivores, pathogens, competitors). These interactions have numerous ecological and physiological implications (Arimura et al., 2005; Kessler and Heil, 2011).

Volatile organic compounds (VOCs) are products emitted into the atmosphere from natural sources (Holopainen and Gershenzon, 2010). VOCs are involved in defense systems, communication, pollinator attraction, and resistance to abiotic stress factors (Dicke and Baldwin, 2010; Wenke et al., 2010). Such compounds are released in small amounts into the environment by undamaged healthy plants, whereas during infestation by herbivores or pathogens, they are released in large amounts, from leaves to roots.VOC emission may be either constitutive or induced as a result of stress factors (Niinemets et al., 2013). Terpenoids, the predominant group of plant secondary metabolites, are particularly abundant in peppermint (*Mentha x piperita*), an aromatic species cultivated worldwide for the production of essential oils (EOs), and used fresh or dried (Lawrence, 2007). Terpenes, the primary constituents of EOs from aromatic plants, are volatile, typically lipophilic compounds with low molecular weight and high vapor pressure (Maffei et al., 2011; Wink, 2003). They are produced exclusively by glandular hairs (Gershenzon et al., 2000). EOs and VOCs indirectly protect the plant via tritrophic interactions. By emitting VOCs from their vegetative tissue, plants create a defense system that may ward off natural predators of the attacking herbivores or ward off microbes and animals (Das et al., 2013; Gershenzon, 2007).

Phenolic compounds are plant secondary metabolites that can be released under the influence of multiple biotic and abiotic stresses (Cheynier, 2012). Generally, the role of phenolic compounds in defense is related to their antibiotic or antinutritional effects. In addition to

Abbreviations: EO, essential oil; PAL, phenylalanine ammonia-lyase; PGPR, plant growth-promoting rhizobacteria; TOC, total phenolic compounds; VOC, volatile organic compound

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their involvement in relationships between plants and −animals and/or plants and microorganisms, plant phenolics also have key roles as pigments; as antioxidants and metal chelators; and as signalling agents both above- and below-ground between plant and other organisms (Lattanzio, 2013). Recently, there has been growing interest in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Zheng and Wang, 2001). It was reported that the antioxidant activity of plant materials correlated well with the content of their phenolic compounds (Moein and Moein, 2010).

Soil bacteria (rhizobacteria) of many genera have demonstrated beneficial effects on plant growth, crop yield, and crop quality. Such bacteria, collectively termed "plant growth-promoting rhizobacteria" (PGPR), promote growth through the production of phytohormones, reduction of plant ethylene level, enhancement of nutrient status, and enhancement of disease-resistance mechanisms, and biocontrol function (Babalola, 2010).

Many of our previous investigations have demonstrated that PGPR inoculation increases biomass and EO production in aromatic plants (Banchio et al., 2010; Cappellari et al., 2015; Santoro et al., 2015). On the other hand, biotic and abiotic stresses, such as high temperature, high light and herbivore attack, are well known to increase the emission of VOCs from plants (Holopainen and Gershenzon, 2010). However, VOC emission and total phenol content by aromatic plants inoculated with PGPR has not yet been thoroughly studied.

Here we describe a comparative study of the influences of *M. piperita* inoculation with three beneficial rhizobacterial strains (singly or in combination) on plant VOC emission and total phenol contents.

2. Material and methods

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

Three bacterial strains previously reported as PGPR were studied: *Pseudomonas fluorescens* WCS417r; *P. putida* SJ04, a native fluorescent strain isolated from rhizospheric soil collected from a commercial crop of *Mentha* × *piperita* (San José) in Córdoba, Argentina, and tested for plant growth-promoting activity (GenBank KF312464.1); and *Bacillus subtilis* GB03 (Banchio et al., 2010). Bacteria 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.

Each bacterial culture was grown overnight at 30 °C with rotation at 120 rpm until reaching the exponential phase, washed twice in 0.9% NaCl by Eppendorf centrifugation (4300 x g, 10 min, 4 °C), resuspended in sterile water, and adjusted to a final concentration of ~10^9 CFU/mL for use as inoculum.

Plants were grown in plastic pots (diameter 12 cm, depth 22 cm) containing sterilized vermiculite. *M. piperita* seedlings were planted (one per pot) in vermiculite and inoculated with 100 µL bacterial suspension. Six experimental treatments were performed: sterile water (control); SJ04; WCS417r; GB03; WCS417r + GB03; WCS417r + SJ04. Ten plants were used for each treatment.

2.2. Greenhouse experiments

Young shoots from *M. piperita* plants grown in Traslasierra Valley (Córdoba Province, Argentina) were surface-disinfected by being soaked for 1 min in 17% sodium hypochlorite solution and rinsed 3 x in sterile distilled water. Disinfected shoots were cultured in 100 mL MS culture medium containing 0.7% (w/v) agar and 1.5% (w/v) sucrose (Murashige and Skoog, 1962). All culture media contained 30 g/L sucrose and 7.5 g/L agar.

Following 30 days of culture as above, the apical meristems with foliar primordia of shoots that did not show contamination were aseptically removed from terminal buds. Explants were cultured in test tubes in 10 mL MS medium with 0.53 µM naphthalene acetic acid (NAA) and 0.26 µM benzyladenine (BA) (Santoro et al., 2011). Plantlets obtained from tips were multiplied by single-node culture in MS medium with 0.53 µM NAA and 0.28 µM BA. pH was adjusted to 5.8, growth regulators were added, and cultures were autoclaved (20 min, 121 °C). Temperature was maintained thereafter at 22 °C, with photoperiod 16 h/d and ~2000 Lux light radiation from cool-white fluorescent tubes. On day 7 of culture, rooting plantlets were obtained at the *in vitro* multiplication stage, transplanted directly into vermiculite in a greenhouse, and watered by a micro-irrigation system (Cappellari et al., 2013).

Plants were grown in a growth chamber under controlled conditions of light (16/8-h light/dark cycle), temperature (22 ± 2 °C), and relative humidity (~70%). Bacterial suspensions as described above were applied to experimental seedlings, and sterile water was applied to control seedlings. All plants received Hoagland's nutrient medium (20 mL/pot) once per week (Cappellari et al., 2013). Experiments were performed under non-sterile conditions.

Experiments were replicated 3 times (10 pots per treatment; 1 plant per pot). Pots were arranged randomly in the growth chamber. Thirty days after inoculation, plants were removed from pots, roots were washed to remove vermiculite, and shoot fresh weight (FW) was measured. The biological material for the collection of VOCs was the same as that used for the extraction of EOs published in (Cappellari et al., 2015), and a new replicate was performed to verify the data.

2.3. Collection of plant VOCs

Plant VOCs were collected 30 days after inoculation, prior to the removal of plants. The collection system consisted of a vacuum pump that created a constant airflow (300 mL/min) through a polyethylene terephthalate (PET) chamber (volume 1500 mL) containing a plant (inoculated or uninoculated). The chamber was closed at one end with a cap pre-drilled to fit the collection trap. At the other end, a cap with a hole through which the plant stem passed separated the bottom of the chamber from the base of the pot. Air exited the chamber through a reusable glass collection trap packet with 30 mg Super-Q absorbent (80–100 mesh; Alltech), which was rinsed 5 x with 10 mL dichloromethane prior to each collection to remove impurities. Headspace VOCs were collected for 2 h and eluted immediately from the absorbent traps with 200 mL dichloromethane, after which internal standard was added (1 µL α-pinene in 50 µL ethanol) (Banchio et al., 2007). Collected VOCs were analyzed by gas chromatography as described below. VOCs emitted by *M. piperita* plants consisted of ~30 different compounds. Thirty percent of the VOC terpene components included (−) menthone, (−) menthol, and (+) pulegone. Following VOC collection, each plant was cut and weighed. VOCs were also collected from control (uninoculated) plants. Collections from an empty chamber showed that the background level of monoterpenes was negligible.

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 (Cappellari et al., 2013).

2.5. Determination of PAL enzyme activity

PAL was extracted from100 mg mint leaves; plant material was homogenized with liquid nitrogen using a mortar and pestle containing appropriate buffer solution (50 mM potassium phosphate and 1 mM