

# Phytohormone production by three strains of *Bradyrhizobium japonicum* and possible physiological and technological implications

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**Abstract** The aim of this work was to evaluate phytohormone biosynthesis, siderophores production, and phosphate solubilization in three strains (E109, USDA110, and SEMIA5080) of *Bradyrhizobium japonicum*, most commonly used for inoculation of soybean and nonlegumes in USA, Canada, and South America. Siderophore production and phosphate solubilization were evaluated in selective culture conditions, which had negative results. Indole-3-acetic acid (IAA), gibberellic acid (GA<sub>3</sub>), and abscisic acid (ABA) production were analyzed by gas chromatography–mass spectrometry (GC-MS). Ethylene and zeatin biosynthesis were determined by GS–flame ionization detection and high-performance liquid chromatography (HPLC-UV), respectively. IAA, zeatin, and GA<sub>3</sub> were found in all three strains; however, their levels were significantly higher ( $p < 0.01$ ) in SEMIA5080 (3.8  $\mu\text{g ml}^{-1}$ ), USDA110 (2.5  $\mu\text{g ml}^{-1}$ ), and E109 (0.87  $\mu\text{g ml}^{-1}$ ), respectively. ABA biosynthesis was detected only in USDA110 (0.019  $\mu\text{g ml}^{-1}$ ). Ethylene was found in all three strains, with highest production rate (18.1  $\text{ng ml}^{-1} \text{h}^{-1}$ ) in E109 cultured in yeast extract mannitol medium plus L-methionine. This is the first report of IAA, GA<sub>3</sub>, zeatin, ethylene, and ABA production by *B. japonicum* in pure cultures, using quantitative physicochemical methodology. The three strains have differential capability to produce the five major phytohormones and this fact may

have an important technological implication for inoculant formulation.

## Introduction

Soil is a natural system colonized by various microorganisms including bacteria, fungi, and actinomycetes (Foster 1988). Soil in which the proliferation of microorganisms is induced by the presence of plant roots is termed the “rhizosphere” (Garate and Bonilla 2000). Bacteria (by far the most common type of soil microorganism) growing in the rhizosphere are called “rhizobacteria.” Rhizobacteria that possess some direct mechanism or capacity to promote plant growth are referred to as “plant growth-promoting rhizobacteria” (PGPR) (Kloepper et al. 1989); those that promote plant growth by some indirect mechanism are referred to as “biocontrol plant growth-promoting bacteria” Bashan and Holguín (1998).

Direct promotion of growth occurs when PGPR provide compounds that affect plant metabolism or when they facilitate acquisition by plants of a nonavailable nutrient from the soil. In PGPR, the most important direct plant growth-promoting mechanism besides biological nitrogen fixation is synthesis of phytohormones or plant growth-regulating compounds. Examples are production of indole-3-acetic acid (IAA) by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* (Bastian et al. 1998); zeatin and ethylene by *Azospirillum* sp. (Strzelczyk et al. 1994); gibberellic acid (GA<sub>3</sub>) by *Azospirillum lipoferum* strain op33 (Bottini et al. 1989); and abscisic acid (ABA) by *Azospirillum brasilense* strains Cd and Az39 (Perrig et al. 2005).

Another important PGPR mechanism is biosynthesis and release of siderophores, acidic compounds that supply iron

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or phosphates to the plant when they are not available in the soil (Seshadri et al. 2000).

PGPR are comprised almost exclusively of free-living bacteria that colonize the rhizosphere or certain tissues within the plant (Kloepper et al. 1989). In addition to PGPR, there are a number of “symbiotic rhizobacteria” that establish a complex, intimate, and mutually beneficial relationship with the plant (Bohlool 1990). This group, particularly the family Rhizobiaceae, was extensively studied in relation to their association with legumes under the “classic” perspective of biological nitrogen fixation (Hablieb and Luden 2000). However, there is a tendency to consider *Rhizobiaceae* species as PGPR, especially when they are inoculated in nonlegumes (Gaur et al. 1980). In field experiments, Höflich et al. (1994) obtained significant shoot dry matter that increases after inoculation of spring wheat and barley with *Rhizobium leguminosarum* strain R39. Yanni et al. (1997) demonstrated that *R. leguminosarum* colonizes roots and improve growth and productivity in rice. Noel et al. (1996) showed that *R. leguminosarum* promotes early root growth in lettuce (*Lactuca sativa* L.) and canola (*Brassica rapa* L.) seedlings under controlled conditions. These authors and Chabot et al. (1996) established plant growth-promoting capacity through direct mechanisms such as siderophore production or phosphorus solubilization for various *R. leguminosarum* strains. Antoun et al. (1998) found the same plant growth-promoting mechanisms in 18 strains of *Bradyrhizobium japonicum*, and also reported IAA production in 6 of the 18 strains. However, these results were not accurate because the methodology employed was qualitative (Bric et al. 1991). Kaneshiro and Kwoleck (1985) showed that inoculation of soybean with spontaneous mutants of *B. japonicum* over-producing IAA resulted in a threefold increase of root nodule volume. *Bradyrhizobium elkanii* mutants deficient in IAA production induced few nodules on soybean roots, but nodulation was promoted by exogenous IAA application (Fukuhara et al. 1994).

Zeatin and 9R-zeatin biosynthesis by *B. japonicum* cultures was reported by Sturtevant and Taller (1989), but quantitative data were not provided.

Regarding gibberellin biosynthesis by these rhizobacteria, there is a single report by Katznelson and Cole (1965), who determined GA<sub>3</sub> production by *B. japonicum* using TLC bioassay. No further studies on reliable identification and quantification of cytokines or gibberellins produced by *B. japonicum* were reported in the 40 years since then. More generally, bioactive molecules (auxins, gibberellins, cytokines, ABA, ethylene, etc.) produced by *B. japonicum* have never been identified or quantified by gas chromatography–mass spectrometry (GC-MS) or other unequivocal methodology. Biosynthesis of ABA or ethylene by *B. japonicum* in defined media was not studied either.

*B. japonicum* strains of choice for soybean inoculant formulation are E109 (USDA 138) in Argentina, USDA110 in USA and Canada, and SEMIA5080 (CPAC7) in Brazil. Numerous field and laboratory experiments have demonstrated their ability to greatly increase soybean productivity (Ressia et al. 2003; Cattelan et al. 1999; Hume and Blair 1992). These strains were also reported to promote plant growth or yield of nonlegumes such as wheat Monteleone et al. (2003).

The purpose of the present study was to evaluate the capability of *B. japonicum* strains E109, USDA110, and SEMIA5080 to biosynthesize phytohormones and other plant growth-promoting compounds such as siderophores and phosphate solubilizers in defined culture media, and to discuss possible technological implications.

## Materials and methods

### Bradyrhizobium strains

*B. japonicum* strains were provided by Ing. Agr. Alejandro Peticari of the Instituto de Microbiología y Zoología Agrícola, Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina (Peticari et al. 1996). Strain E109 (USDA138 of ARS collection, USA), USDA110 of ARS collection (USA), and SEMIA5080 (CPAC7 of Mircen collection, Brazil) are the strains most commonly used for soybean inoculation in Argentina, USA/Canada, and Brazil, respectively.

### Culture media

Bacteria were grown in yeast extract mannitol medium (YEM) (Vincent 1970) composed of (g l<sup>-1</sup>): K<sub>2</sub>HPO<sub>4</sub> (0.5), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2), NaCl (0.1), mannitol (10.0), and yeast extract (0.4), pH 6.8. For ethylene determination, this formulation was modified by addition of 0.1 g l<sup>-1</sup> methionine (YEM<sub>m</sub>). For determination of phosphate solubilizing compounds, pentacalcium phosphate agar (TPA) (g l<sup>-1</sup>) (Katznelson and Bose 1959) composed of soybean trypticase broth (10.0), Ca<sub>5</sub>(PO<sub>4</sub>)<sub>3</sub>OH (4.0), and agar (15.0), pH 6.8, was used. For siderophore determination, chrome-azurol-S (CAS) medium (Schwyn and Neilands 1987) was used.

### Culture conditions

*B. japonicum* strains E109, USDA110, and SEMIA5080 were grown in 250 ml flasks containing 100 ml of YEM at 30°C and 80 rpm shaking until exponential growth phase, OD<sub>600</sub> nearly 1, equivalent to 1.12, 1.23, and 1.18×10<sup>9</sup> colony-forming units (c.f.u.) ml<sup>-1</sup> in YEM-agar, respec-

tively. From each pure culture, an aliquot of biological material was taken for the evaluation of phosphate solubilization, siderophore production, and phytohormones identification and quantification.

#### Phosphate solubilization

Phosphate solubilization was measured on TPA plates by the method of Katznelson and Bose (1959). Plates were sown with 1  $\mu\text{l}$  of YEM pure bacterial culture in halfway points of a petri dish plate containing (0.1 $\times$ ) trypticase soya agar medium added with mannitol (10.0 g  $\text{l}^{-1}$ ) and  $\text{Ca}_5(\text{PO}_4)_3\text{OH}$  (4.0 g  $\text{l}^{-1}$ ). Experiments were performed in triplicate. Plates were incubated at 30°C and observed daily for formation of transparent halos around each colony for up to 7 days.

#### Siderophore production

Siderophore production was determined on agar CAS medium (Schwyn and Neilands 1987). Plates were sown with 1  $\mu\text{l}$  of YEM pure bacterial culture in halfway points of a petri dish plate containing agar CAS medium. Experiments were performed in triplicate. Plates were incubated at 30°C and observed daily for orange color formation around each colony for up to 7 days.

#### Identification and quantification of IAA, ABA, $\text{GA}_3$ , and zeatin

Bacterial cultures (YEM) in exponential growth phase, as defined in the “Phosphate solubilization” section, were separated into several 20-ml fractions for determination of IAA, ABA,  $\text{GA}_3$ , and zeatin. Fractions were centrifuged at 8,000 rpm for 20 min at 4°C, and supernatants were acidified at pH 2.5 with acetic acid solution (1% v/v). Individual samples were then added with 100 ng of the corresponding  $^2\text{H}_6$ -ABA (kindly supplied by Professor Richard P. Pharis, University of Calgary, Canada),  $^2\text{H}_5$ -IAA, or  $^2\text{H}_2$ - $\text{GA}_3$  (OChemIm, Czech Republic) deuterated internal standard, and kept at 4°C for 2 h. No deuterated internal standard was used for zeatin. Each sample was partitioned four times with the same volume of acetic acid-saturated ethyl acetate (1%, v/v). After the last partition, acidic ethyl acetate was evaporated to dryness at 36°C. Dried samples were diluted in 100  $\mu\text{l}$  of acetic acid/methanol/water (1:30:70) for ABA determination, acetic acid/acetonitrile/water (1:15:85) for IAA determination, and methanol/water (30:70) for  $\text{GA}_3$  and zeatin determination. They were injected into a reverse phase  $\text{C}_{18}$  high-performance liquid chromatography (HPLC) column ( $\mu\text{Bondapak}$ , 300 $\times$ 3.9 mm, Waters Associates, Milford, MA, USA) in a Konik 500 (Konik Instruments) system

coupled to a diode-array spectrometer UV-Vis Konik 3000. For each sample, elution was performed at 1 ml  $\text{min}^{-1}$  flow rate, and fractions eluting at the retention time corresponding to each pure standard were collected.

Zeatin was identified and quantified by HPLC-UV at 254 nm (Tien et al. 1979). IAA, ABA, and  $\text{GA}_3$  were identified and quantified by GC-MS with selective ion monitoring (GC-MS-SIM). UV-absorbing fractions at 254, 262, and 220 nm were grouped for IAA, ABA, and  $\text{GA}_3$  determination, respectively, then methylated with ethereal diazomethane and silylated with 1:1 pyridine: BSTFA [bis(trimethylsilyl) trifluoroacetamide] plus 1% trimethylchlorosilane (Fluka Chemika, Switzerland) to obtain methyl-trimethylsilyl derivatives of IAA, ABA, and  $\text{GA}_3$ . Aliquots of each sample were injected directly into a DB1-15N (15 m $\times$ 0.25 mm, 0.25  $\mu\text{M}$  methyl silicone) capillary column (J&W Scientific) fitted in a Hewlett-Packard 5890 Series II GC with a capillary direct interface to a 5970B Mass Selective Detector. The GC temperature program was 60 to 195°C at 20°C  $\text{min}^{-1}$ , then 4°C  $\text{min}^{-1}$  to 260°C. Carrier gas (He) flow rate was 1 ml  $\text{min}^{-1}$ , interface temperature was 280°C, and data acquisition was controlled by a HP 300 Series computer. By comparison of the peak areas of the ion at a mass/charge ( $m/z$ ) 196 (molecular ion for [ $^2\text{H}_6$ ]ABAMeTMSi) and the ion at  $m/z$  190 (molecular ion for [ $^1\text{H}$ ]ABAMeTMSi) at the corresponding time (Kobats 1958), the amount of free ABA was calculated. Similarly, the amount of free IAA was calculated by comparison of peak areas for the parent ion ( $m/z$ ) 194 and ( $m/z$ ) 189, and parent ion ( $m/z$ ) 506 and ( $m/z$ ) 504 for free  $\text{GA}_3$ . Data from three experiments were analyzed by ANOVA followed by post hoc Tuckey test.

#### Identification and quantification of ethylene

Ethylene production was measured by GC-flame ionization detection (GC-FID) (Strzelczyk et al. 1994). One milliliter of YEM bacterial culture in exponential growth phase ( $\text{OD}_{600}$  approximately 1) was transferred to 250-ml Erlenmeyer flasks containing 100 ml of YEM or YEM*m*. Flasks were fitted with rubber plugs tightened with metal cowls and incubated for 7 days at 30°C and 80 rpm, corresponding to 1.25, 1.31, and 1.28 $\times 10^9$  c.f.u.  $\text{ml}^{-1}$  in YEM and 1.34, 1.37, and 1.31 $\times 10^9$  c.f.u.  $\text{ml}^{-1}$  in YEM*m* for E109, USDA110, and SEMIA5080, respectively. To test ethylene production, a 500- $\mu\text{l}$  air sample was taken from the flasks after 7 days, and analyzed by a Konik KNK 3000 gas chromatograph equipped with Kromapak column (2 m in length) packed with chromosorb W-AW-DCMS, operated isothermally at 110°C with nitrogen as gas carrier and a flame ionization detector (170°C). Pure ethylene was used as standard (Air Liquid Group, Argentina). Experiments were performed in triplicate.

## Results

Our results indicate that *B. japonicum* strains E109, USDA110, and SEMIA5080 show differential patterns of plant growth regulator biosynthesis. Production and release of phytohormones in defined media was the only direct putative plant growth promoter mechanism detected in these strains and quantitative differences in phytohormone production were found among them. Three strains grew satisfactorily in these experimental conditions; however, no production of siderophores or phosphate solubilizers was observed. IAA production in defined YEM was significantly higher ( $p < 0.01$ ) for SEMIA5080 ( $3.8 \mu\text{g ml}^{-1}$ ) than for USDA110 ( $2.1 \mu\text{g ml}^{-1}$ ) or E109 ( $0.91 \mu\text{g ml}^{-1}$ ) (Fig. 1a). Meanwhile, zeatin synthesis in YEM was significantly higher ( $p < 0.01$ ) for USDA110 ( $2.5 \mu\text{g ml}^{-1}$ ) than for E109 ( $0.82 \mu\text{g ml}^{-1}$ ) or SEMIA5080 ( $0.75 \mu\text{g ml}^{-1}$ ) (Fig. 1b). Moreover,  $\text{GA}_3$  synthesis was higher ( $p < 0.01$ ) for E109 ( $0.87 \mu\text{g ml}^{-1}$ ) and USDA110 ( $0.79 \mu\text{g ml}^{-1}$ ) than for SEMIA5080 ( $0.59 \mu\text{g ml}^{-1}$ ) (Fig. 1c).

ABA production was measured as  $0.019 \mu\text{g ml}^{-1}$  for USDA110. No ABA synthesis was detected for E109 or SEMIA5080 under our experimental conditions (Fig. 1d).

Ethylene biosynthesis was much higher in YEMm than in YEM for each of the three strains (Fig. 2). In the YEMm experiments, ethylene production rate was significantly higher ( $p < 0.01$ ) for E109 ( $18.1 \text{ ng ml}^{-1} \text{ h}^{-1}$ ) than for USDA110 ( $3.95 \text{ ng ml}^{-1} \text{ h}^{-1}$ ) or SEMIA5080 ( $2.02 \text{ ng ml}^{-1} \text{ h}^{-1}$ ).

## Discussion

*Bradyrhizobium* sp. were widely studied because of their symbiotic association with legumes and biological nitrogen fixation capacity. However, some members of this genus are capable of inducing plant growth and development through biochemical or physiological mechanisms other than nitrogen fixation. Our present findings indicate that *B. japonicum* strains E109, USDA110, and SEMIA5080 are potentially capable of regulating plant growth through phytohormone production and releases in biological formulations.

IAA levels produced by E109 and USDA110 in our experiments are similar to those reported by Hunter (1987) for other *B. japonicum* strains in soybean nodules. In contrast, IAA production was much higher for SEMIA5080 (Fig. 1a). IAA was correlated with stimulation of physiological processes such as nodule formation and root growth (Kaneshiro and Kwoleck 1985; Fukuhara et al. 1994). Coinoculation of *Rhizobium* sp. with an IAA-producing PGPR such as *Azospirillum* sp. on *Medicago polymorpha* (Yahalom et al. 1990), or *Bacillus* sp. on *Phaseolus vulgaris* (Srinivasan et al. 1996), significantly increased

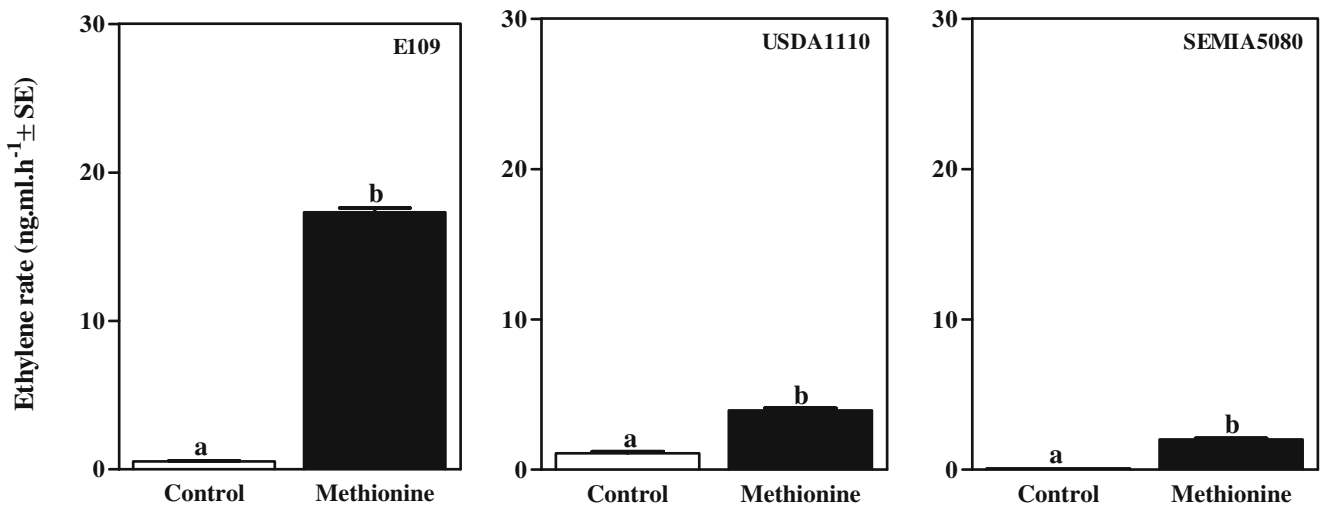
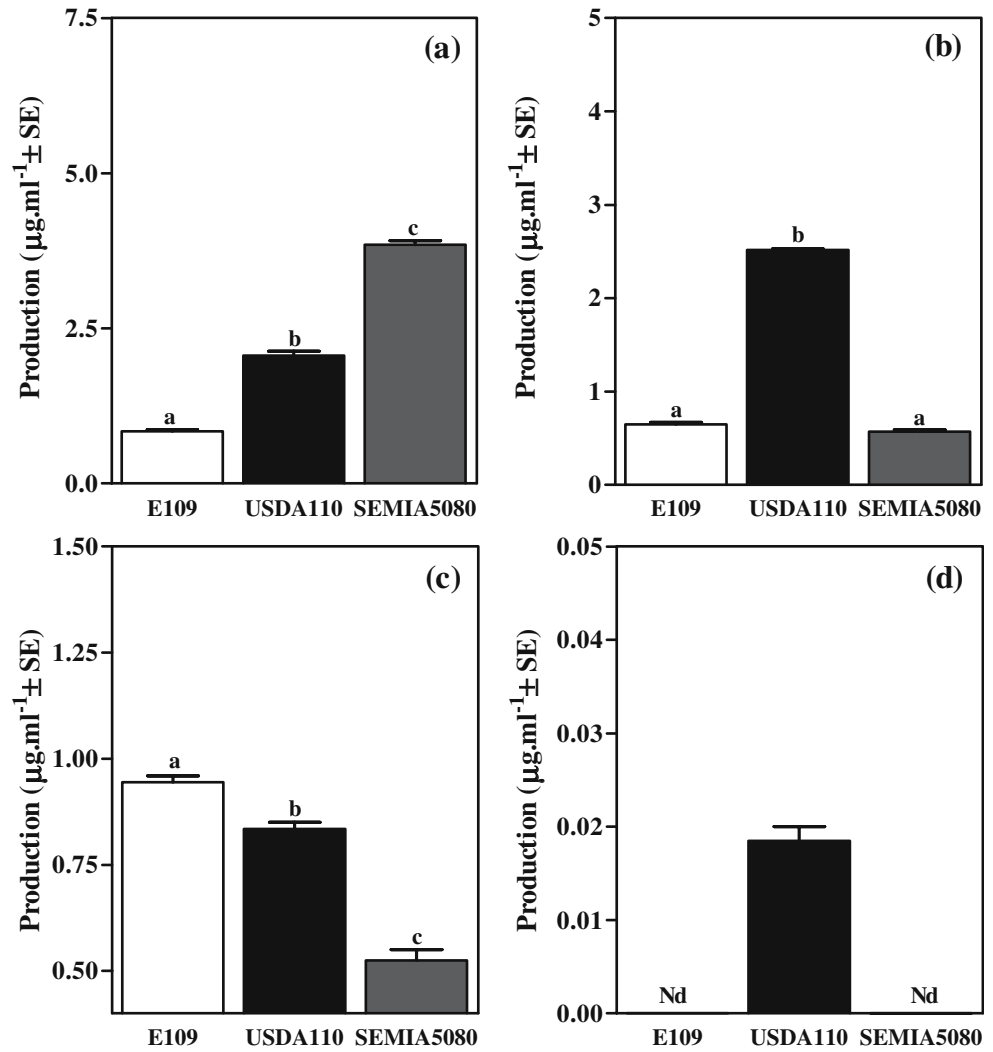
number, weight, and nitrogenase activity of root nodules in comparison with *Rhizobium* sp. mono-inoculated plants. On the other hand, exogenous application of IAA on maize (*Zea mays* L.) seedling roots at concentrations of  $0.00175$ – $0.175 \mu\text{g ml}^{-1}$  significantly increased lateral root number and root hair number (Zelena et al. 1988), which would greatly enhance the possibility of plant–microorganism interaction. Because E109, USDA110, and SEMIA5080 synthesize IAA at higher concentrations than the strains used in the above-cited experiments, they could be used as natural sources of this growth-promoting hormone, eliminating the need for synthetic sources.

Zeatin and some derivative compounds from *B. japonicum* grown in defined medium were characterized by Taller and Sturtevant (1989), who estimated the equivalent of  $0.001$ – $0.005 \mu\text{g ml}^{-1}$  production of kinetin by biological assays. We found higher concentrations in the present study, particularly for USDA110 (Fig. 1b). Jaiswal et al. (1982) found that exogenous zeatin increased nitrogenase activity in nodules of *Vigna mungo*. Zeatin production capacity could be advantageous for *B. japonicum* USDA110 as a legume partner, in view of the important role of this phytohormone plays at the beginning of nodulation.

Synthesis of gibberellin in rhizobia was first demonstrated by Atzorn et al. (1988) in *Rhizobium phaseoli*, where the bioactive molecules  $\text{GA}_1$  and  $\text{GA}_4$  were found at concentrations of  $0.03$  and  $0.7 \mu\text{g ml}^{-1}$ , respectively. We observed high  $\text{GA}_3$  production in all three *B. japonicum* strains, particularly E109 and USDA110 (Fig. 1c). Radley (1961) described high production of gibberellin-like substances in nodules of *P. vulgaris* and *Pisum sativum* in comparison to roots without nodules. Although the role of gibberellins in nodule formation is unclear, we expect that the concentrations of  $\text{GA}_3$  produced by E109, USDA110, and SEMIA5080 could be effective at least in promoting plant aerial growth. Along this line, Lucangelli and Bottini (1996) and Cassán et al. (2001) demonstrated that shoot growth in maize and rice dwarf mutants was promoted by gibberellin-like substances excreted or metabolized by *Azospirillum* sp.

ABA production was detected for USDA110 ( $0.02 \mu\text{g ml}^{-1}$ ), but not for E109 or SEMIA5080 (Fig. 1d). This is the first demonstration of ABA synthesis by any *B. japonicum* strain. Suzuki et al. (2005) reported recently that exogenous application of  $0.01$ – $5 \mu\text{g ml}^{-1}$  ABA reduced the number of established nodules in *Trifolium repens* and *Lotus japonicus* roots. However, ABA production capability could be an advantageous trait of USDA110 for inoculation of nonleguminous species in restrictive soil conditions, such as salinity or drought stress; i.e., ABA biosynthesis could help alleviate the plant stress by inducing a more tolerant plant response. Preliminary

**Fig. 1** Identification and quantification of phytohormones by HPLC-UV-GC-MS-SIM and obtained from chemically defined medium of *B. japonicum* E109 (with bars), USDA110 (black bars), and SEMIA5080 (gray bars): **a** IAA, **b** zeatin, **c** GA<sub>3</sub>, and **d** ABA obtained from 7 days of YEM cultures at 30°C and 80 rpm. Experiment was analyzed with ANOVA and Tuckey test. Values with the same letter are not significantly different at  $p < 0.01$ . Nd Not determined



**Fig. 2** Identification and quantification of ethylene by FID-HPLC obtained from YEM (white bars) and YEM plus methionine ( $0.1 \text{ g l}^{-1}$ ) (black bars) cultures of *B. japonicum* E109, USDA110,

and SEMIA5080 at 30°C and 80 rpm for 7 days. Experiment was analyzed with ANOVA followed by post hoc Tuckey test. Values with the same letter are not significantly different at  $p < 0.01$

results along this line in our laboratory Cassán et al. (2005) led us to propose a third category of beneficial bacteria: plant stress homeo-regulating rhizobacteria.

We also observed, for the first time, ethylene production by *B. japonicum* in culture medium with or without the addition of L-methionine (Fig. 2). The highest level ( $18.1 \text{ ng ml}^{-1} \text{ h}^{-1}$ ) was found for E109 in YEMm. This capacity of E109 could have a positive or negative effect on plant growth promotion, depending on the species to be inoculated. Ethylene functions as a nodulation inhibitor in legumes in which nodule establishment is controlled by ethylene biosynthesis (Nukui et al. 2000) through restriction of new nodulation zones by a feedback mechanism (Pierce and Bauer 1997). We found that *Bradyrhizobium* ethylene biosynthesis is mediated by the presence of L-methionine in culture medium. Similarly, if L-methionine is present in apoplast or rhizosphere exudates, association with *Bradyrhizobium* could increase the endogenous level of plant ethylene and thereby suppress new formation of nodules. In contrast, Ribaudo et al. (2006) suggest that IAA-mediated ethylene production could promote root growth in nonleguminous species. These authors show that ethylene production by bacteria or plants, induced by bacterial IAA biosynthesis, increases root biomass, root hair number, and consequently root surface area of inoculated plants.

In this work, we have identified and quantified the phytohormones IAA,  $\text{GA}_3$ , zeatin, ABA, and ethylene produced and released by *B. japonicum* strains E109, USDA110, and SEMIA5080 cultured in defined media. Such phytohormone production provides a mechanism other than nitrogen fixation by which these rhizobacteria may promote plant growth. Our findings also show differential capacities by different strains of the same species to synthesize and release various plant growth regulators, i.e., phytohormones.

An inoculant is a complex biological formulation that combines two elements: cultured microorganisms and compounds secreted to their growing medium under controlled conditions. This means that a commercially available inoculant should not be considered as a carrier of microorganisms but as a complex resulting from the biotransformation by the bacteria of the components added to the growing medium into different metabolites (phytohormones). Their biological activity may greatly influence processes such as early germination events, early seedling growth, plant colonization by bacteria, and bacterial establishment. Most inoculant factories sell the microorganism bottled together with the medium in which they were grown, and the whole complex is applied to the seeds. Thus, it would be very important to evaluate all the inoculant components for each microorganism strain to make accurate quality control.

Our results constitute an important technological contribution to *B. japonicum* strains selection for inoculant formulations to be used with leguminous and nonleguminous species, showing different phytohormone profile excreted by different strains.

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