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## Original article

# ***Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.)**

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## ABSTRACT

Inoculants are biological formulations that combine a stable microorganism population and various types of compounds produced and released during fermentation, such as phytohormones and plant growth regulators. *Azospirillum brasilense* strain Az39 and *Bradyrhizobium japonicum* strain E109 were previously shown to produce indole 3-acetic acid (IAA), gibberellic acid (GA<sub>3</sub>) and zeatin (Z). We tested the hypothesis that such compounds are responsible for early growth promotion in inoculated corn (*Zea mays* L.) and soybean (*Glycine max* L.) seedlings. Seeds were inoculated with Az39, E109, or both, and kept in a chamber at 20–30 °C under a controlled photoperiod to evaluate seed germination. To evaluate root and shoot length and dry weight, and number of nodules and percentage of nodulated seedlings, in soybean, seedlings were kept in a growth chamber for 14 days under similar photoperiod and temperature conditions. Az39 and E109, singly or in combination, showed the capacity to promote seed germination, nodule formation, and early development of corn and soybean seedlings. Both strains were able to excrete IAA, GA<sub>3</sub> and Z into the culture medium, at a concentration sufficient to produce morphological and physiological changes in young seed tissues.

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## 1. Introduction

Soil is a natural ecosystem in which a large variety and number of microorganisms proliferate. The term “rhizosphere” is used to describe the portion of soil in which such proliferation is induced by the presence of plant root systems

[8]. Bacteria in the rhizosphere, called “rhizobacteria”, have the ability to colonize plant roots and/or their immediate environment, in many species. Rhizobacteria are classified into two major groups, those which form symbiotic relationships with plants, and those which do not, termed *free-living* rhizobacteria. The *free-living* rhizobacteria are

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closely associated (epiphytic or endophytic) with plant roots [10]. Symbiotic or free-living rhizobacteria which are considered beneficial for plant growth and/or development have been termed plant growth-promoting rhizobacteria (PGPR) [9]. Many members of the Rhizobiaceae family have been extensively studied as symbiotic bacteria because they perform biological nitrogen fixation in association with legumes. However, certain free-living members of this family which establish different rhizospheric relationships with non-legumes can also be considered as PGPR [4,18]. In 1998, Bashan and Holguin [2] proposed dividing the PGPR group into bacteria which promote plant growth (a) through direct physiological or biochemical mechanisms (PGPB), and (b) indirectly through pathogen biocontrol or competition (biocontrol-PGPB).

Identification and possible manipulation of these relationships between PGPR and higher plants has been considered a basic strategy of modern agriculture in developing countries. In this regard, the most successful relationships studied are those between symbiotic Rhizobiaceae sp. with legumes, and those of free-living PGPR genera such as *Pseudomonas*, *Bacillus*, *Azotobacter*, and *Azospirillum*, which colonize the rhizosphere or root tissues of grasses or non-legumes [7]. Many reports have documented the successful use of *Azospirillum* sp. for improved growth, development, and yield of agriculturally important crop species throughout the world [1,11].

The growth-promoting capacity of rhizobacteria has been correlated with several mechanisms which have been extensively reviewed [3]. In particular, these mechanisms have been evaluated in bacterial strains commonly used for inoculant formulation in South American agriculture [13]. There is evidence that symbiotic nitrogen-fixing rhizobacteria could also be applied as free-living rhizobacteria in certain economically important grasses and non-legume crop species. For example, it has been shown that *Rhizobium* sp. can develop on the surface of monocots similarly to dicotyledons [15], and that *Bradyrhizobium* sp. can grow efficiently on grass or legume seeds during germination, stimulating root development in a similar manner to free-living rhizobacteria [12]. Other studies have documented direct plant growth-promoting capacity through siderophore production or phosphorus solubilization by various *R. leguminosarum* strains [19], and through these mechanisms plus IAA production in 18 strains of *B. japonicum* [18].

### 1.1. Overview of inoculant formulation in Argentina

When expansion of soybean (*Glycine max* L.) agricultural production in Argentina and other South American countries occurred during the 1970s, seed inoculation with *Bradyrhizobium* sp. was considered as a possible alternative to indiscriminate use of chemical fertilizers. After an intensive selection program initiated in 1980 by the Strains Collection Laboratory of the Agricultural Zoology and Microbiology Institute–Agricultural Technology National Institute (INTA-IMYZA), *B. japonicum* strain E109 was selected as the most suitable strain for soybean inoculant formulation in Argentina. Numerous field and laboratory studies since then have demonstrated the ability of E109 to significantly increase

soybean productivity [20,21]. However, there are no reports on its potential capacity to promote germination or early plant growth in non-legumes, individually or in combination with other PGPR, under controlled experimental conditions. When putative plant growth promoter compounds present in E109 culture medium were evaluated [22], phytohormone production was identified as the most important bacterial mechanism, besides symbiotic nitrogen fixation, for potential growth promotion. This mechanism could be applied for growth promotion in both legume and non-legume plant species.

In the 1980s, another intensive agricultural program to select and identify *Azospirillum* sp. strains able to improve productivity of wheat and corn (*Zea mays* L.) crops in Argentina was started by INTA-IMYZA. *Azospirillum brasilense* strain Az39 was selected as the most effective PGPR, and was recommended for use in inoculant formulation. Numerous field experiments since then have demonstrated the ability of Az39 to increase the productivity of grain crops such as wheat, corn, and sorghum [22,23]. However, there are no reports on its capacity to promote seed germination and/or early seedling growth of soybean, alone or co-inoculated with *Bradyrhizobium* sp. When putative plant growth promoter mechanisms present in Az39 grown in chemically-defined medium were evaluated [13], phytohormone production was again identified as the most important factor. Thus, phytohormone production and nitrogen fixation could effectively induce positive growth response in various crop species, alone or in co-inoculation systems, particularly in early-stage plant development.

In the present study, we tested the hypothesis that inoculation with *B. japonicum* E109 and *A. brasilense* Az39, either separately or in combination, may promote seed germination and early seedling development in soybean and corn through production of bacterial plant growth regulators.

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## 2. Materials and methods

### 2.1. Bacterial strains

*Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109 strains were provided by the Agriculture Collection Laboratory of the Instituto de Microbiología y Zoología Agrícola (IMYZA), Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, Argentina.

### 2.2. Soybean and corn seeds

Soybean (*Glycine max* L.) cv. Don Mario and corn (*Zea mays* L.) var. Mass 484 (Morgan) seeds were used. Quality control parameters were established by the International Seed Test Association (ISTA) (<http://www.ista.org>).

### 2.3. Bacterial growth and seed inoculation

Seeds were inoculated with individual or combined *B. japonicum* E109 and *A. brasilense* Az39 cultures. E109 titer was adjusted to  $5 \times 10^9$  UFC ml<sup>-1</sup>, obtained at exponential growth phase on Yeast Extract–Mannitol broth (YEM), as described by Terouchi and Syono [16]. The Az39 titer was adjusted to

$3 \times 10^8$  UFC ml<sup>-1</sup>, obtained at exponential growth phase on NFb broth, according to Döbereiner [6], and modified by addition of yeast extract (1.0 g l<sup>-1</sup>). Inoculation doses were adjusted to a final volume of 6 ml for soybean and 20 ml for corn individual seed inoculations; equal volumes of each strain were used for co-inoculation. After inoculation, seeds were maintained under sterile laminar air flow for 2 h at 30 °C. Four treatments were used: (1) non-inoculated seeds (control) treated with a final volume of buffer solution; (2) seeds inoculated with E109; (3) seeds inoculated with Az39; (4) seeds co-inoculated with E109 and Az39.

#### 2.4. Soybean germination test

One hundred seeds were sowed in plastic containers (10 × 30 cm), with double Whatman no. 2 filter paper soaked with sterile distilled water. To maintain humidity, the containers were wrapped in transparent plastic bags, hermetically sealed, and placed in a germination chamber for 8 days with a photoperiod 16 h light (30 °C)/8 h dark (20 °C), at 80% RH. The following parameters were measured: (a) percentage of germinated seeds (roots 5 mm long) on day 5 and day 8; (b) length (cm) of hypocotyls and roots on day 8.

#### 2.5. Soybean early growth promotion test

Ten soybean seedlings were grown hydroponically in plastic pots (1 l volume) containing vermiculite as substrate, and with nitrogen-deficient sterile Hoagland's solution (25% v/v) [24] provided by capillary watering. Seedlings were maintained for 14 days in a growth chamber with the same photoperiod as above. The following parameters were measured, as indicators of early growth promotion [5]: (a) shoot and root dry weight; (b) shoot and root length; (c) number of nodules per plant; (d) percentage of nodulated plants with three or more nodules in the main root.

#### 2.6. Corn germination test

The procedure was the same as for soybean seeds. The following parameters were measured: (a) percentage of germinated seeds (roots 5 mm long) on day 4 and day 7; (b) length (cm) of hypocotyls and roots on day 7.

#### 2.7. Corn early growth promotion test

The procedure was the same as for soybean seeds. Shoot and root length, and shoot and root dry weight, were measured as indicators of early growth promotion.

#### 2.8. Production of indole 3-acetic acid, zeatin, and gibberellic acid in defined cultures of *A. brasilense* Az39 and *B. japonicum* E109

Four 20 ml fractions of each bacterial culture were taken in exponential growth phase, and centrifuged separately at 8000 rpm, 4 °C, for 15 min. Supernatants were acidified at pH 2.5 with acetic acid solution (1% v/v), added to 100 ng corresponding <sup>2</sup>H<sub>5</sub>-IAA, or <sup>2</sup>H<sub>2</sub>-GA<sub>3</sub> (OlChemIm Ltd, Czech Republic) deuterated internal standard, and kept at 4 °C for 2 h. No deuterated

internal standard was used for zeatin determination. 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 μl acetic acid:acetonitrile:water (1:15:85) for IAA determination and methanol:water (30:70) for GA<sub>3</sub> and zeatin determination. Samples were injected into a reversed phase C<sub>18</sub> HPLC column (μBondapak, 300 × 3.9 mm, Waters Associates, Milford, MA) 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 min<sup>-1</sup> flow rate, and fractions eluting at the retention time (RT) corresponding to each pure standard were collected. Zeatin was identified and quantified by HPLC-UV at 254 nm [26]. IAA and GA<sub>3</sub> were identified and quantified by gas chromatography–mass spectrometry with selective ion monitoring (GC-MS-SIM). UV-absorbing fractions at 254 and 220 nm were grouped for IAA and GA<sub>3</sub> determination, respectively, and then methylated with ethereal diazomethane and silylated with 1:1 pyridine: BSTFA [bis(trimethylsilyl)trifluoroacetamide] plus 1% trimethyl-chlorosilane (Fluka Chemika, Switzerland) to obtain methyl-trimethylsilyl derivatives of IAA and GA<sub>3</sub>. Aliquots of each sample were injected directly into a DB1-15N (15 m × 0.25 mm, 0.25 μM methyl silicone) capillary column (J&W Scientific Inc.) 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 min<sup>-1</sup>, then 4 °C min<sup>-1</sup> to 260 °C. The carrier gas (He) flow rate was 1 ml min<sup>-1</sup>, interface temperature was 280 °C, and data acquisition was controlled by a HP 300 Series computer. By comparison of peak areas of the ion at mass/charge (m/z) 194 (molecular ion for [<sup>2</sup>H<sub>5</sub>]AIAMeTMSi) and the ion at m/z 189 (molecular ion for [<sup>1</sup>H]AIAMeTMSi) at the corresponding time, the amount of free AIA was calculated. The amount of GA<sub>3</sub> was calculated similarly by comparison of peak areas for the parent ion (m/z) 506 and (m/z) 504.

#### 2.9. Statistical analysis

Experiments were performed in triplicate. Values shown represent mean ± standard error of mean (SEM). Data were analyzed for variance by ANOVA followed by Tukey's post hoc analysis at *p* < 0.05. Analyses were performed using the PRISM V 4.0 statistical package for Windows.

## 3. Results

### 3.1. Soybean and corn germination

Seed germination percentage and early seedling growth in soybean inoculated with *B. japonicum* E109 or *A. brasilense* Az39, alone or in combination, are shown in Fig. 1. Both single inoculation and co-inoculation enhanced germination at 5 and 8 days, compared with control seeds. Germination percentage of inoculated seeds reached its maximal value by day 5 and maintained this value up to day 8. Maximal germination percentage of control seeds was lower. Final germination percentage values were not significantly different for the treatments, suggesting that promoting effect

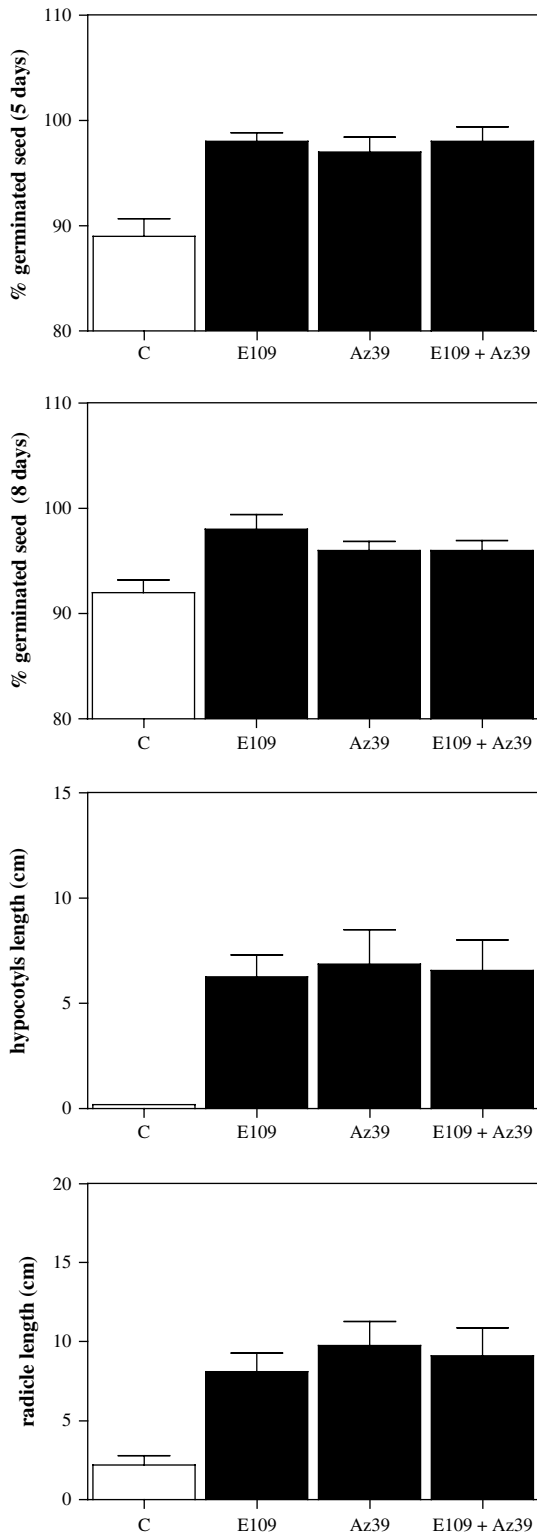


Fig. 1 – Percentage (%) of germinated soybean seeds at day 5, and percentage of germinated seeds, and length (cm) of hypocotyls and roots at day 8 after sowing. White bar, non-inoculated seeds; black bars, seeds inoculated with E109, Az39, or both. Data represent mean ± standard error of mean (SEM).

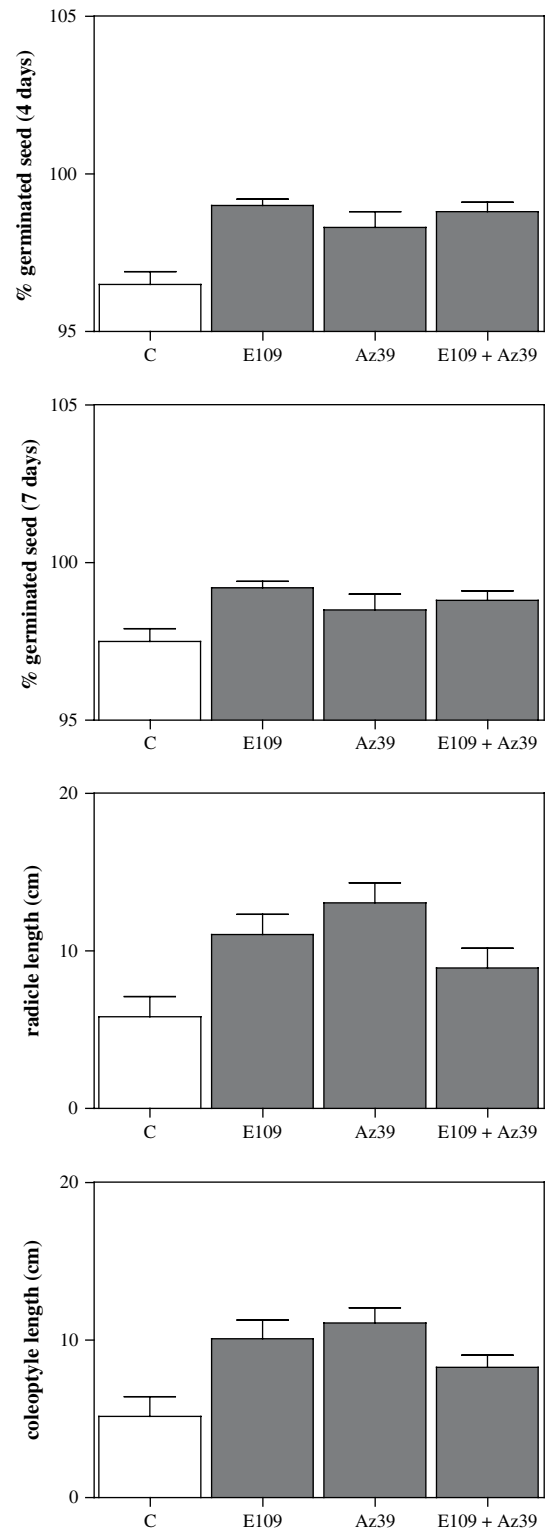


Fig. 2 – Percentage (%) of germinated corn seeds at day 4 and % of germinated seeds, and length (cm) of hypocotyls and roots at day 7. White bar, non-inoculated seeds; gray bars, seeds inoculated with E109, Az39, or both. Data represent mean ± standard error of mean (SEM).

in early growth stages may not depend on bacteria, but rather may involve growth regulators present in the culture medium. For root and hypocotyl growth, a similar but more pronounced difference was observed for inoculated vs. non-inoculated seedlings, supporting the potential role of bacterial plant growth regulators in enhancing germination rate and early development of plant structures.

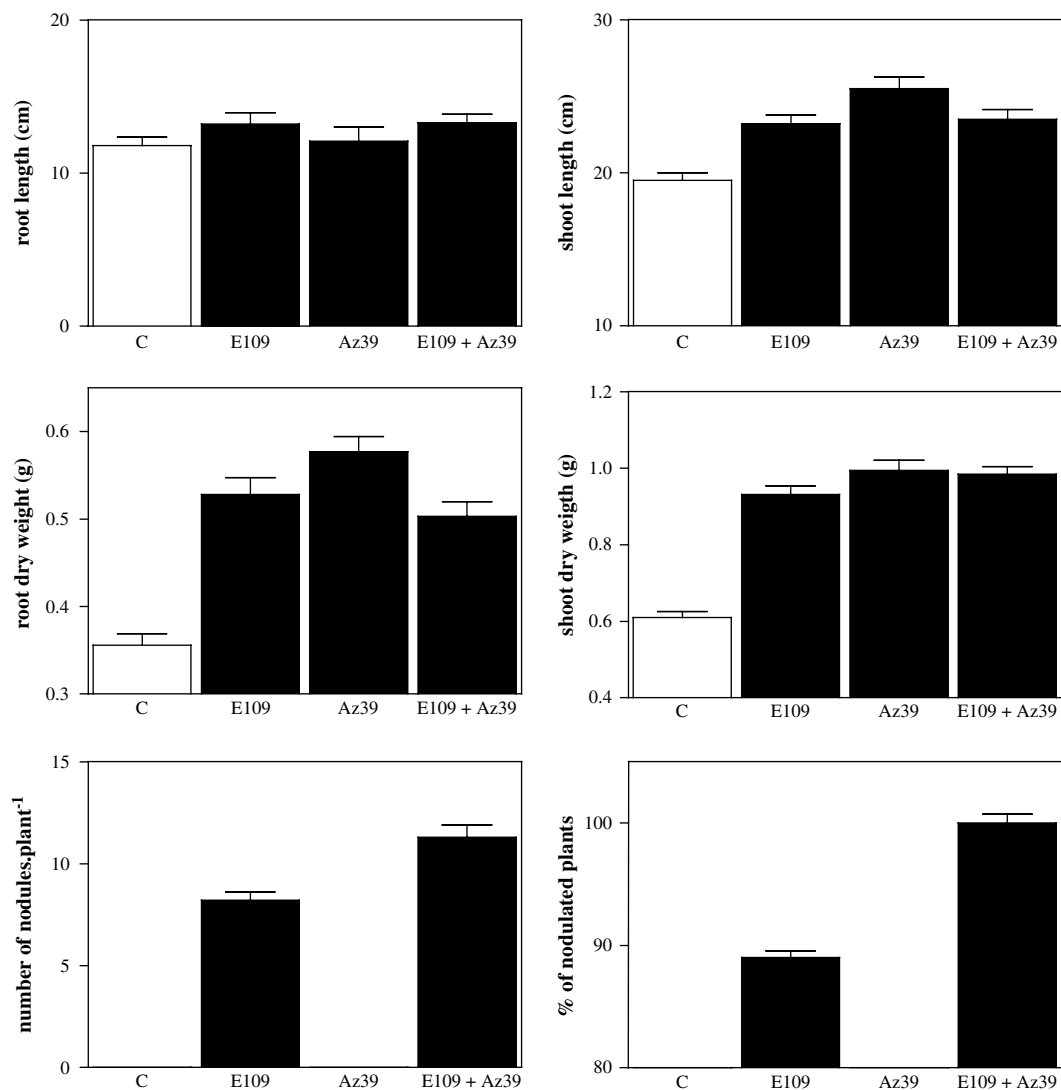
Seed germination percentages and early seedling growth in corn seeds inoculated with E109 and Az39, singly or in combination, are shown in Fig. 2. Single or combined inoculation enhanced germination up to day 7, compared with control seeds. For soybean, germination percentage of inoculated seeds reached maximal value at day 4 and stayed constant until day 7, while control seeds reached a lower final germination percentage.

Inoculation with Az39 alone caused a significant increase in root and hypocotyl early growth compared to co-inoculation

treatment. Such effect was not observed when E109 was inoculated alone.

### 3.2. Early growth in soybean and corn seedlings

Fig. 3 shows early seedling growth in terms of root and shoot length, root and shoot dry weight, number of nodules per plant, and percentage of nodulated plants [5] in 14-day-old soybean seedlings inoculated with E109, Az39, or both. Single and combined inoculations significantly increased shoot length and shoot and root dry weight. Root length in control seedlings was similar to that in inoculated seedlings. Root dry weight and shoot length in Az39-inoculated seedlings were significantly higher than in E109-inoculated or co-inoculated seedlings. Symbiosis-related parameters (number of nodules per plant; percentage of nodulated plants) were also determined in seedlings inoculated with E109, Az39, or both.



**Fig. 3 – Early growth promotion indicated by root and shoot length (top); root and shoot dry weight (middle); and nodulation (as number of nodules per plant and percentage of nodulated plants) (bottom) of soybean seedlings at day 14. White bar, non-inoculated seeds; black bars, seeds inoculated with E109, Az39, or both. Data represent mean  $\pm$  standard error of mean (SEM).**

Numbers of newly-formed nodules and percentage of nodulated plants (having three or more nodules in the main root), were both higher in co-inoculated seedlings, perhaps because of excretion of metabolic products from Az39 during bacterial fermentation.

Root and shoot length and dry weight in 14-day-old corn seedlings inoculated with E109, Az39, or both, are shown in Fig. 4. Again, shoot length and dry weight increased significantly for both single and combined inoculation treatments, whereas roots showed increase in dry weight but not in length. In fact, root length of non-inoculated seedlings was higher than that for any inoculation treatment, perhaps due to root morphogenesis regulation by bacterial IAA [25]. Root and shoot dry weight for co-inoculation treatments was higher than for control or single-inoculation treatments.

### 3.3. Phytohormones and production of plant growth regulators

Table 1 summarizes production of indole 3-acetic acid (IAA), zeatin (Z), and gibberellic acid ( $GA_3$ ) by cultures of E109 and Az39 in exponential growth phase, determined by GC-MS or HPLC-UV as described in Section 2. Phytohormone production was tested as previously described for E109 [4] and for Az39 [13]. Az39 culture medium, compared to E109 medium, showed higher IAA and Z levels and lower  $GA_3$  level.

## 4. Discussion

Our results indicate that *A. brasilense* Az39 and *B. japonicum* E109, inoculated singly or in combination, have the capacity to

promote seed germination and early seedling growth in soybean and corn. This capacity could be due, at least in part, to bacterial phytohormone biosynthesis during culture. Az39 and E109 are both evidently able to excrete plant growth regulator compounds into the culture medium, at a concentration sufficient to produce morphological and physiological changes in young seed tissues. Such “phytohormonal shock” would be the first contact between the bacterial formulation and the seed, and would not necessarily depend on bacterial cell presence as has been proposed previously [26,27]. However, the presence of live bacteria may contribute to *in situ* phytohormone production, since it was shown, that e.g. *A. brasilense* induces the key gene of indole-acetic acid production (*ipdC*-gene) when colonizing the wheat root surface [40]. Similarly, increase of biomass during the seedling stage could be due in part to differential embryo development induced by bacterial growth regulators which penetrate the seed coat along with water, and accelerate root growth with concomitant increases in water and mineral uptake. This concept is consistent with the “additive hypothesis” of Bashan et al. [3], and emphasizes the importance of biological nitrogen fixation for biomass increase in later developmental stages, and even for grain production, once a significant number of bacteria have become established in plant tissues. According to this concept, bacterial phytostimulation would be crucial in early developmental stages such as germination and seedling growth.

We observed differences in the intrinsic ability of the two tested strains to produce phytohormones in defined medium. Az39 was a better producer of the root growth-regulating compounds IAA and zeatin, whereas E109 was a better producer of the shoot growth promoter  $GA_3$ . Single or

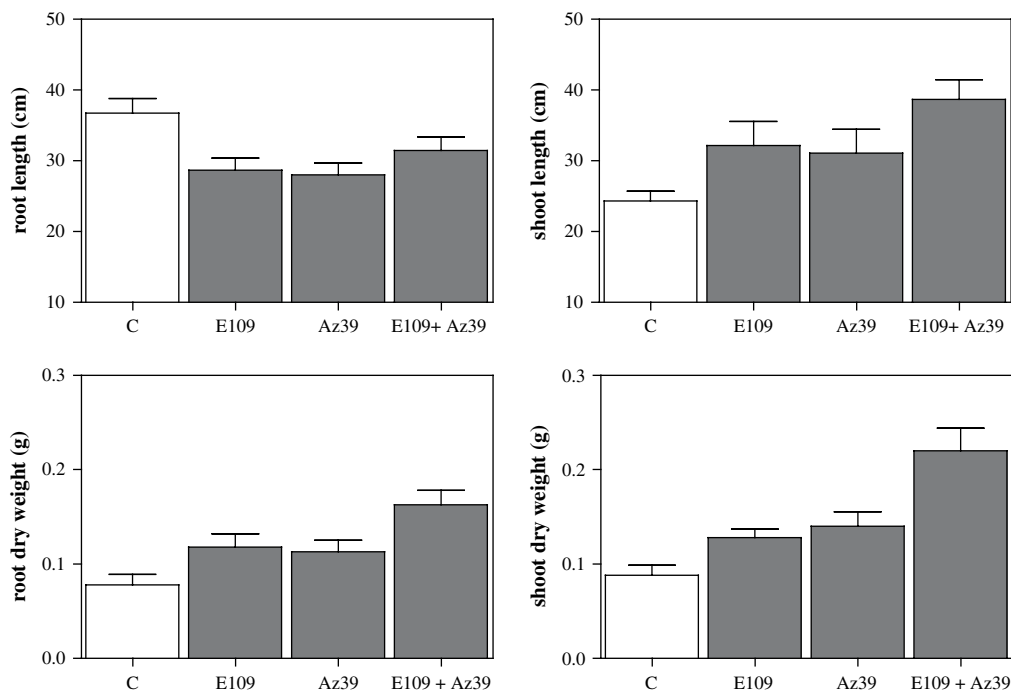


Fig. 4 – Early growth promotion indicated by root and shoot length (top) and root and shoot dry weight (bottom) of corn seedlings at day 14. White bar, non-inoculated seeds; gray bars, seeds inoculated with E109, Az39, or both. Data represent mean  $\pm$  standard error of mean (SEM).

**Table 1 – Phytohormone production ( $\mu\text{g ml}^{-1}$ ) of indole 3-acetic acid (IAA), zeatin (Z) and gibberellic acid ( $\text{GA}_3$ ) in exponential growth culture of *B. japonicum* E109 and *A. brasilense* Az39, identified by GC-MS or HPLC-UV, as described in Section 2**

Strain	IAA production ( $\mu\text{g ml}^{-1}$ )	Z production ( $\mu\text{g ml}^{-1}$ )	$\text{GA}_3$ production ( $\mu\text{g ml}^{-1}$ )
<i>B. japonicum</i> E109	6.62 $\pm$ 0.37	0.65 $\pm$ 0.02	0.95 $\pm$ 0.02
<i>A. brasilense</i> Az39	13.16 $\pm$ 0.33	0.88 $\pm$ 0.03	0.39 $\pm$ 0.03

All experiments were carried out in triplicate and the values shown are mean  $\pm$  standard error of mean (SEM). *B. japonicum* E109 titer:  $5.2 \times 10^9$  UFC  $\text{ml}^{-1}$ ; *A. brasilense* Az39 titer:  $3.4 \times 10^8$  UFC  $\text{ml}^{-1}$ .

combined inoculation treatments increased seed germination by almost 100%. The inoculation treatments also caused significant increase of hypocotyl and coleoptile length in soybean and corn respectively, and of root length in both species. Consistent with this observation, Barbieri et al. [28] described the ability of *Azospirillum* sp. to modify root growth in grasses through phytohormone production, and Burdman et al. [29] reported a similar effect by inoculation of legume seedlings with *Azospirillum* sp. Noel et al. [30] reported significant enhancement of early seedling root growth in non-legumes by seed inoculation with a Rhizobiaceae family member, *Rhizobium leguminosarum*, and attributed this effect to bacterial phytohormone production.

In the present study, single and combined inoculations promoted early dry weight and shoot length in both soybean (Fig. 3) and corn (Fig. 4) seedlings. In contrast, root length was not increased in inoculated seedlings. In regard to this point, Dobbelaere et al. [25] showed that inhibition of root length together with increase of root volume and weight are typical responses to bacterial IAA production, and can be mimicked by exogenous application of IAA on the seed. Zelena et al. [34] found that exogenous application of IAA on corn seedling roots significantly increased lateral root number. Kolb and Martin [32] showed that inoculation of beet (*Beta vulgaris*) with *A. brasilense* increased the number and length of lateral roots, and these effects were correlated with IAA concentration in the bacterial culture medium used for seed inoculation.

The increases of seed germination percentage and seedling shoot length observed here are considered typical gibberellin-like responses. They mimic the effect of exogenous  $\text{GA}_3$  application, and the typical promoter effect caused by seed inoculation with PGPR [31].

Regarding nitrogen fixation in soybean seedlings, we found that co-inoculation with Az39 and E109 significantly increased the number of nodules per plant, and percentage of nodulated plants, compared to single inoculation with E109. This finding could be explained by the capacity of Az39 to synthesize and release phytohormones into the culture medium applied to the seeds.

Several studies have suggested that auxins and cytokines play essential roles in nodule development [36]. Prinsen et al. [37] proposed that both *nod* factors and IAA biosynthesis are triggered in *Rhizobium meliloti* by plant *nod*-derivatives such as flavonoids. Schmidt et al. [38] showed that co-inoculation of *Medicago sativa* seeds with an *R. meliloti* inefficient IAA producer strain and *A. brasilense* significantly increased the number of nodules on the main root, and that co-inoculation of *M. polymorpha* with *Rhizobium* sp. and *Azospirillum* sp. increased the number, weight, and nitrogenase activity of root

nodules in comparison with single-inoculated plants [33]. Jaiswal et al. [35] reported that exogenous zeatin application (at a concentration similar to that found in the present study) increased nitrogenase activity in nodules of *Vigna mungo*, suggesting that this phytohormone may play an essential role at the start of the nodulation process. Very recently, Giraud et al. [39] demonstrated that *nod* factors are not strictly necessary in certain legume species because their *Bradyrhizobium* sp. partner strain can use an alternative communication pathway, in which a cytokine-like compound is responsible for triggering root nodule formation in the host plant.

Taken together, these findings illustrate the importance of knowing the precise “hormonal profile” of each bacterial strain involved in inoculation for improved crop production, in order to clarify the complex biochemical and physiological processes underlying germination and early plant growth.

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