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Original Article

New insights into auxin metabolism in *Bradyrhizobium japonicum*

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ABSTRACT

Bacterial metabolism of phytohormones includes several processes such as biosynthesis, catabolism, conjugation, hydrolysis and homeostatic regulation. However, only biosynthesis and occasionally catabolism are studied in depth in microorganisms. In this work, we evaluated and reconsidered IAA metabolism in *Bradyrhizobium japonicum* E109, one of the most widely used strains for soybean inoculation around the world. The genomic analysis of the strain showed the presence of several genes responsible for IAA biosynthesis, mainly via indole-3-acetonitrile (IAN), indole-3-acetamide (IAM) and tryptamine (TAM) pathways. However; *in vitro* experiments showed that IAA is not accumulated in the culture medium in significant amounts. On the contrary, a strong degradation activity was observed after exogenous addition of 0.1 mM of IAA, IBA or NAA to the medium. *B. japonicum* E109 was not able to grow in culture medium containing IAA as a sole carbon source. In YEM medium, the bacteria degraded IAA and hydrolyzed amino acid auxin conjugates with alanine (IAAla), phenylalanine (IAPhe), and leucine (IAPhe), releasing IAA which was quickly degraded. Finally, the presence of exogenous IAA induced physiological changes in the bacteria such as increased biomass and exopolysaccharide production, as well as infection effectiveness and symbiotic behavior in soybean plants.

1. Introduction

Symbiotic rhizobacteria belonging to the genus *Bradyrhizobium* have been extensively studied from the perspective of their association with legumes and the biological nitrogen fixation process [1]. Some members of this genus have also shown capacity to colonize certain non-legumes, survive in their rhizosphere and promote their growth in a *free-living* plant–microbe interaction model [2]. These are considered plant growth-promoting rhizobacteria (PGPR) [3]. The plant growth-promoting activity was primarily correlated with nitrogen fixation but the presence of other stimulatory actions was also assumed. They include the effects on (a) phosphorus availability [4], (b) siderophore production [5], and (c) phytohormone metabolism – auxins [6], cytokinins [7] or gibberellins [8], among other molecules with biological activity in plants, such as lipochitooligosacharides (LCOs) [9]. Auxin is a generic name for a group of chemical compounds characterized by their ability to induce plant cell growth, affecting both cell division and cellular expansion in the subapical region of the stem, and to reproduce the physiological effects of the naturally occurring auxin indole-3-acetic acid (IAA). These compounds have been associated with different plant processes such as (a) gravitropism and phototropism, (b) vascular tissue differentiation, (c) apical dominance, (d) lateral and adventitious root initiation, (e) stimulation of

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cell division, and (f) stem and root elongation [10]. Ever since Thimann [11] proposed that auxins play an important role in the ontogeny (formation and development) of the nodule in *Rhizobium*-legume symbiosis, many studies have indicated that changes in the concentration of this phytohormone or its balance with cytokinins are a mandatory pre-requisite for nodule organogenesis [7]. Therefore, inoculation with auxin-producing rhizobacteria can influence the symbiotic outcome by altering phytohormonal homeostasis.

Several molecules have been classified as auxins, but IAA is one of the most important in nature. The IAA metabolism in higher plants and fungi comprises several mechanisms, such as the biosynthesis, catabolism (i.e. oxidation and assimilation), conjugation and hydrolysis of IAA conjugates [12], which globally regulate the IAA levels in these biological systems. The IAA metabolism *per se* is still poorly understood in bacteria, since the focus is mainly on IAA biosynthesis. At least six metabolic pathways for IAA biosynthesis have been proposed in bacteria and most of them use tryptophan (Trp) as a precursor. These routes have been named mostly according to a key intermediate of the given pathway, such as indole-3-pyruvate (IPyA), indole-3-acetamide (IAM), tryptamine (TAM) and indole-3-acetonitrile (IAN) pathway, although one pathway was named after the key enzyme tryptophan side-chain oxidase (TSO) and briefly described in [56]. Moreover, a tryptophan-independent pathway has been suggested [13]. Despite this diversity of pathways, prokaryotic IAA biosynthesis seems to follow predominantly two major routes: the IAM and IPyA pathways [14]. In addition to *de novo* biosynthesis, plants produce IAA by hydrolysis of IAA conjugates containing sugars, high molecular weight glycans, amino acids and peptides [15]. These metabolites usually serve as a reservoir of IAA that may be hydrolyzed to quickly provide the plant with hormones in their active forms. These compounds have diverse roles, such as protection against degradation, storage and transport, and are important to maintain IAA homeostasis in the cells [16]. Thus, their hydrolysis must be taken into account along with *de novo* synthesis when considering input into the free IAA pool [17]. The role of IAA conjugates in microorganisms is unknown. Certain strains of *Pseudomonas syringae* subsp. *savastano*i conjugate IAA with lysine (IALys) [18]. The gene responsible for the synthesis of IALys is indole-3-acetic acid-lysine synthetase (*iaaL* gene), which encodes an ATP-dependent enzyme that catalyzes the formation of an amide bond between the carboxyl group of IAA and the amino group of lysine [18,19]. It has been demonstrated that variations in the size of the IAA "pool" of *P. syringae* subsp. *savastanoi* are related to the synthesis and conversion of IAA in this conjugated form. It was reported that *iaaL* gene function may be related to pathogenesis, but the mechanism of expression and the environmental conditions in which this occurs remain unclear [20]. Chou et al. [21] identified a hydrolase, an enzyme that cleaves IAA conjugates with amino acids in a strain of *Enterobacter agglomerans* with apparent specificity for N-acyl aspartate. Nevertheless, other compounds of amide structure could induce the synthesis of this enzyme. Catabolism is characterized by the loss of biological activity of the hormone through oxidation, decarboxylation or assimilation, creating a source of carbon or nitrogen. *Arthobacter* spp. [22] and *Pseudomonas* spp. are examples of IAA catabolism by oxidation. In "isolates obtained from air samples" degradation occurs via formation of acetic o-formaminobenzoyl acid, o-aminobenzoyl, and anthranilic acid [23]. Furthermore, *Pseudomonas putida* strain 1290 is able to degrade IAA and use it as a source of C and N for growth due to its high nutritional value and easy digestibility. *P. putida* 1290 catabolizes IAA to catechol due to the existence of a cluster of 10 genes (*iac* genes) that encodes a set of necessary enzymes for IAA catabolic assimilation [24]. All these examples demonstrate a great versatility of the IAA metabolism in nature and especially in microorganisms, and show a wide range of mechanisms and factors which regulate the phytohormone levels in the rhizosphere.

Bradyrhizobium sp. has the ability to produce IAA and this capacity has been demonstrated *in vitro* and *in planta* after soybean seed inoculation [6,18]. Nodules caused by an IAA-overproducing strain of *Bradyrhizobium japonicum* contained more IAA than nodules induced by a wild strain. This indicates that the bacteroid in the nodule is able to synthesize the phytohormone [25,26]. Minamisawa and Fukai [27] observed that the rhizobitoxine-producing strains of *B. japonicum* also excreted IAA into the culture medium. Lamont et al. [28] showed greater IAA production by *Bradyrhizobium* after soybean root infection. Egebo et al. [29] showed that *Bradryhizobium diazoefficiens* strain USDA 110 is able to degrade IAA and that this reaction might be dependent on oxygen availability in the culture medium. They hypothesized that anthranilic acid could be a degradation product of this catabolic pathway. Jensen et al. [30] isolated metabolites of IAA degradation in USDA 110. The products identified indicated a new metabolic pathway in which IAA is metabolized to anthranilic acid, which is then further metabolized. Donati et al. [31] evaluated transcriptional activity of *B. diazoefficiens* USDA110 after exogenous treatment with 1 mM IAA. They observed a general increase in stress response genes, such as those involved in response to heat, cold, oxidative, osmotic, and desiccation stress and in exopolysaccharide (EPS) biosynthesis. However, they could not identify the enzyme(s) responsible for such degradation and proposed an indole pyruvate ferredoxin oxidoreductase and tryptophan 2,3-dioxygenase as the enzymes responsible for the hormone degradation. Recent results showed that the *Bradyrhizobium* strain RJS9-2 exhibited higher salt tolerance than other *Bradyrhizobium* sp. strains evaluated, and the presence of salt in the culture medium induced higher IAA production. These results suggest that the presence of IAA could regulate RJS9-2 tolerance to salt stress [32]. There are no further reports confirming or refuting this hypothesis. At present, there is little information about the full set of processes involved in IAA metabolism in *B. japonicum*, its homeostatic control, and the effects of exogenous auxin on the symbiotic interaction with soybean.

The aim of this study was to elucidate the metabolism of IAA and its conjugates in *B. japonicum* E109, the bacterial homeostatic response to exogenous auxin applications and their effect on bacterial growth and soybean symbiosis under environmentally controlled conditions.

2. Materials and methods

2.1. Bacterial strains

B. japonicum strain E109 was provided by the Instituto de Microbiología y Zoología Agrícola, INTA-IMyZA, Castelar, Buenos Aires, Argentina. *B. japonicum* E109 is the most recommended strain for soybean inoculation in the last fifty years in Argentina due to its capacity to effectively colonize the plant, to fix nitrogen efficiently and increase crop productivity [33]. Torres et al. [34] recently published the complete annotated genome sequence of *B. japonicum* E109. *Bradryhizobium elkanii* strain SEMIA 5019, an IAA producer, was obtained from the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" located in Londrina, Paraná, Brazil.

2.2. Culture conditions

B. japonicum strain E109 and *B. elkanii* SEMIA 5019 were grown in 250 ml flasks containing 100 ml of Yeast Extract Mannitol (YEM) culture medium, according to Vincent [35] at 30 °C and 80 rpm

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shaking until exponential growth phase, $OD_{595} \approx 1$, equivalent to $\approx 1.0 \times 10^9$ colony-forming units (cfu.ml^{-1}) in agar YEM plates. From each culture, an aliquot of biological material was taken for optical density (OD_{595}) and cell counting (cfu.ml^{-1}) measurements, as well as for determination of exopolysaccharide (EPS) production, and/or for auxin quantification, according to the treatment. The level of purity of each culture was determined by agar TSA plating (Trypticase Soybean Agar) at 10% (v/v). The agar TSA inhibits the development of *Bradyrhizobium* sp. but allows the development of a wide range of bacteria.

2.3. Quantification of auxins in bacterial culture by HPLC

An HPLC methodology was used to quantify IAA. Briefly, 1000 μl of *B. japonicum* E109 and *B. elkanii* SEMIA 5019 cultures were taken and placed in an Eppendorf type plastic tube of 1.5 ml capacity. The samples were centrifuged at 10,000 rpm for 10 min and then filtered (0.2 μm). The supernatant was injected with a final volume of 20 μl in an HPLC Waters 600-MS device (Waters Inc., USA) equipped with an U6K injector and C_{18} reverse phase column (Purospher STAR RP C-18 3 μm, Lichrocart 55-4) heated to 30 °C, coupled to a system with UV-VIS Waters 486 detector (Waters Inc., USA) set at 265 nm. Elutions were performed with a mixture of ethanol: acetic acid: water (12: 1: 87) as mobile phase at a flow rate of 1 ml min^{-1}. The retention time for IAA was 10.5 min, and it was previously identified using appropriate standards (Sigma–Aldrich, Germany and Olchemin, Czech Republic). Quantification was performed by integration of the peak area corresponding to retention time (RT) with Empower® integration software (Waters Inc. USA). A similar methodology was developed to identify and quantify amino acid IAA conjugates (IAA-L-phenylalanine, IAPhe; IAA-L-alanine, IAAla; IAA-L-leucine, IALeu), IBA and 1-naphthalene acetic acid (NAA) in *B. japonicum* supernatants.

2.4. Identification and quantification of endogenous IAA and related metabolites

A methodology combining a one-step solid phase extraction (SPE) purification method with sensitive and selective liquid chromatography–multiple reaction monitoring-mass spectrometry (LC-MRM-MS) analysis was used [36]. Briefly, cultures of *B. japonicum* E109 and *B. elkanii* SEMIA 5019 in exponential growth phase ($OD_{595} = 0.8–1.0$, respectively) were obtained. Then, 750 μl of each culture were taken and inoculated in a glass bottle (250 ml capacity) containing 150 ml of YEM medium [35] modified by addition of 10 mg l^{-1} of L-Trp. The treatments were grown to late exponential and stationary phase ($OD_{595} = 1.4–1.6$, respectively). Finally, the culture was centrifuged and filtered to obtain a clean supernatant, which was used to identify and quantify all auxin metabolites. The original sample was divided into 3 replicates of equivalent volume. For quantification, 1 ml of supernatant of each treatment was taken. To validate the quantification of endogenous auxin metabolites, the following stable isotope-labelled internal standards were added: [$^2H_4$]ANT, [$^{13}C_6$]IAA, [$^2H_5$]IAM, [$^2H_4$]IPyA, [$^{13}C_6$]oxIAA, [$^{13}C_6$]IAAsp, [$^{13}C_6$]IAGlu, [$^{13}C_6$]IAGlc (10 pmol per sample), [$^2H_4$]IAN (20 pmol per sample and [$^2H_5$]Trp (100 pmol per sample). The supernatants were incubated at 4 °C with continuous shaking (15 min), centrifuged (15 min, 23,000 g at 4 °C), and then divided in two. In one half, the sample was diluted 5 times with distilled water and the pH was adjusted to 2.7 with 1 M hydrochloric acid. The sample was purified by solid-phase extraction using Oasis™ HLB columns (60 mg/3 ml, Waters, http://www.waters.com) conditioned with 2 ml methanol and 2 ml water, and equilibrated with 0.5 ml sodium phosphate buffer (acidified with 1 M HCl to pH 2.7). After sample application, the column was washed with 4 ml of 5% methanol and then eluted with 3 ml 80% methanol. The eluate was evaporated to dryness *in vacuo* and stored at −20 °C until LC-MS analysis. The second half of the supernatant (approximately 0.5 ml) was derivatized using cysteamine to convert labile IPyA to its thiazolidine derivate IPyA-TAZ. Three milliliters of a 0.25 M cysteamine solution (adjusted with NH_3 pH 8.0) were added to each extract. The samples were incubated for 1 h at room temperature, acidified with 3M HCl to pH 2.7 and purified as described above.

2.5. Quantification of exopolysaccharides (EPS)

For quantification of EPS, the samples obtained from different *B. japonicum* E109 and *B. elkanii* SEMIA 5019 treatments were centrifuged at 16,000 × g for 30 min at 4 °C. The supernatants were filtered using 0.45 μm filter (Millipore, USA) and then treated with DNase I and proteinase K as previously described [37,38]. The EPS precipitated after application of 3-fold volume of ethanol at −20 °C and overnight incubation. A second ethanol precipitation was performed to ensure proper recovery of EPS [31,39]. EPS was dried at room temperature before being resuspended in deionized water. Total carbohydrate content (EPS.mg^{-1} biomass) was measured with the phenol-sulfuric acid method, following DuBois et al. [40] with glucose as a standard.

2.6. Analysis of IAA metabolism and homeostasis

2.6.1. Genomic analysis of IAA biosynthetic pathways

The aim of this analysis was to examine *in silico* whether E109 has coding sequences in its genome related to the biosynthesis of IAA. We compared the results with an *in vitro* metabolite profile in section 2.6.2. The *in silico* analysis of biological systems was done through KEEG (Kyoto Encyclopedia of Genes and Genomes) [41] and NCBI (National Center for Biotechnology Information). Briefly, predicted and annotated gene sequences obtained from the genome of E109 were analyzed for similarity with the enzyme database of KEGG and NCBI, followed by the assignment of each gene to KEGG pathway chart for Trp metabolism and IAA biosynthesis. Based on this analysis, integrated biochemical pathways were proposed and compared with the metabolite profile obtained *in vitro* as mentioned in section 2.6.2.

2.6.2. IAA biosynthesis and metabolic profile

The aim of these experiments was to evaluate the capacity of *B. japonicum* E109 to produce IAA and its metabolites in liquid culture medium, and to define the possible IAA biosynthetic pathway in the strain. For this, a pure culture of E109 was obtained in the conditions mentioned in § 2.2. Five ml aliquots from early exponential growth phase ($OD_{595} \approx 0.6$) were transferred to 10 ml sterile borosilicate tubes and then either (1) 10 mg ml^{-1} L-Trp solution (IAA precursor), or (2) an equivalent volume of distilled water were added. Tubes were incubated at 30 °C and 80 rpm until stationary growth phase. Samples for the evaluation of optical density (OD_{595}), cell number (cfu.ml^{-1}) and IAA, ANT, IAM, and IPyA concentrations (μg ml^{-1}), as described in § 2.4, were collected after 24 h and 48 h incubation. The metabolite profiles were compared with the IAA-producing strain of *B. elkanii,* SEMIA 5019.

2.7. Biosynthesis of IAA conjugates

The aim of these experiments was to evaluate the ability of *B. japonicum* E109 to produce IAA conjugates with amino acids. For that, 100 mL of *B. japonicum* E109 from YEM culture were obtained in exponential growth phase and then divided aseptically by triplicate into 5 ml tubes as follows: (1) *B. japonicum* E109 (control),

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(2) *B. japonicum* E109 culture supplemented with 0.1 mM of IAA, (98%, Sigma–Aldrich, USA) and 10 mg ml^{-1} L-phenylalanine (L-Phe), (3) *B. japonicum* E109 with 0.1 mM of IAA and 10 mg ml^{-1} L-leucine (L-Leu), and (4) E109 with 0.1 mM of IAA and 10 mg ml^{-1} L-alanine (L-Ala). A set of negative controls was prepared using YEM culture medium supplemented with 0.1 mM of IAA and 10 mg ml^{-1} of each amino acid and YEM culture medium supplemented with 10 mg ml^{-1} of each amino acid. Tubes were incubated at 30 °C and shaken at 80 rpm, and samples were collected after 24 h and 48 h incubation to evaluate bacterial growth (OD_{595}), cell counts ($cfu.ml^{-1}$), exopolysaccharide content ($EPS.mg^{-1}$ biomass), and the concentration of IAA and its conjugates ($\mu g.ml^{-1}$).

2.8. IAA conjugate hydrolysis

Our aim here was to determine the ability of *B. japonicum* E109 to hydrolyze IAA conjugates. For that, 100 ml YEM culture from exponential phase were obtained, and then divided aseptically by quintuplicate into 5 ml tubes and used for the following treatments: (1) *B. japonicum* E109 (control), (2) *B. japonicum* E109 culture supplemented with 0.1 mM of IAPhe (98%), (3) 0.1 mM IALeu (98%) and (4) 0.1 mM of IAAla (98%) (Sigma–Aldrich, USA). A set of negative controls was prepared using YEM culture medium supplemented with 0.1 mM of each IAA conjugate. Tubes were incubated at 30 °C and shaken at 80 rpm, and then triplicate samples were collected at 24 and 48 h to evaluate bacterial growth (OD_{595}), cell count ($cfu.ml^{-1}$), exopolysaccharide production ($EPS.mg^{-1}$ biomass), and the concentration of IAA and its conjugates ($\mu g.ml^{-1}$).

2.8.1. Hydrolysis of IAA conjugates and IAA degradation

We evaluated the hydrolysis of IAA amino acid conjugates and the consequent degradation of free IAA at different stages of *B. japonicum* E109 growth. Free IAA content was quantified by HPLC after addition of IAA conjugate solutions to the E109 cultures. The following treatments were evaluated: (1) *B. japonicum* E109 (control), (2) *B. japonicum* E109 supplemented with 0.1 mM IAA (98%), (3) 0.1 mM IAPhe (98%), (4) 0.1 mM IALeu (98%), and (5) 0.1 mM IAAla (98%) (Sigma–Aldrich, USA). A set of negative control treatments was also performed using YEM culture medium supplemented with 0.1 mM of each IAA conjugate or IAA. The tubes were incubated at 30 °C with 80 rpm shaking, and samples were collected at 15 min, 6 h, 24 h and 48 h in order to consider different stages in the culture growth. In all cases, bacterial growth (OD_{595}), cell counts ($CFU.ml^{-1}$) and IAA concentrations ($\mu g.ml^{-1}$) were measured.

2.9. Non-assimilative degradation of auxins

The aim of these experiments was to examine the non-assimilative degradation of IAA and related auxins by *B. japonicum* E109. Briefly, E109 grew until exponential growth phase ($OD_{595} \approx 0.6$) and then 5 ml aliquots of the culture were transferred into 10 ml sterile borosilicate tubes for the following treatments: (1) *B. japonicum* E109 (control), (2) E109 supplemented with 0.1 mM of IAA (98%), (3) 0.1 mM indole-3-butyric acid (IBA) (98%), and (4) 0.1 mM of NAA (technical grade) (Sigma–Aldrich, USA). A set of negative controls was prepared using YEM culture medium supplemented with 0.1 mM of each IAA, IBA or NAA. The tubes were incubated at 30 °C and shaken at 80 rpm, and samples were taken after 24 and 48 h incubation to evaluate bacterial growth (OD_{595}), cell counts ($cfu.ml^{-1}$), exopolysaccharide production ($EPS.mg^{-1}$ biomass) and auxin concentrations ($\mu g.ml^{-1}$). Similar treatments were performed with *B. elkanii* SEMIA 5019.

2.10. IAA assimilative degradation

The ability to use IAA as a sole carbon source in a chemically defined culture medium was analyzed in *B. japonicum* E109. A pure IAA culture was pre-incubated in 250 ml flasks containing 100 mL of YEM medium without addition of L-Trp (30 °C and 200 rpm orbital shaking). After cultures reached $OD_{595} \approx 0.6$, the cells were centrifuged, washed and re-suspended in fresh YEM medium or minimal medium (MM-IAA) containing 0.1 mM-1.0 mM IAA (98%) (Sigma–Aldrich, USA) as a sole carbon source, sodium glutamate (0.05 g/l) as a sole nitrogen source, K_2HPO_4 (0.5 g/l), $MgSO_4.7H_2O$ (0.2 g/l), NaCl (0.1 g/l), 10% $FeCl_3.6H_2O$ (0.1 ml/l) and 10% SO_4Mn (0.1 ml/l). The cultures were incubated for 24 and 48 h at 30 °C and shaken at 80 rpm, with the aim to evaluate the decrease in IAA concentration due to assimilative degradation. A minimal medium containing 1.0 mM mannitol as carbon source was used as a control to confirm bacterial growth capacity. Biomass production (OD_{595}), cell viability ($cfu.ml^{-1}$), exopolysaccharide (EPS) production, and IAA concentration ($\mu g.ml^{-1}$) were measured.

2.11. IAA homeostasis and Bradyrhizobium-soybean symbiosis

The *Bradyrhizobium*-soybean symbiosis is considered to be one of the most efficient in fixing N_2 and is probably the most economically important around the world [42]. The use of inoculants is evaluated by the number and physiological state of the cells contained in the formula [43], the number of bradyrhizobia recovered from the inoculated seeds [44], and the bacterial capacity to establish symbiosis with soybean seedlings under the controlled conditions. Therefore, the objective of this experiment was to evaluate if the presence of exogenous IAA in *B. japonicum* E109 culture can modify its capacity to survive on soybean seeds after inoculation and to establish an effective symbiosis with soybean seedlings. For that, *B. japonicum* E109 grew until exponential growth phase ($OD_{595} \approx 0.6$) in 200 ml flasks, and then 5 ml aliquots in quadruplicates were placed in sterile tubes as follows: (1) *B. japonicum* E109 (control) and (2) E109 supplemented with IAA (0.1 mM). The tubes were incubated at 30 °C and shaken at 80 rpm until late exponential growth phase. After 24 h incubation, bacterial growth (OD_{595}), cell count ($cfu.ml^{-1}$), IAA concentration ($\mu g.ml^{-1}$) and exopolysaccharide (EPS) production were measured. These cultures were used to inoculate soybean seeds cv. Don Mario 4800 with an agronomic dose of 3 ml kg^{-1}. Bacterial viability on soybean seeds after inoculation was examined according to Penna et al. [44], as explained in section 2.11.1, while the nodulation percentage was evaluated according to Burton et al. [45], as explained in section 2.11.2.

2.11.1. Bacterial viability on soybean seeds

The effects of the exogenous addition of IAA to the *B. japonicum* E109 culture medium and the bacterial capacity to tolerate stress desiccation were evaluated. For that, inoculated soybean seeds (according to the treatment described in section 2.11) were maintained at room temperature and aseptic conditions (≈25 °C) for 6 days. At each selected time (4 h, 1, 2, 3, 4, 5 and 6 days), bacterial cell counts and survival factor percentage (SFP) were determined following Penna et al. [44], using a sub-sample of 100 seeds rinsed and soaked in 250 ml Erlenmeyer flasks containing 100 ml sterile saline solution, and agitated at 450–500 rpm for 15 min. Decimal dilutions were performed in 20 ml tubes containing 9 ml of saline solution until 10^{-4}. Finally, 0.1 ml of each dilution was inoculated in triplicate on YEM agar plates containing 0.2.g l^{-1} pentachloronitrobenzene (PCNB) and 1.0 mg l^{-1} vancomycin (PV-YEM) to reduce the fungal and grampositive populations on the seed, respectively, and to improve the rhizobia cell counts [44]. Plates

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were incubated in an inverted position at 30 °C for 7 days. Controls were performed on non-inoculated soybean seeds. Results were expressed as the number of viable cells recovered from soybean seeds (cfu.seed^{-1}) and survival factor percentage (SFP) as the cfu.seed^{-1} after 4 h inoculation, according to Penna et al. (2011).

2.11.2. Nodulation test

According to the treatment described in section 2.9, a triplicate of 9 soybean seeds (n = 27) was sown in three separate plastic pots (300 ml volume capacity) containing vermiculite as a solid substrate, irrigated with nitrogen-deficient sterile N-free Hoagland's solution (25% v/v) [46]. The seedlings were maintained for 21 days in a growth chamber with a photoperiod of 16 h light (30 °C) and 8 h darkness (20 °C) at 80% humidity. At the end of the experiment, the following parameters were measured: (1) number of nodules on main root per plant, (2) number of nodules on secondary roots per plant, (3) number of nodules on roots per plant, (4) percentage of plants with three or more nodules in the main root, following Burton et al. [45], and (5) shoot and root dry weight.

2.12. Statistical analysis

Treatments were performed in triplicate from three independent experiments. 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. IAA biosynthesis

Table 1 (IAA biosynthesis) summarizes bacterial growth, EPS production, and concentrations of IAA in *B. japonicum* E109 YEM culture medium or YEM modified by addition of L-Trp as a precursor. Both cultures, *B. japonicum* E109 (Table 1) and *B. elkanii* 5019 (data not shown) had no significant differences at the level of bacterial growth (OD_{595}) and cell number (CFU.ml^{-1}) in the presence or absence of the precursor L-Trp in the culture medium. Concerning IAA production, we could not find this molecule by HPLC in the *B. japonicum* E109 cultures, even after application of L-Trp. In *B. elkanii* SEMIA 5019 cultures, the media concentrations reached a maximal production of 40 μg ml^{-1} IAA at late exponential growth phase ($DO_{595} = 0.972$) and maintained this level until the end of this experiment at late stationary growth phase (data no shown).

3.1.1. Genomic analysis of IAA biosynthesis pathways

The bioinformatic analysis of the *B. japonicum* E109 genome sequence obtained by Torres et al. (2015) reveals that it contains the genetic information necessary to synthesize IAA, mainly the genes involved in indole-3-acetonitrile (IAN), indole-3-acetamide (IAM), tryptamine (TAM) and indole-3-pyruvate (IPyA) pathways. However, because the *B. japonicum* E109 genome does not contain the *ipdC* gene sequence, which is considered to code the key enzyme of the indole-3-pyruvate pathway (IPyA), we should dismiss this biosynthesis route. Table SI (supplementary material) shows the bioinformatic analysis of the coding sequences related to IAA biosynthesis identified in the genome of *B. japonicum* E109, in comparison with those identified in other strains of the same species and genus. We also added strain *Azospirillum brasilense* Az39 because it was already considered as a model of plant growth-promoting rhizobacteria (PGPR) due to its capacity to produce IAA [47]. Figure S1 (supplementary material) shows the genes related to the putative IAA biosynthesis pathways in *B. japonicum* E109 according to KEGG pathway. In the case of the unidentified genes related with the four IAA pathways proposed in Figure S1, these sequences were exhaustively searched by Blast-N and Blast-P in all databases available with negative results. According to the genomic analysis, the most probable pathways for IAA biosynthesis in E109 might include: (A) the conversion of tryptophan to tryptamine by a L-tryptophan decarboxylase (EC. 4.1.1.105), the subsequent conversion of tryptamine to indole-3-acetaldehyde by a monoamine oxidase (EC. 1.4.3.4) and finally the conversion to indole-3-acetic acid by an aldehyde dehydrogenase (EC. 1.2.1.3) or (B) the conversion of tryptophan to indole-acetamide by a tryptophan 2-monooxygenase (EC. 1.13.12.3) and the subsequent transformation to indole-3-acetic acid by an amidase (EC. 3.5.1.4). However, both the L-tryptophan decarboxylase and the tryptophane 2-monooxygenase are missing in the genome of E109. These results show that *B. japonicum* E109 has the necessary genomic information to synthesize IAA, as occurs in the model bacterium *Azospirillum* sp., which is able to produce IAA even though some genes are not present in its genome [47].

3.1.2. Quantitative analysis of IAA biosynthesis pathways

Figure S2 summarizes the IAA metabolite profile for *B. japonicum* E109 and *B. elkanii* 5019. The supernatant of *B. japonicum* E109 analyzed by sensitive and selective liquid chromatography–multiple reaction monitoring-mass spectrometry (LC-MRM-MS) showed the presence of anthranilate (11.61 pmol ml^{-1}), IPyA (0.28 pmol ml^{-1}), IAM (0.55 pmol ml^{-1}) and IAA (0.67 pmol ml^{-1}). In the case of *B. elkanii* SEMIA5019 we identified IAM (0.30 pmol ml^{-1}) and IPyA (0.05 pmol ml^{-1}), besides anthranilate (18.58 pmol ml^{-1}) and IAA (1515.17 pmol ml^{-1}). With this information, we can confirm that *B. japonicum* E109 is able to synthesize IAA, but the molecule was not accumulated in the culture medium in a concentration significant enough to be detected by HPLC. The IAA levels are far below those determined in *B. elkanii* SEMIA5019, probably because of the degradation activity in E109 and its biological ineffectiveness to produce IAA (see section 3.3 for further details about IAA degradation by *B. japonicum* E109). Similarly, low IAA levels were detected in cultures of *B. japonicum* USDA 6 and SEMIA5079, as well as *Bradryhizobium diazzoefficiens* USDA110 and SEMIA5080 (Torres D. personal communication). In the case of *B. elkanii* SEMIA587 and *B. elkanii* 5019, IAA was released into the culture medium and measured by HPLC. The levels determined fluctuated around 40 μg ml^{-1} IAA (data not shown).

3.2. Biosynthesis of IAA conjugates

Table 1 (IAA conjugate biosynthesis) summarizes bacterial growth, EPS production and concentrations of IAA or its conjugates in *B. japonicum* E109 YEM culture medium or YEM modified by addition of 0.1 mM IAA in combination with 10 mg ml^{-1} L-phenylalanine (L-Phe), L-leucine (L-Leu) or L-alanine (L-Ala). The simultaneous addition of IAA and amino acids to the culture medium increased the bacterial biomass and EPS production. The number of viable cells (cfu.ml^{-1}) increased slightly, with the exception of the simultaneous addition of IAA and L-Ala, but without statistical differences. In relation to the auxin concentrations measured, we were unable to identify IAA either in free or conjugated form with amino acids in any of the experimental combinations. This was probably due to the ability of *B. japonicum* E109 to degrade IAA before conjugation (see section 3.3 for further details about IAA degradation by *B. japonicum* E109). No significant differences were observed by addition of amino acids to the culture medium in comparison with YEM base medium (data not shown). Even very sensitive LC-MRM-MS of the E109 supernatants (section 2.6.2) was unable to identify the usual natural conjugates with L-aspartate L-glutamate (IAAsp, IAGlu), or with glucose in the

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Table 1
Biomass production (OD_{595}), cell number (cfu.ml^{-1}), EPS production (μg.ml^{-1}) and IAA or auxins concentration (μg.ml^{-1}) of *B. japonicum* E109 in YEM medium or YEM modified by addition of L-Trp during lag (L) or exponential (E) growth phases and incubated for 24 and 48 h in similar experimental conditions (IAA biosynthesis), or YEM modified by addition of free IAA and the amino acids L-phenylalanine, L-leucine and L-alanine (IAA conjugates biosynthesis), or YEM modified by addition of the IAA-amino acid conjugates IAPhe, IALeu and IAAla (IAA conjugates hydrolysis), or YEM modified by addition of IAA, IBA and NAA (auxin degradation) during exponential growth phase (OD_{595} 0.613) and incubated for 24 and 48 h in similar experimental conditions.

	Culture conditions	T	Biomass OD_{595}	Cell number cfu.ml^{-1}	EPS μg.ml^{-1}	IAA μg.ml^{-1}
Control	**YEM media**	**24**	1.445 ± 0.010[a] **0**	5.21E + 08	2.38 ± 0.13[a] **0**	*
		48	1.854 ± 0.006[b] **0**	1.23E + 09	4.17 ± 0.39[b] **0**	*
Biosynthesis of IAA	**YEM + L-Trp** (E)	**24**	1.455 ± 0.018[a] **± 0.7**	5.42E + 08	2.41 ± 0.16[a] **± 1.2**	*
		48	1.858 ± 0.036[b] **± 0.2**	1.24E + 09	4.31 ± 0.18[b] **± 3.3**	*
	YEM ± L-Trp (L)	**24**	1.466 ± 0.038[a] **± 1.4**	5.17E + 08	2.34 ± 0.21[a] **−1.6**	*
		48	1.862 ± 0.012[b] **± 0.4**	1.29E + 09	4.20 ± 0.09[b] **± 0.7**	*
Biosynthesis of IAA conjugates	**YEM ± IAA ± L-phe**	**24**	1.526 ± 0.021[b] **± 5.6**	5.66E + 08	5.88 ± 0.41[c] **± 147.0**	**
		48	1.996 ± 0.006[d] **± 7.6**	1.30E + 09	10.04 ± 0.37[d] **± 140.7**	**
	YEM ± IAA + L-leu	**24**	1.531 ± 0.015[b] **± 5.9**	5.37E + 08	5.71 ± 0.24[c] **± 139.9**	**
		48	1.985 ± 0.010[d] **± 7.0**	1.27E + 09	9.99 ± 0.78[d] **± 138.5**	**
	YEM + IAA + L-ala	**24**	1.529 ± 0.049[b] **± 5.8**	5.20E + 08	6.01 ± 0.51[c] **± 152.5**	**
		48	2.001 ± 0.021[d] **± 7.9**	1.31E + 09	10.01 ± 0.22[d] **± 132.7**	**
Hydrolysis of IAA conjugates	**YEM ± IAphe**	**24**	1.563 ± 0.011[b] **± 8.3**	5.44E + 08	7.13 ± 0.76[c] **± 99.5**	**
		48	2.073 ± 0.031[d] **± 12.0**	1.12E + 09	12.13 ± 0.96[e] **± 190.8**	**
	YEM + IAleu	**24**	1.574 ± 0.035[b] **± 8.9**	5.19E + 08	6.47 ± 0.26[c] **± 71.8**	**
		48	2.090 ± 0.008[d] **± 12.7**	1.17E + 09	10.47 ± 0.96[d] **± 151.0**	**
	YEM + IAla	**24**	1.581 ± 0.030[b] **± 9.7**	5.27E + 08	7.47 ± 0.84[c] **± 113.8**	**
		48	1.99 ± 0.037[d] **± 7.3**	1.16E + 09	10.33 ± 0.63[d] **± 147.7**	**
Degradation of IAA and auxins	**YEM + IAA**	**24**	1.51 ± 0.011[b] **± 4.8**	5.78E + 08	6.54 ± 0.24[c] **± 174.7**	*
		48	1.98 ± 0.022[d] **± 7.0**	1.45E + 09	10.43 ± 0.61[d] **± 150.1**	*
	YEM + IBA	**24**	1.42 ± 0.018[a] **−1.3**	5.39E + 08	2.47 ± 0.16[a] **± 15.1**	**
		48	1.84 ± 0.021[c] **−0.5**	1.61E + 09	5.02 ± 0.95[b] **± 20.3**	**
	YEM ± NAA	**24**	1.44 ± 0.012[a] **0.0**	5.09E + 08	2.71 ± 0.54[a] **± 13.8**	**
		48	1.91 ± 0.024[d] **± 3.0**	1.21E + 09	5.34 ± 0.17[b] **± 37.6**	**

Different letters represent significant differences according to Tukey test $p < 0.05$.
*IAA not identified.
**IAA, IAA-amide conjugates, auxins not identified.
Bold numbers represent the percentage of difference against the control treatment (YEM media).

metabolic profile (Figure S2). On the other hand, in the case of *B elkanii* SEMIA 5019, 0.33 pmol·$^{-1}$ IAGlc (IAA-1-glucosylester) were quantified by LC-MRM-MS (Figure S2). No conjugates were identified in the uninoculated YEM culture medium modified by addition of IAA and amino acids. These findings allow us to reconsider the ability of *B. elkanii* to produce IAA conjugates with sugars. There are no previous reports about such capacity in other rhizobia studied.

3.3. Hydrolysis of IAA conjugates

Table 1 (IAA conjugates hydrolysis) summarizes bacterial growth, EPS production and concentrations of IAA or its conjugates by *B. japonicum* E109 in YEM culture medium modified by addition of the amino acid IAA conjugates IAPhe, IALeu, and IAAla. The addition of the conjugates increased production of bacterial biomass and EPS, in comparison with the untreated control. At 24 h, there was an increase in the production of EPS by addition of IAAla, while at 48 h the addition of IAPhe increased this parameter four times in comparison to the control. In the case of cell number, there were no significant differences in relation to the untreated control. As shown in Fig. 1, the addition of IAAla to *B. japonicum* E109 culture in exponential growth phase produced the full disappearance of both IAA or IAA conjugates after 24 h incubation, indicating that IAA (free or conjugated) was quickly degraded by the microorganism. In the IAPhe treatment, there was 64% of the added amino

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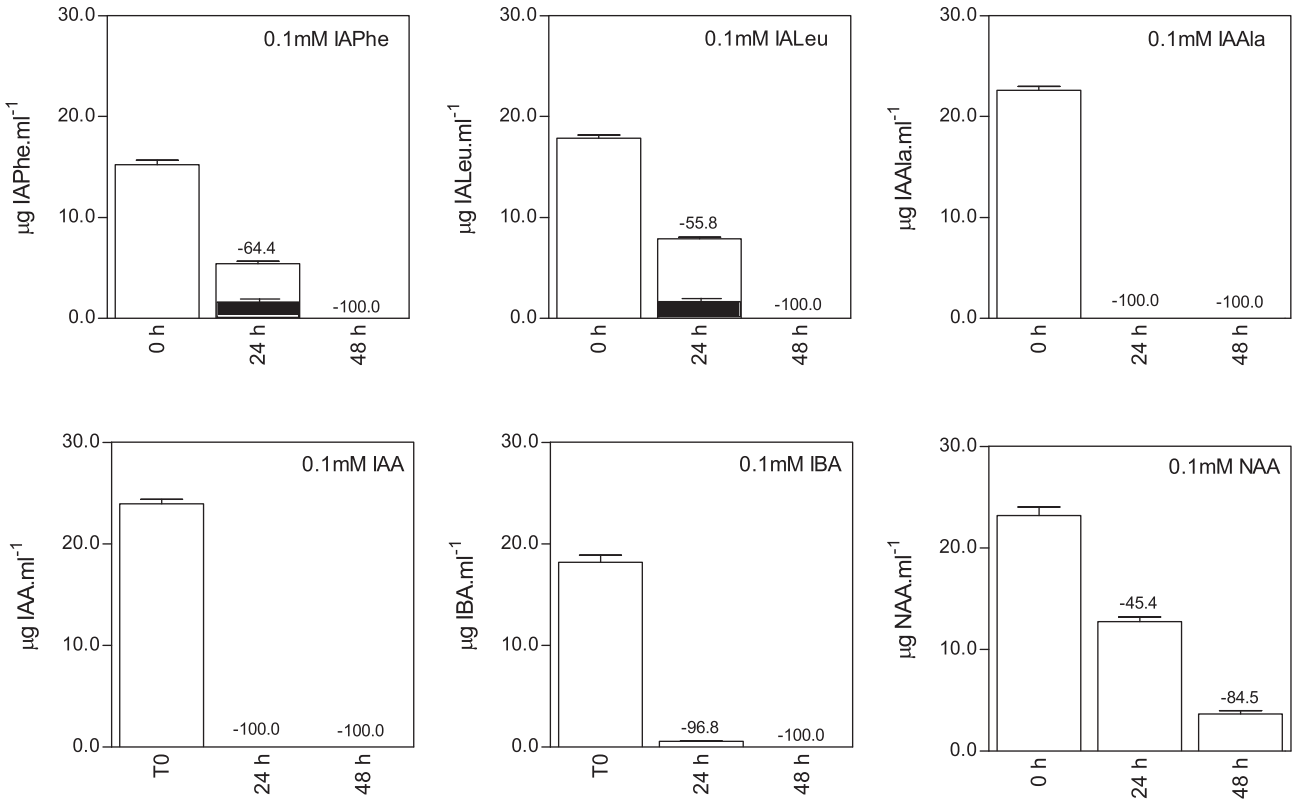

Fig. 1. Concentration of IAA conjugates (upper row), IAA and auxins (botton row) quantified (µg.ml^{-1}) in supernatants of *B. japonicum* E109 cultured in YEM media or YEM modified by addition of 0.1 mM IAPhe, IALeu or IAAla (IAA conjugates), IAA or IBA and NAA (auxins) during exponential growth phase (OD_{595} ≈ 0.6) and incubated for 24 and 48 h in similar experimental conditions. Treatments with percentages above bars show the difference in comparison with the control treatment (T0). Black bars represent IAA concentration (µg.ml^{-1}) in the same samples. Different letters represent significant differences according to Tukey test $p < 0.05$. Values shown are mean ± SD.

acid conjugate, and approximately 2% of free IAA (black bars) appeared due to hydrolysis of the conjugate after 24 h. At 48 h, both compounds could not be detected in significant concentrations in the culture medium. *B. japonicum* E109 hydrolyzed IALeu similarly to the IAPhe conjugate. At 24 h, there was 50% of the conjugate and 2% of IAA while at 48 h we did not find any of these compounds. To summarize these results, *B. japonicum* E109 has the simultaneous or sequential capacity to hydrolyze amino acid conjugates and degrade IAA, as shown in experiments with IAAla, IAPhe and IALeu in liquid culture medium.

3.3.1. Hydrolysis of IAA conjugates and IAA degradation

Table SII summarizes the *B. japonicum* E109 biomass production (OD_{595}), cell number (CFU.ml^{-1}) and biomass duplication time in YEM media or YEM modified by addition of IAA, IAPhe, IALeu or IAAla during lag, early exponential, exponential or stationary growth phases after 48 h incubation in presence of the hormone. During the lag phase, no significant differences were observed after the addition of free IAA or IAA conjugates. The presence of such exogenous molecules increased the production of biomass with a slight increase in the number of cells, but decreased the bacterial growth rate between 1 and 2 h in the early exponential growth phase. Biomass production and cell number increased by addition of IAA and IAA-amino acid conjugate treatments, while biomass duplication time decreased between 3 and 5 h with respect to the control in the exponential growth phase. No significant differences were detected in stationary phase in terms of biomass production, biomass duplication time or viable cell number after addition of either IAA or IAA conjugates. Similarly, Figure S3 summarizes the capacity of *B. japonicum* E109 to hydrolyze IAPhe, IALeu and IAAla and/or degrade free IAA or IAA conjugates during lag, exponential or stationary growth phases. After an incubation period of 6 h, the microorganism degraded 50% of the free IAA added exogenously. After 24 h exposure, we found traces of the free hormone, whilst the hormone was undetectable after 48 h. In treatments with addition of IAPhe and IALeu, only a fraction of the conjugates was hydrolyzed to free IAA (Figure S3, black bars) 6 h after the application. However, the "released" molecule of IAA was degraded to undetectable levels after 24 h incubation. At the same time, the conjugate levels decreased between 40 and 70%, and at 48 h there were only traces of these compounds. Treatment with addition of IAAla differed from the other two treatments because the conjugate, as well as free IAA, were not found at 48 h. During exponential growth phase E109 degraded IAA much faster, 6 h after its application. The strain was also able to hydrolyze IAPhe and IALeu completely after 48 h. IAAla was hydrolyzed entirely after 24 h with no free IAA being detected. During the stationary growth phase, the only compound degraded and completely hydrolyzed was IAA after 48 h incubation. Free IAA was not quantified in any of the treatments examined. The data obtained in these experiments show that the capacity of *B. japonicum* E109 to hydrolyze IAA conjugates and to subsequently degrade this molecule is dependent on the "physiological state" of the bacterial culture, and is also much higher in the exponential than in the lag or stationary growth phases.

3.4. Degradation of IAA and other auxins

In addition to IAA, we also evaluated elimination of indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) from the

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culture medium by *B. japonicum* E109. Table 1 (IAA and auxin degradation) summarizes bacterial growth (OD_{595} and cfu.ml^{-1}), EPS production and concentrations of IAA or other auxins by *B. japonicum* E109 in YEM culture medium modified by addition of IAA, IBA or NAA. According to these results, *B. japonicum* E109 growth (OD_{595}) significantly increased with the addition of IAA after 24 and 48 h incubation, whereas in the treatments with IBA and NAA there were no significant growth differences compared to the control. In addition, cell counts (cfu.ml^{-1}) did not change significantly for IAA, IBA and NAA compared to the control. Exopolysaccharide (EPS) content increased after IAA application at 24 and 48 h compared to the control. However, NAA and IBA treatments maintained similar EPS concentrations to the control. These results suggest that the addition of a natural auxin (IAA) is very beneficial to *B. japonicum* E109 cultures because it increases bacterial growth (biomass) and EPS content, while the addition of other auxins like IBA and NAA has no detected effect on this microorganism. When we evaluated the kinetics of degradation in these auxins, we observed that the IAA molecule was fully degraded after 24 h incubation, as seen in Fig. 1. IBA was present in the culture medium at 3.2% after 24 h, and fully degraded after 48 h incubation. In the case of NAA, there was 54.6% of the molecule present after 24 h incubation and 15.5% after 48 h incubation. These results show that IAA was more easily degraded than IBA and NAA, which are not produced by *B. japonicum* E109 as natural auxins.

In summary, although E109 possesses the coding sequences for IAA biosynthesis in its genome, we only detected very low concentrations of this phytohormone under our experimental conditions by LC-MRM-MS. As shown above, this fact probably originates from the bacterial capacity to degrade IAA quickly. In our experiments, the exogenous addition of IAA, IBA or NAA to *B. japonicum* E109 cultures caused a strong degradative activity on all three compounds. There was also a change in bacterial physiology after IAA application in comparison with the untreated control. E109 was capable of degrading auxin at all stages of its growth but it performed a fast elimination in the exponential growth stage. Our hypothesis is that the enzymes responsible for carrying out these degradation reactions are synthesized in relation to the bacterial growth curve and the maximal activity is reached during exponential growth phase.

3.5. Assimilative degradation of IAA

As previously observed in section 3.4, strain *B. japonicum* E109 has the capacity to degrade IAA within a few hours after addition to the culture medium. Leveau and Gerards [24] confirmed the ability of *P. putida* 1290 to use IAA as a source of C, N and energy due to the presence of genes grouped in a cluster called *iac*. *B. japonicum* E109 does not have the full *iac* cluster in its genome, but it has some genes with homology to *iacA*, *iacC* and *iacD* from *P. putida* 1290 which encode an acyl-CoA dehydrogenase and a 3-phenylpropionate dioxygenase (α and β sub-units), respectively. Because the purpose of the *iac* cluster in *P. putida* 1290 is related to IAA assimilation, we evaluated whether *B. japonicum* E109 had the same enzymatic capacity despite having only some genes of the above mentioned cluster.

As highlighted in Table 2, *B. japonicum* E109 grew in YEM medium of the original formulation but did not grow in minimal medium (MM-IAA) modified by addition of IAA and glutamate as a sole source of carbon and nitrogen, respectively. Nevertheless, we observed that the cells survived in the culture medium without any toxicity effects. For this reason, we increased the phytohormone concentration from 0.1 to 1.0 mM in the MM-IAA culture medium (data not shown). *B. japonicum* E109 was again unable to use IAA as a carbon source, as there was no biomass growth or changes in cell number in this culture medium. With these data, we confirmed that *B. japonicum* E109 is unable to use IAA as a source of carbon and energy in an assimilative pathway.

Table 2
Biomass production (OD_{595}) and cell number (cfu.ml^{-1}) of *B. japonicum* E109 in the YEM or minimal medium modified by the addition of 0.1 mM IAA and sodium glutamate as carbon and nitrogen source (MM-IAA).

Parameter	YEM media		MM-IAA
	T0	72 h	72 h
Biomass production OD_{595}	0,56[a]	1,546[b]	0,58[a]
Cell number cfu.ml^{-1}	$7{,}37 \times 10^8$	$2{,}33 \times 10^9$	$7{,}46 \times 10^8$

Different letters are significant differences according to Tukey test with $p < 0.05$.

3.6. IAA degradation and the Bradyrhizobium-soybean symbiosis

We evaluated the effect of exogenous IAA on bacterial physiology (homeostasis) and its ability to establish an effective symbiosis with the macro-symbiont soybean under environmentally controlled conditions. Table SIII (supplementary material) shows the effects of IAA added during the exponential growth phase of *B. japonicum* culture in YEM medium (after 24 h incubation). E109 was able to degrade the phytohormone 24 h after addition to the culture medium. *B. japonicum* E109 showed an increase in the biomass and EPS production in comparison with the control treatment. Regarding viable cell count (cfu.ml^{-1}), there were no significant differences between both treatments. Comparing both control and IAA treatments, we evaluated the recovery of the microorganism from inoculated soybean seeds and the establishment of effective symbiosis within soybean seedlings at 4 h, 1, 2, 3, 4, 5 and 6 days. These experimental conditions were established following Penna et al. [44] and in relation to the bacterial capacity to tolerate the desiccation process after inoculation on seed surface. For this, we calculated the survival factor percentage or SFP following Penna et al. [44].

Table SIII in the supplementary material shows the number of viable cells of *B. japonicum* E109 obtained in YEM culture medium or YEM modified by addition of IAA (cfu.ml^{-1}) and used for an inoculation bioassay. Fig. 2 shows the number of viable cells generated with the previously mentioned conditions and recovered from soybean seeds after different times of inoculation (cfu.seed^{-1}).

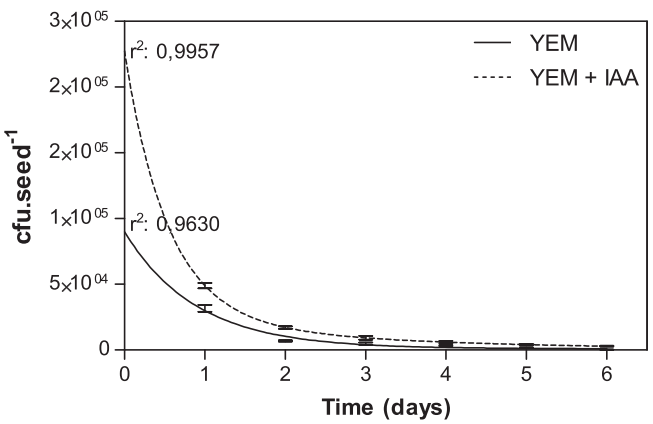

Fig. 2. Exponential decay curve (cfu.seed^{-1}) of viable *B. japonicum* E109 cells obtained from YEM (line) or YEM modified by addition of 0.1 mM IAA (dotts) and recovered from soybean seeds at different times after inoculation, according to Penna et al. (2011). Values shown are mean ± SD and r^2 adjusts to a typical exponential decay function.

The recovery of viable cells in terms of SFP was greater across time (typical death curve) in *B. japonicum* E109 cultures modified by exogenous addition of IAA, in comparison to the untreated control. For the nodulation test, as can be seen in Table SIV (supplementary material), all the symbiotic and plant growth parameters of soybean plants increased in seeds inoculated with cultures of *B. japonicum* E109 pre-treated with IAA, in comparison with bacterial cultures without exposure to IAA. The nodule number in the main root increased by 14% at 4 h and 32% 6 days after inoculation with cultures pre-treated with IAA, in comparison with the same treatment without exposure to the hormone. Furthermore, secondary root nodules increased 8% and 19% in seeds inoculated with pre-treated cultures after 4 h or 6 days of inoculation, in comparison with seeds which had not been previously treated. The number of nodules per plant was also greater for seeds inoculated with pre-treated cultures in comparison with those seeds inoculated with ones which had not been pre-treated, with an increase of 13% and 22% at 4 h and 6 days after inoculation, respectively. These data were in accordance with the percentage of nodulated plants, since in the case of *B. japonicum* E109 cultures pre-treated with IAA there was a 100% nodulation percentage at 4 h and 70% at 6 days, in comparison with the controls that had not been pre-treated, where the percentages were 94% and 56%, respectively. In summary, the mortality of *B. japonicum* E109 inoculated on soybean seeds, which was previously explained with a mathematical function by Penna et al. (2011), was reduced in our conditions because of the pre-treatment with IAA. As for dry shoot and root weight, both increased in seeds inoculated with cultures of *B. japonicum* E109 pre-treated with IAA. Shoot dry weight increased from 9% to 34% at the times evaluated, in comparison with the seeds inoculated with cultures not previously treated. Root dry weight increased 27% at 4 h and 13% at 6 days after inoculation when soybean seeds were inoculated with cultures of *B. japonicum* E109 pre-treated with IAA.

4. Discussion

The ability of the *Bradyrhizobium* genus to synthesize IAA has been reported in previous studies. Genomic analysis of *B. japonicum* E109 showed that the strain might be able to synthesize IAA either by indole-3-acetamide (IAM) or by indole-3-acetonitrile (IAN) pathways. Our results show that *B. japonicum* E109 has the genetic information necessary to synthesize IAA, as occurs in the model bacterium *Azospirillum* sp. [47], but in comparison with this microorganism, the hormone was detected by LC-MRM-MS only in an extremely low concentration (less than a pmol.ml^{-1}), suggesting either a slow biosynthetic or a strong degradative activity in *B. japonicum*. We were able to find IAM, IPyA and their metabolites using the same methodology, even though *B. japonicum* E109 does not possess the full genetic information for the biosynthesis of IAA biosynthesis through the IPyA pathway. The presence of the IAM pathway was thus unambiguously confirmed by 2 independent approaches. In short, only partial information for the biosynthesis of IAA has been detected in the *B. japonicum* E109 genome, but the metabolite analysis indicates the presence of some intermediates and a low concentration of IAA. This suggests there are at least two possible pathways for the biosynthesis of the hormone, although some of the genes for these pathways are not detected in the bacterial genome, as occurs in another well-characterized IAA-producing bacteriumn, *A. brasilense* [47]. The analysis becomes even more complex if we consider that *B. japonicum* E109 has the capacity to degrade IAA, as was also described by Nielsen et al. [48]. In line with this, Minamisawa and Fukai [27] proposed the classification of *B. japonicum* into two genotypes with different capacity to produce IAA, EPS and toxins. The strains belonging to Genotype II have the ability to accumulate IAA in the culture medium, while the strains belonging to Genotype I do not. Currently, we know that strains within Genotype II should be those corresponding to the *B. elkanii* species, while those in Genotype I belong to *B. japonicum* and/or *B. diazoefficiens*. Donati et al. [31] evaluated the behavior of *B. diazoefficiens* USDA 110 in culture medium supplemented with IAA. They observed that with an increase in hormone concentration (from 0.25 to 5 mM) in the culture medium, the growth rate decreased while EPS production increased after 48 h incubation. In general, EPS production has been shown to increase because of the exposure of the bacterium to stress, such as desiccation [49,50], oxidation via superoxide production [31] and cold [51], among others. In our experiments, addition of IAA into the E109 cultures led to an increase in biomass (OD_{595}) and EPS production after 24 and 48 h incubation, respectively. When IBA and NAA were added, no significant differences in growth were observed in comparison to the untreated control. *B. japonicum* E109 was able to degrade 100% of IAA, 90% of IBA and 50% of NAA after 24 h incubation, and it was also able to degrade NAA almost completely after 48 h incubation. When IAA was added to the *B. japonicum* E109 culture, significant physiological changes occurred at the level of biomass in terms of OD_{595} and EPS production. Thus, IAA seems to be a very important growth regulation factor for *B. japonicum* E109.

Amino acid IAA conjugates are reservoirs of free IAA molecules that play a key role in homeostasis in higher plants [16]. The function, production, accumulation and hydrolysis of auxin conjugates in microorganisms are unknown, but these compounds have been detected in some bacteria. Research studies have determined the existence of some bacterial strains with the ability to hydrolyze IAA conjugates, such as *E. agglomerans* and *Arthrobacter ilicis*, which are capable of hydrolyzing IAAsp and IAAla, respectively [21,52]. Moreover, Glass and Kosuge [53] reported the ability of *Pseudomonas savastanoi* to hydrolyze IALys. In the case of conjugate hydrolysis, we confirmed the metabolic capacity of *B. japonicum* E109 to degrade IALeu, IAPhe, and IAAla and/or IAA in such experimental conditions. The addition of free or conjugated auxin to pure cultures of E109 induced some changes at the level of biomass production and EPS synthesis in comparison to the control. Thus, with our work we can confirm that E109 can hydrolyze IAA-amino acid conjugates and quickly catabolize IAA released from that cleavage. The biosynthesis of auxin conjugates may play an important role as a storage form of IAA in plants, but we need more information about the function and ecological role of different IAA-amino acid species in bacteria. *P. syringae* subsp. *savastanoi* was able to transform IAA into IALys [18]. *B. japonicum* E109 does not have the capacity to conjugate IAA with amino acids. However, it was possible to observe significant changes in cell biomass and EPS production after application of IAA conjugates, similar to what occurred with the addition of free IAA. We therefore conclude that bacterial production of biomass and/or EPS were generated by the presence of exogenous free IAA in the culture medium.

In our experiments, IAA degradation capacity was initially evaluated at an early exponential growth phase ($OD_{595} \approx 0.6$), when the microorganism is metabolically more active. Previous degradation experiments using other strains of *Bradyrhizobium* were conducted in early or exponential growth phase [29–31]. We observed that hydrolysis of amino acid IAA conjugates was followed by fast IAA degradation at all stages of the *B. japonicum* E109 growth curve. However, the rate of degradation was not the same. In the lag phase of the culture (24 h), almost 90% of exogenous IAA was degraded, while IAA-amino acid conjugates were degraded at a slower rate and approximately only 30–40% were degraded. A small amount of free IAA was consequently detected as a result of IAA conjugate hydrolysis. In the early exponential growth phase, only IAAla was degraded completely, while about 90% of IAPhe and IALeu was degraded after 48 h. In the exponential growth phase, all

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the auxins were completely degraded 48 h after their supplementation to the culture medium. Finally, in stationary growth phase, IAA was degraded in 48 h but IAA-amino acid conjugates were degraded by about 50–70% at this point in time. We can conclude that the degradation of IAA-amino acid conjugates was different when compared to exogenously applied IAA. These experiments also indicate that different growth stages of *B. japonicum* E109 influenced the speed of degradation of IAA as well as its amino acid conjugates.

Bradyrhizobium sp. has been widely studied because of its symbiotic association with legumes and its biological nitrogen fixation capacity. *B. japonicum* strain E109 is widely used to inoculate soybean seeds because it has the ability to increase the performance of the legume under agronomic conditions [54]. In our experiments, we observed that the addition of IAA to a *B. japonicum* E109 culture in the exponential growth phase, followed by the application of the samples withdrawn for inoculation, increased biomass and EPS production. There were no significant differences in the cell numbers in comparison to the control. The recovery of *B. japonicum* E109 from soybean seeds after inoculation was higher for cultures pre-treated with IAA for 24 h, in comparison with the control treatment. *B. japonicum* E109 has the capacity to promote seed germination and early seedling growth in soybean [2]. This capacity could be due at least in part to bacterial phytohormone biosynthesis in the culture medium. The effect of IAA was determined on the basis of stimulation of nodule formation and root growth [6,55]. In this paper we showed that *B. japonicum* E109 preincubated with IAA was able to increase the number of nodules and dry weight of shoots and roots in soybean plants, as well as the number of nodulated soybean plants. These parameters were remarkably higher in the seeds sown 4 h after inoculation, in comparison to those sown 6 days after inoculation. In similar experiments, Donati et al. (2013) examined the effect of pretreatment with IAA on the ability of *B. diazoefficiens* USDA110 to nodulate soybean roots. According to them, none of the pretreatments affected the number of nodules, nodule weight, or plant weight. However, they inoculated 1 ml of *B. japonicum* cell suspension pretreated with IAA on roots of soybean seedlings, whereas we inoculated 3 ml of pretreated *B. japonicum* E109 culture medium per kg of soybean seeds, where desiccation stress is higher than in roots. To sum up, IAA metabolism and homeostasis were evaluated in *B. japonicum* E109. No evidence for IAA conjugation with amino acids or sugars was observed. The IAA biosynthetic capacity was confirmed *in vitro* and proposed *in silico,* but it is significantly lower than in IAA producer *B. elkanii* 5019, probably due to the higher capacity to degrade the IAA molecule in the culture medium. The presence of the IAM pathway was confirmed by several approaches. TAM and IPyA pathways should, however, also occur. The strong degradation activity was confirmed in all treatments after exogenous addition of IAA, other auxins, and auxin conjugates. We found that *B. japonicum* E109 had the capacity to hydrolyze IALeu, IAPhe and IAAla and to then quickly degrade the released IAA. The presence of exogenous IAA in culture medium triggered several physiological parameters in the *B. japonicum* E109 cells, in particular, biomass and EPS production as well as symbiotic capacity in soybean.

Conflicts of interest

The authors report no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.resmic.2018.04.002.

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