Research in Microbiology 168 (2017) 283–292

www.elsevier.com/locate/resmic

Original Article

Indole-3-acetic acid production via the indole-3-pyruvate pathway by plant growth promoter *Rhizobium tropici* CIAT 899 is strongly inhibited by ammonium

Eddie Luidy Imada [a], Amanda Alves de Paiva Rolla dos Santos [b], André Luiz Martinez de Oliveira [c], Mariangela Hungria [b], Elisete Pains Rodrigues [a,*]

[a] *Laboratório de Genética de Microrganismos, Departamento de Biologia Geral, Universidade Estadual de Londrina, PR-445, Km 380, Campus Universitário, PO Box 6001, CP 86.051-970, Londrina, PR, Brazil*

[b] *Laboratório de Biotecnologia do Solo, Embrapa Soja, PO Box 231, CP 86.001-970, Londrina, PR, Brazil*

[c] *Departamento de Bioquímica e Biotecnologia, Universidade Estadual de Londrina, PR-445, Km 380, Campus Universitário, PO Box 6001, CP 86.051-970, Londrina, PR, Brazil*

Received 10 July 2016; accepted 31 October 2016
Available online 11 November 2016

Abstract

Like many rhizobia, *Rhizobium tropici* produces indole-3-acetic acid (IAA), an important signal molecule required for root hair infection in rhizobia-legume symbioses. However, the IAA biosynthesis pathway and its regulation by *R. tropici* are still poorly understood. In this study, IAA synthesis and the effects of mineral N in IAA production by *R. tropici* CIAT 899 were verified by ultraperformance liquid chromatography-mass spectrometry (UPLC-MS). Furthermore, expression of genes related to IAA biosynthesis and metabolism were evaluated by RT-qPCR. Results indicated that IAA production by CIAT 899 was 12 times lower in the presence of NH_4^+. Moreover, it was found that indole-3-pyruvate (IPyA) is the major IAA biosynthesis intermediate. Genes *y4w*E, *lao* and *ior*A were identified by analysis of *R. tropici* genome in silico and were upregulated by tryptophan, indicating a possible role of these genes in IAA biosynthesis by CIAT 899. In conclusion, we show that IPyA is the major pathway for IAA biosynthesis in CIAT 899 and that its production is strongly inhibited by NH_4^+. Although present results arose from in vitro experiments, they provide new insight into the role of nitrogen in early events related to legume nodulation.

Keywords: Plant growth regulators; Tryptophan; Indoles; Symbiosis; Nitrogen fixation; Fertilizers

1. Introduction

Bacteria from the group rhizobia represent one of the most frequently studied plant growth-promoting bacteria because of their capacity to establish symbiosis with leguminous plants (Fabaceae), including economically important crops such as soybean (*Glycine max*) and the common bean (*Phaseolus vulgaris*). Symbioses between rhizobia-legumes generate root nodules, whereby the bacterium provides an assimilable nitrogen source to the host plant as a result of its biological nitrogen fixation (BNF) activity. In turn, the host plant provides photosynthetically-fixed carbon to the nodule-residing bacteria, allowing rhizobia to survive and continue their diazotrophic activity, closing a cooperation cycle [1,2].

The initial establishment of rhizobia-legume symbiosis is regulated by molecular signals, mostly flavonoids, secreted by the host plant, which attract the rhizobia close to the root hairs, starting a cascade of expression of several genes from both partners involved in the nodulation process [3]. While initial symbiosis stages are regulated by signal exchanges between

* Corresponding author.
E-mail addresses: eddie.imada@gmail.com (E.L. Imada), amandaaapr@gmail.com (A.A.P. Rolla dos Santos), almoliva@uel.br (A.L.M. Oliveira), mariangela.hungria@embrapa.br (M. Hungria), elisete@uel.br (E.P. Rodrigues).

http://dx.doi.org/10.1016/j.resmic.2016.10.010

the rhizobia and the host legume, NH_4^+ acts as the main regulatory signal for nodule and symbiosome maintenance at later stages. In this sense, regulation of bacterial genes related to NH_4^+ assimilation is necessary for symbiosome differentiation and efficient symbiosis [4]. Another important factor influencing symbiosis is the synthesis of the phytohormone indole-3-acetic acid (IAA) by rhizobia. IAA has been reported to act during the early stages of the infection process, e.g. root hair curling, development of infection thread, nodule initiation and nodule differentiation [5,6]. Moreover, genetically engineered strains with increased IAA biosynthetic activity show higher BNF capacity and greater plant nodulation [5,7].

Microbial biosynthesis of IAA can occur through four different tryptophan(TRP)-dependent pathways named (according to intermediate compounds) indole-3-acetamide, indole-3-pyruvic acid, indole-3-acetonitrile and tryptamine. The indole-3-pyruvic acid (IPyA) pathway represents the main route of IAA biosynthesis in plants, plant pathogens and plant growth-promoting bacteria such as *Pseudomonas*, *Azospirillum*, *Bacillus*, *Bradyrhizobium* and *Enterobacter cloacae* among others [8–10]. Although IAA has shown great importance for the constitution of successful legume-rhizobia symbiosis, its role in nodule formation is not fully understood.

Given the importance of IAA in legume-rhizobia signaling, we postulate that IAA biosynthesis by rhizobia is influenced under high nitrogen availability conditions, which also repress BNF activity and plant nodulation [11–13]. To address this hypothesis, *Rhizobium tropici* CIAT 899, a nitrogen-fixing bacterium able to establish symbiotic relationships with a variety of legume hosts [14], was studied so as to determine pathways for IAA synthesis and inorganic N source effects in regulating production of this phytohormone. Due to its effectiveness in fixing nitrogen, as well as its adaptability to stressful conditions such as high temperature and acidity, CIAT 899 is widely used in commercial inoculants recommended for the common bean crop in Brazil [15] as well as in other African countries (http://www.n2africa.org/). Like many rhizobia strains, *R. tropici* CIAT 899 synthesizes IAA [16]; however, until now, no pathway or genes involved in IAA synthesis have been described in this bacterium. Here we suggest a major pathway for IAA biosynthesis and demonstrate the inhibitory effect of NH_4^+ upon IAA production by CIAT 899.

2. Materials and methods

2.1. Strains, media and growth conditions

The *R. tropici* CIAT 899 (=SEMIA 4077) strain, obtained from the "Diazotrophic and Plant Growth-Promoting Bacteria Culture Collection of Embrapa Soja" (WDCM#1054, Londrina, Brazil) was used throughout the experiments presented here. Cultures were maintained in agarized YM medium [17] with Congo red (0.025%) at 4 °C. The pre-inoculum used for auxin assays and gene expression analysis was obtained by inoculation of a loopful of the pure colony into 100 mL of liquid YM incubated under orbital shaking at 120 rpm and 28 ± 2 °C for 24 h. The bacterial cells density in the pre-inoculum was estimated spectrophotometrically at 600 nm.

2.2. Auxin production assays

Production of IAA and related compounds by *R. tropici* CIAT 899 was evaluated in vitro by growing the bacterium in modified YM medium in two independent experiments. In experiment 1, CIAT 899 was grown in YM without TRP or in YM supplemented with 100 μg mL^{-1} TRP (YM-TRP medium); in experiment 2, bacterial cultures were grown in YM-TRP, YM-TRP with KNO_3 (1.0 g L^{-1}) or in YM-TRP with $NH_4H_2PO_4$ (1.0 g L^{-1}). In both assays, an aliquot of 1 mL of a pre-inoculum at $OD_{600nm} = 0.5$ (prepared as stated above) was used as inoculum of 100 mL bacterial cultures in YM or YM-TRP media modified according to the experimental design. Bacterial cultures were incubated at 28 ± 2 °C under orbital shaking at 120 rpm for 30 h and 54 h, considering experiments 1 or 2, respectively. Bacterial growth and auxin production were monitored during the experimental period by sampling of culture broths at 6 h intervals for experiment 1 and 12 h intervals for experiment 2. At each sampling time, an aliquot of 2 mL of culture was collected and absorbance (OD_{600nm}) was determined following a centrifugation step (14,000 rpm, 10 min) to obtain the culture supernatant. To perform indolic compound quantification, supernatants were mixed with Salkowski reagent [18] in a 1:4 (v/v) ratio, followed by incubation for 30 min in the dark and determination of absorbance at 530 nm. A standard curve was constructed using synthetic indole-3-acetic acid (Sigma–Aldrich) and the regression equation obtained was used to estimate the auxin concentration in the supernatants. Each experiment was performed with three biological replicates and data were analyzed statistically using the Scott–Knott test ($p \leq 0.05$).

2.3. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)

The composition of metabolites in culture supernatants of experiment 2 was determined by UPLC-MS using an LC20AD UPLC system (Shimadzu, Japan) coupled with a compact triple-quadrupole mass spectrometer (Bruker, Germany) equipped with an electrospray ion source. Aliquots of 20 mL of culture grown in YM or YM-TRP media (modified as defined in the experiment 2) were collected after 24 h of cultivation and centrifuged for 10 min at 9000 rpm to obtain the supernatants. Supernatant was acidified with 1 volume of 0.1 M HCl and then metabolites were extracted using solid phase extraction (SPE) cartridge Strata-X ® (200 mg, 3 mL, Phenomenex) according to the following procedure: the cartridge was activated with 10 mL methanol and equilibrated with 5 mL of HCl 0.05 M. After loading 5 mL of the acidified supernatant, the cartridge was washed with 10 mL of HCl 0.05 M and the metabolites were eluted with 3 mL methanol.

Chromatographic separation was achieved by gradient elution at a flow rate of 0.2 mL min^{-1} in a KINETEX 5 μM EVO C18 (100 Å, 100 × 2,1 mm, Phenomenex) column with