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The key role of indole-3-acetic acid biosynthesis by *Bacillus thuringiensis* RZ2MS9 in promoting maize growth revealed by the *ipd*C gene knockout mediated by the CRISPR-Cas9 system

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ABSTRACT

The bacterial biosynthesis of indole-3-acetic acid (IAA) is often related to the beneficial effects of plant growth-promoting rhizobacteria (PGPR) on plant development. In PGPR belonging to the *Bacillus* genus, the synthesis of IAA may occur through different metabolic pathways that are still poorly understood. *B. thuringiensis* (*Bt*) is well known for its insecticidal properties; however, its beneficial features are not limited to pest control. Our group has been studed the beneficial effects of *Bt* strain RZ2MS9 as growth promoter in a range of plant crops, including soybean, tomato, and maize. We recently demonstrated that bacterial IAA biosynthesis plays an important role in the ability of RZ2MS9 to benefit plant development. However, the molecular involved mechanisms in the IAA biosynthesis by this bacterium in the beneficial interaction with plants remain unclear. Here, we investigated the genetic basis of IAA biosynthesis by RZ2MS9. We knocked out the *ipdC* gene, involved in IAA biosynthesis via the tryptophan-dependent IPyA pathway, using the CRISPR-Cas9 system. Our results showed that, by disrupting the IPyA pathway, the amount of IAA synthesized by the mutant RZ2MS9 (Δ*ipdC*) in the presence of tryptophan drops 57%. The gene knockout did not affect the bacterial growth, but it did affect its ability to colonize maize. Moreover, deactivating the *ipdC* gene in RZ2MS9 significantly reduces its ability to promote maize growth. Δ*ipdC* performed worse than RZ2MS9 in almost all evaluated plant parameters, including total root length, projected root area, lateral roots, aerial part dry matter, and germination speed index. Therefore, we demonstrated that tryptophan-dependent IAA biosynthesis via the IPyA pathway by RZ2MS9 is strongly influenced by the *ipd*C gene. Furthermore, IAA biosynthesis by RZ2MS9 is a major mechanism used by this PGPR to promote maize growth.

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) are microorganisms that positively influence the metabolism and development of plants and, therefore, can sustainably increase crop yields. The benefits of applying beneficial microbes to plants have been known for over 120 years; however, only technological advances that happened mainly in the last decade allowed researchers to understand and explore the full potential of these microorganisms (Batista and Singh, 2021). As gene-editing tools such as the CRISPR-Cas9 system become available, new insights into the molecular basis of plant-microbe interaction can be obtained (Prabhukarthikeyan et al., 2020; Figueredo and Quecine et al., 2021), which can greatly support the development of optimized microbial products for agricultural application.

Bacillus spp. are one of the most studied PGPR and have been widely used as plant growth promoters and/or as biocontrol agents of pests and diseases (Pérez-García et al., 2011; Borriss, 2016). Bacteria of this genus can produce endospores, making them highly resistant to abiotic stresses

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such as high temperature, drought, and nutrient limitation (Blake et al., 2021). In addition, endospore production facilitates the bioformulation of *Bacillus*-based products, increasing their shelf-life (Toyota, 2015).

Bacillus thuringiensis (*Bt*) are well known for their insecticidal properties and currently represent about 75% of biopesticides marketed worldwide (Samada and Tambunan, 2020). However, *Bts* have an underexplored potential to promote plant growth, recently reported by some researchers (Vidal-Quist et al., 2013; Armada et al., 2016; Azizoglu, 2019). Our group has been investigating the beneficial effects of applying the PGPR *Bt* RZ2MS9 on a range of plant crops including soybean, tomato, and maize (Batista et al., 2018, 2021; Almeida et al., 2021). We have recently demonstrated that the bacterial production of indole-3-acetic acid (IAA) plays an important role in the ability of RZ2MS9 to promote plant growth (Batista et al., 2021). However, the molecular involved mechanisms in the production of IAA by RZ2MS9 in its beneficial interaction with plants remain unclear.

Bacterial IAA may influence plant growth, affecting processes such as cell division, elongation, tropism, apical dominance, senescence, flowering, and response to stress (Lambrecht et al., 2000; Taghavi et al., 2009; Facella et al., 2012). IAA synthesized by PGPR, including by *Bacillus* strains, may originate from five tryptophan-dependent metabolic pathways: indole-3-acetamide (IAM), indole-3-acetonitrile (IAN), tryptophan side chain oxidase (TSO), tryptamine (TAM) and indole-3-pyruvate (IPyA) (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011; Lin et al., 2015).

RZ2MS9 harbours in its genome the complete set of genes required in two of these pathways: TAM and IPyA (Bonatelli et al., 2020; Batista et al., 2021). The IPyA pathway is the best reported in beneficial bacteria and involves the following steps: i) the conversion of L-tryptophan to indole-3-pyruvate (IPyA) by the action of an aminotransferase; ii) the conversion of IPyA to indole-3-acetaldehyde (IAAd) by the action of the indole-3-pyruvate decarboxylase enzyme (ipdC), which is encoded by the *ipdC* gene (Carreño-Lopez et al., 2000; Patten and Glick, 2002); iii) the oxidation of IAAd to IAA, which can occur spontaneously or by the action of an aldehyde oxidase (Broek et al., 1999; Spaepen et al., 2007; Bulgarelli et al., 2013; Jasim et al., 2014). The second step appears to be the rate-limiting stage of the IPyA pathway, and some researchers have linked the beneficial effects of PGPR in plants to the *ipdC* gene. For instance, IAA biosynthesis by *Azospirillum brasilense* SMIT568s10 was reduced by 50% when its *ipdC* gene was knocked out, which affected the bacterial ability to promote root growth in *Sorghum bicolor* (Malhotra and Srivastava, 2008). Other researchers have demonstrated varied effects on plant root morphology after silencing the *ipd*C gene of beneficial bacteria (Brandl and Lindow, 1998; Dobbelaere et al., 1999; Patten and Glick, 2002; Suzuki et al., 2003). However, it is incipient the studies correlating the IAA production and *Bt* plant growth promotion ability.

In this study, we used the CRISPR-Cas9 system to knockout the *ipdC* gene of *Bt* RZ2MS9 to decipher the molecular basis involved in bacterial IAA biosynthesis and to understand the contribution of this mechanism in the beneficial bacterial-maize interaction.

2. Material and methods

2.1. Bacterial strains and culture conditions

Bacillus thuringiensis RZ2MS9 and *Escherichia coli* DH5α used in this study have been routinely grown in Lysogeny Broth (LB) culture medium (Sambrook and Russell, 2001) at 28 °C and 37 °C, respectively, from stocks maintained at − 80 °C. Different concentrations of antibiotics (100 μg/ml of kanamycin, 100 and 500 μg/ml of spectinomycin) were added to the LB medium for the cultivation of the transformants. Plasmids and genomic DNA were extracted from bacterial cell cultures using the QIAprep® Spin Miniprep (Qiagen) and DNeasy® Blood & Tissue (Qiagen) kits, respectively, following the manufacturer's instructions. DNA fragments were purified using an Illustra ™ GFX ™ PCR DNA and Gel Band Purification Kit. All primers used in the experiments are listed in the Supplementary Table 1.

2.2. CRISPR-Cas9-mediated knockout of the RZ2MS9 ipdC gene

The knockout of the RZ2MS9 *ipd*C gene was performed using the CRISPR-Cas9 system according to the methodology described by Jiang et al. (2015). Plasmids pCas and pTargetF were obtained from Addgene (https://www.addgene.org). pCas carries the sequence encoding Cas9 of *Streptococcus pyogenes* (MGAS5005) with its native P_{cas} promoter, the Kan^R gene, the replicon (Ts) *repA101*, the *λ-Red* gene under the control of a P_{arab} promoter and a *sgRNA* with a N_{20} sequence target in the pMB1 replicon of plasmid pTargetF under the control of a P_{trc} promoter (Addgene 62225). pTargetF carries sgRNA sequences *tracrRNA*::*crRNA*, with N_{20} target DNA, the *pJ23119* promoter, the spectinomycin resistance gene (*aadA*), the pMB1 replicon and multiple restriction sites (Addgene 62226).

2.3. $pTarget_{ipdC}$ assembly

The *ipd*C N_{20} sequence containing the sgRNA was constructed based on the RZ2MS9 *ipd*C gene sequence (GenBank acession n. QIZ42416.1) (Bonatelli et al., 2020) flanked by a PAM sequence NGG (Jiang et al., 2013). The tools CRISPR RGEN Tools/Cas-OFFinder v. 2.4 (http://www.rgenome.net/cas-offinder/) (Bae et al., 2014), CHOPCHOP (https://chopchop.rc.fas.harvard.edu/dev/) (Labun et al., 2016) and CRISPOR v. 4.2 (http://tefor.net/crisporDev/crisporBeta/crispor.py) (Haeussler et al., 2016) were used to generate sequences and identify potential *off-target* sites using the RZ2MS9 complete genome (GenBank acession n. CP049978.1) (Bonatelli et al., 2020). Sequence selection was performed based on the following criteria according to the platforms used: GC content close to or equal to 40%, specificity score close to 100%, out-off frame score ≥ 50%, and low number of *off-target sites* (Moreno-Mateos et al., 2015; Doench et al., 2016).

For the construction of $pTarget_{ipdC}$, the plasmid pTargetF was digested using the restriction enzymes *Bam*HI and *Eco*RI (*New England Biolabs* -NEB®) in a double digestion reaction. The insert was obtained from the synthesis of 144 bp oligos containing the complementary sequences of restriction sites *Bam*HI, on the 5′− 3′-strand, and *Eco*RI on the 3′− 5′ strand, the pJ23119 promoter, the sgRNA *scaffold*, and the N_{20} sequence targeting the *ipdC* gene. The phosphorylation of the vectors was performed with T4 polynucleotide kinase (NEB®). Insert-vector ligation was performed using the T4 DNA ligase (NEB®) (Fig. 1A). The ligation product was electroporated into *E. coli* DH5α and the cells spreaded onto LB solid medium. Transformants were identified by colony PCR with the primers aadA_D-F and ipdC_N20-R (Supplementary Table 1) using the following reaction: 0.2 μM of each primer; 0.2 mM dNTP; 1x Taq Buffer; 3.5 mM $MgCl_2$; 0.06 U of the enzyme Taq DNA polymerase (Thermo Scientific®), and water to a final volume of 25 μl. A loop of each colony was used to provide the DNA template. The thermal cycling conditions were as follows: 2 min at 94 °C for initial DNA denaturation; followed by 30 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 5 min. To confirm the construction of $pTarget_{ipdC}$, the plasmid was sequenced (Fig. 1B) using the primer MoClo-F (Jiang et al., 2015) (Supplementary Table 1).

2.4. Construction and assembly of the donor DNA

The donor DNA template was obtained from upstream and downstream sequences of the *ipdC* gene, excluding approximately 200 bp of the central region. It was performed by Splicing by Overlapping Extension – SOEing-PCR (Horton et al., 2013). Briefly, in the first round of the PCR, the upstream and downstream regions of the *ipd*C gene were amplified separately. The reactions were performed using the following components: 10 ng of RZ2MS9 DNA as template; 0.2 μM of each primer (ipdC_up-F and ipdC_up-R for the upstream region and ipdC_dw-F and ipdC_dw-R for the downstream region) (Supplementary Table 1);

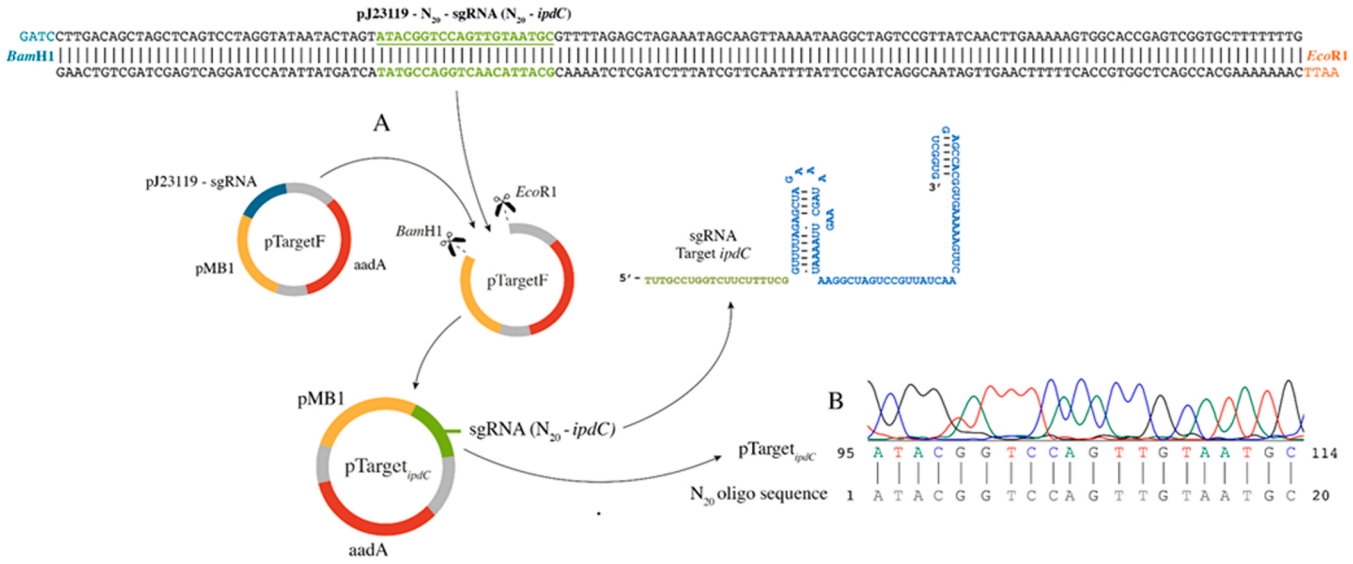

Fig. 1. The strategy adopted to knockout the *ipdC* gene from *Bacillus thuringiensis* RZ2MS9 using the CRISPR-Cas9 system double plasmid. **A.** Construction of pTarget$_{ipdC}$ by plasmidial digestion with *Bam*HI and *Eco*RI and insertion of the fragment obtained from annealing the oligonucleotide sequences containing the pJ23119 promoter and the N_{20} sequence and sgRNA. The oligo terminals were cohesive with the sites of the restriction enzymes used in digestion. **B.** Confirmation of the cloning of N_{20} in the sgRNA by aligning the inserted sequences with those of the N_{20} of the cloned pTarget$_{ipdC}$ plasmid.

0.2 mM dNTP; 1x Taq Buffer; 3.5 mM $MgCl_2$; 0.06 U.µl of the enzyme Taq DNA polymerase (Thermo Scientific®), and water to a final volume of 25 µl. The thermal cycling conditions were as follows: 5 min at 94 °C for initial denaturation, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 7 min. In the second round of the PCR (the assembly step), 50 ng of the product from the first round were used as template in a new reaction with the same conditions and reagents previously described. The three initial thermocycling rounds were performed without the addition of any primers. Then, the reaction was paused, the primers ipdC_up-F and ipdC_dn-R were added, and the thermocycling continued to the end. The amplicons containing the donor DNA were observed by electrophoresis in an agarose gel, and the sequence of nucleotides was checked by Sanger sequencing using the primers ipdC_up-F and ipdC_dn-R.

2.5. CRISPR-Cas9-mediated knockout

The *ipd*C knockout using the CRISPR-Cas9 system was performed with a double plasmid system according to Jiang et al. (2015), with modifications. Firstly, RZ2MS9 was electroporated with pCas (30 ng), according to the method described by Schurter et al. (1989), with modifications (Almeida et al., 2021). Electroporation was carried out in 0.2 mm electroporation cuvettes (Bio-Rad) at 1.3 kV. The transformants were grown for 3 h, then inoculated onto LB solid medium and incubated overnight at 28 °C. The transformants were confirmed by colony PCR using the primers: pCas-F and pCas-R (Supplementary Table 1). Subsequently, RZ2MS9::pCas electrocompetent cells, previously grown in the presence of L-arabinose for the induction of λ-Red, were electroporated with 300 ng of pTarget$_{ipdC}$ plasmid and 600 ng of the donor DNA cassette. Then, the transformants were spread onto LB plates and incubated at 28 °C for 24 h. To increase the transformation efficiency, the mechanism of λ-Red contained in pCas was induced by the addition of L-arabinose (10 mM) in the culture media, and the cells were coelectroporated with the donor DNA. The confirmation of the transformants was performed by PCR using DNA of colonies from transformants and DNA sequencing with the primers ipdC01A -F; ipdC02A-R; ipdC01B-F; ipdC02B-R; ipdC01C-R, and ipdC02C-F (Supplementary Table 1). The confirmation of the deleted region in the *ipdC* was performed using the *ClustalW2 Pairwise Sequence Alignment* (https://www.ebi.ac.uk/Tools/psa/) comparing the sequences found in the transformants with the original *ipd*C gene sequence. One selected transformant, named Δ*ipdC,* was used in the further analyses.

2.6. Effects of the RZ2MS9 ipdC gene knockout on bacterial growth and IAA biosynthesis

We compared the growth patterns of RZ2MS9 and Δ*ipdC* and their IAA biosynthesis by growing both strains in LB broth with different compositions: LB full-strength, LB 10%, LB supplemented with tryptophan (5 mM), LB supplemented with synthetic IAA (10 µg/ml), LB supplemented with tryptophan plus synthetic IAA (5 mM and 10 µg/ml respectively), and LB 10% supplemented with maize exudates (final concentration 40%).

To obtain the maize exudates, seeds of hybrid 30A37PW® (Morgan Seeds and Biotechnology®) were surface disinfected by washing with 70% ethanol for 3 min; 5% sodium hypochlorite (v/v) for 3 min; 70% ethanol for 3 min, and a final wash in sterile distilled water. The seeds were then placed in Petri dishes with three sheets of sterile Germitest® paper moistened 2.5 times their weight with sterile distilled water. The plates were kept in sealed plastic bags and placed in a growth chamber at 25 °C for 4 d, with a photoperiod of 12 h light/12 h dark. After germination, seeds with seminal roots and visible epicotyl were transferred to erlenmeyers containing 50 ml of sterile distilled water and incubated for 7 d at 25 °C with a photoperiod of 12 h light/12 h dark. After the cultivation period, the exudates secreted by the plants were collected, filtered through a 0.22 µm membrane, and immediately used in the experiments described below.

2.7. Bacterial growth

RZ2MS9 and Δ*ipdC*, were grown in LB broth overnight. Subsequently, the bacterial cultures' Optical Densities ($OD_{600\ nm}$) were adjusted to 0.1 in a final volume of 10 ml of LB broth with the different compositions described above. The inocula were incubated at 28 °C under rotation (180 rpm) in the dark. We measured $OD_{600\ nm}$ of the bacterial cultures every 3 h after inoculation (HAI) for a period of 12 h, and then at 24 HAI. The assay was performed using 3 replicates.

2.8. IAA biosynthesis

IAA biosynthesis was evaluated according to the method described by Bric et al. (1991) and adapted for the quantitative method described by Husen (2013) with modifications. Briefly, RZ2MS9 and Δ*ipd*C were grown in tubes containing 10 ml of LB broth with the different compositions described above and incubated at 28 °C, 180 rpm, in the dark. After 24 HAI, 2 ml of the bacterial cultures were centrifuged and 1.4 ml of the supernatant was added to 0.6 ml of Salkowsky reagent (Bric et al., 1991) and incubated in the dark for 30 min. The indole compounds were quantified in a spectrophotometer at a wavelength of 520 nm, and the auxin concentration was estimated from a standard curve previously established with known amounts of commercial IAA. LB broth with the different described compositions without any bacterial growth were used as controls. The assay was performed using 3 replicates.

2.9. Effects of the ipdC gene knockout on maize colonization by RZ2MS9

To evaluate the colonization of maize by RZ2MS9 and Δ*ipdC*, seeds of hybrid maize 30A37PW® were surface disinfected as previously described and germinated in moist Germitest® paper incubated at 25 °C with a photoperiod of 12 h light/12 h dark. After germination, the sedlings were transferred to tubes containing 15 ml of sterile distilled water. In parallel, the RZ2MS9 and Δ*ipdC* strains were grown overnight in LB broth. Aliquots of the bacterial suspensions were inoculated in the tubes containing the seedlings to a final $OD_{600\ nm}$ of 0.1 in a 15 ml final volume. Non-inoculated seedlings were used as control.

For the qPCR quantification, the seedlings inoculated with RZ2MS9 and Δ*ipdC* were collected at 24 and 48 HAI and washed three times using sterile distilled water. Subsequently, the aerial part and the root system were separated and immediately stored at − 80 °C for later DNA extraction.

Total DNA from plant samples were extracted using the PowerSoil® DNA isolation Kit (Qiagen) following the manufacturer's instructions. The DNA integrity was verified using agarose gel (1%) electrophoresis stained with 0.5x SYBR® Green (Invitrogen) and observed under UV light. The DNA quantification was performed using a spectrophotometer (BioDrop® - Biochrom).

To quantify the RZ2MS9 and Δ*ipdC* colonization in maize seedlings, we used the primers 6682 F and 6682 R (Longatto, 2020). Briefly, primers 6682 F (5′ TGGTGTGAAAACAAAAGCAGC 3′) e 6682 R (5′ TGCGTTAGAAGGAAGTGGAAG 3′) were designed from the sequence of a hypothetical protein (GenBank acession n. WP_070757031.1) using the Primer 3 software version 0.4.0 (http://bioinfo.ut.ee/primer3–0.4.0/). This gene was chosen because its sequence had no significant similarity to any other sequence available in GenBank and is not present in the genome of closely related *Bacillus* spp. The software Primer3 (https://primer3.org/) was used to assess the possibility of hairpin and dimers formation. The specifitiy and sensibilitywere evaluated according to methodology proposed by Quecine et al. (2012), using the DNA from different *Bacillus* spp. and different concentration of RZ2MS9 genomic DNA respectively.

The qPCR was performed in a 25 µl final volume, containing 12.5 µl of the Platinum® qPCR superMix-UDG (Invitrogen), 0.5 µl of each primer, and 0.24 µl of CxR buffer. Aliquots of the master mix (21 µl) were distributed in the wells of a 96-well plate, and 2 ng of DNA was added as template. The qPCR cycles consisted of a denaturation step at 95 °C for 6 min, 40 cycles at 95 °C for 30 s, and a final step at 60 °C for 45 s. The quantification was performed in the iCycler iQ real-time PCR instrument. Three biological replicates and two technical replicates were used. The standard curve was obtained for each run using a known copy number of the genomic DNA of RZ2MS9. The bacterial density (Log_{10} cells/ng of total DNA) was estimated by interpolation with the standard curve.

2.10. Effects of the RZ2MS9 ipdC gene knockout on maize growth promotion

The ability of RZ2MS9 and Δ*ipdC* to promote maize growth was evaluated using the seed of hybrid 30A37PW. Bacterial inocula containing approximately 10^8 CFU/ml of each strain were prepared by growing RZ2MS9 and Δ*ipdC* strains in LB broth at 28 °C under agitation (180 rpm). Then, 1 ml of each bacterial suspension was added to 100 maize seeds, which were homogenized and mixed manually for 1 min. Seeds inoculated with the bacteria were distributed on moist sterile Germitest® sheets and acclimated in a germination chamber at 28 °C with 90% relative humidity. The experimental unit of treatments consisted of 4 sheets (replicates) containing 25 seeds each. The control treatment consisted of seeds inoculated with LB broth without any bacterial growth.

The seed germination was assessed daily and the seed germination speed index (GSI) was calculated by using the formula GSI = Σ (ni / ti), where: ni = number of seeds germinating at time 'i'; ti = time after test installation, following the 24-hour time scale. On the seventh day, seed germination rate (G %) was estimated by counting the seedlings presenting root system (primary root and seminal roots) and aerial part (complete and developed epicotyl). The seeds that did not have such structures were considered as abnormal plants or unviable seeds and were not counted (Andrade et al., 2020).

Seven days after inoculation (DAI), 16 plants of each treatment were randomly selected, and their aerial parts were separated from their root system. The roots were scanned in an Epson® Expression 11000XL scanner and evaluated for total root length, projected root area, and lateral root length using the WinRHIZO™ software (Regent Instruments Inc.). Eight plants of each treatment were used to estimate the dry weight. For this, roots and aerial parts of these plants were incubated in a dry oven at 70 °C for 3 d and their dry weight was verified using an analytical balance.

2.11. Statistical analyses

Bacterial IAA biosynthesis, qPCR quantification, and maize growth assay data were submitted to the Shapiro-Wilk and Kolmogorv-Smirnov tests to verify the normality of the data and homogeneity of the variances. To quantify the bacteria, qPCR data were log-transformed to stabilize the variance. Bacterial IAA production and qPCR quantification data were submitted to analysis of variance (ANOVA) and Tukey's test ($p \leq 0.05$). Maize growth parameters were evaluated by the Scott Knott test ($p \leq 0.05$). An ANOVA of the regression curve for bacterial growth was obtained by a stepwise regression used to determine which variables provided the best fit. A completely randomized design was used for all assays and the statistical tests were performed using the software R (RStudio Team, 2019).

3. Results

3.1. Development and molecular characterization of ΔipdC

The Δ*ipdC* was obtained in this study by using CRISPR-Cas9 double plasmid system. *In silico* analyses using the programs CHOP CHOP RGEN Tools Cas-Offinder v. 2.4 and CRISPOR v. 4.2 generated 115 protospacers associated with PAM sequences (5′−3 'NGG) with potential target regions to knock-out the *ipdC* gene (Supplementary Fig. 1). Using our strategy, the selected N20 (GCATTACAACTGGACCGTAT) was cloned in the pTarget, which was named $pTarget_{ipdC}$ (Fig. 1). A donor DNA with a deletion of ~200 bp was obtained to improve the homologous recombination (Supplementary Fig. 2). A total of 3×10^4 transformants were obtained using the CRISPR-Cas9 system. One mutant was randomly selected, named Δ*ipd*C, and its *ipdC* gene was sequenced showing a deletion of 211 bp (Supplementary Fig. 3).

3.2. RZ2MS9 IpdC gene knockout has no effect on bacterial growth but affects the bacterial tryptophan-dependent IAA biosynthesis

RZ2MS9 and Δ*ipdC* presented similar growth patterns in the different media compositions tested (Fig. 2). The Δ*ipdC* cell density was slightly lower than that of RZ2MS9 at all evaluated times and media compositions. In the absence of tryptophan in the medium, Δ*ipdC* synthesized 4.59 μg/ml of IAA, which was not significantly different from the amount of IAA produced by RZ2MS9 (4.11 μg/ml). However, when tryptophan was added to the medium (5 mM), Δ*ipdC* synthesized 7.67 μg/ml of IAA, roughly half of the amount produced by RZ2MS9 (13.4 μg/ml). IAA biosynthesis by RZ2MS9 or by Δ*ipdC* was not detected in the presence of exogenous IAA in the medium (Table 1).

Bacterial production of IAA in LB medium (10%) was significantly different between the strains. RZ2MS9 synthesized 1.99 μg/ml of IAA, while Δ*ipdC* produced only 0.08 μg/ml. However, the addition of maize

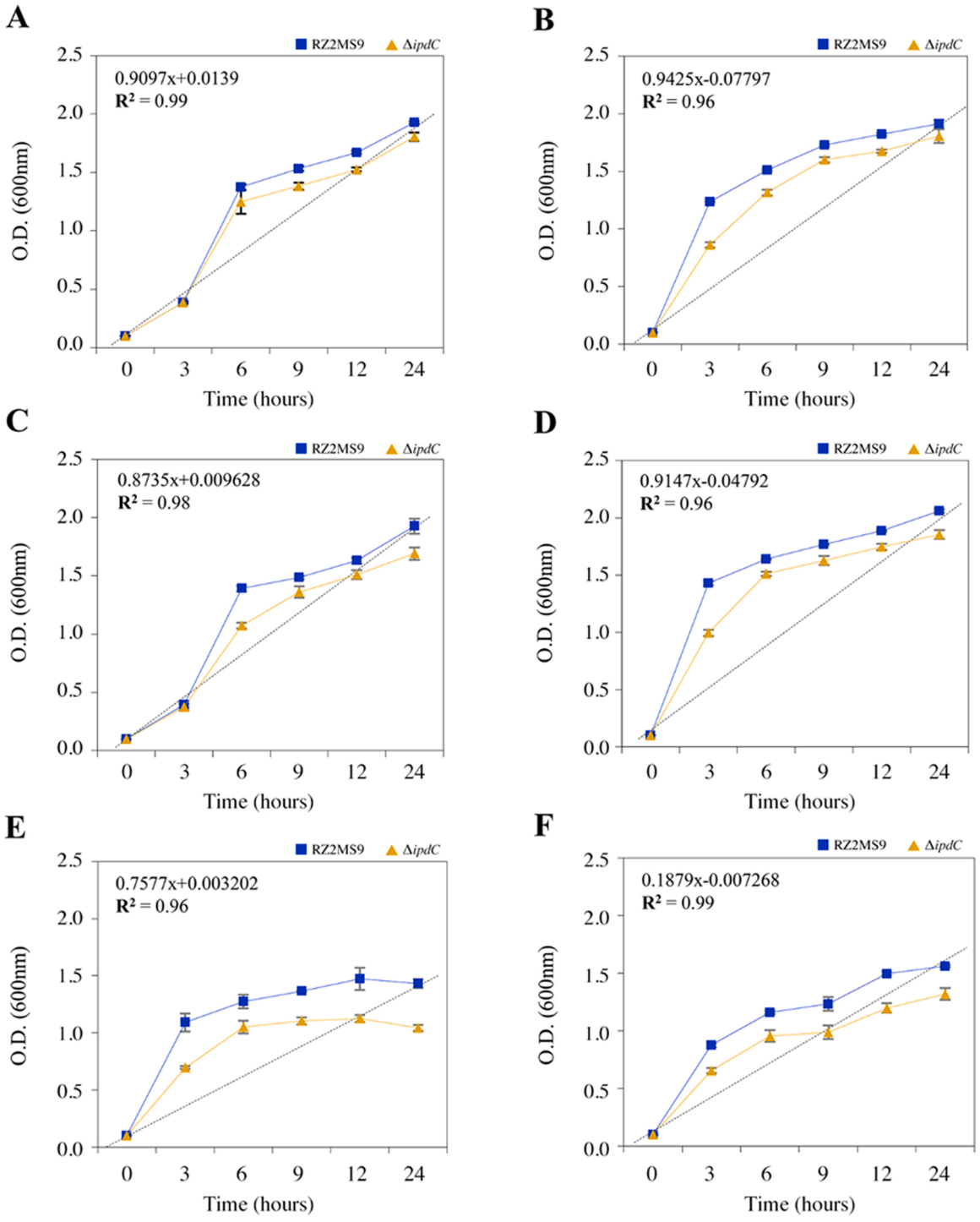

Fig. 2. *Bacillus thuringiensis* RZ2MS9 and Δ*ipdC* growth curves into LB medium with the following compositions: **A**. LB medium, **B**. LB medium supplemented with synthetic IAA (10 μg/ml), **C**. LB medium supplemented with L-tryptophan (5 mM), **D**. LB medium supplemented with L-tryptophan and synthetic IAA (5 mM and 10 μg/ml, respectively), **E**. 10% LB medium. F. 10% LB medium supplemented with 40% maize exudate. Standard deviations from experiments performed in 3 replicates are shown by bars. R^2 values represents the regression coefficient of the curves with $p < 0.001$.

Table 1
Indole-3- acetic acid (IAA) biosynthesis by *Bacillus thuringiensis* RZ2MS9 and mutant Δ*ipdC* in LB medium with different compositions.

Media composition	IAA biosynthesis (μg/ml)	
	RZ2MS9	Δ*ipdC*
LB	4.11 ± 0.89	4.59 ± 0.54
LB + Trp (5 mM)	13.40 ± 1.54	7.67 ± 0.80 *
LB + IAA (10 μg/ml)	N/D	N/D
LB + IAA (10 μg/ml) + Trp (5 mM)	N/D	N/D
LB 10%	1.99 ± 0.31	0.08 ± 0.02 *
LB 10% + Exd 40%	3.32 ± 0.89	1.45 ± 0.28 *

The asterisks in the same line indicate statistical differences according to the Student t test ($p < 0.05$). Average and standard deviation of independent experiments performed using 3 replicates are shown.
LB – Lysogenic Broth
Trp – tryptophan
Exd – maize exudate
N/D: Not Detected

exudates to this medium increased IAA biosynthesis in both strains. In this condition, RZ2MS9 produced 3.32 μg/ml of IAA, more than double the amount produced by Δ*ipdC* (1.45 μg/ml) (Table 1).

3.3. RZ2MS9 ipdC gene knockout influences bacterial colonization of maize, seed germination, and growth promotion

The abundance of both bacterial strains was lower in the aerial part of maize compared to the roots at 24 and 48 HAI (Fig. 3). At 24 HAI, no significant differences were observed between RZ2MS9 and Δ*ipdC* colonization of maize roots or aerial part. At 48 HAI, the abundance of RZ2MS9 was also not significantly different from that of Δ*ipdC* in the aerial part, but maize roots presented a significantly higher abundance of Δ*ipdC* compared to RZ2MS9. We did not detect any bacteria in the non-inoculated control treatment.

All treatments presented a G% of 100%, however; seeds treated with RZ2MS9 had the highest GSI, followed by those treated with Δ*ipdC*, and then by the control (Fig. 4).

In the maize growth promotion trial, inoculation with RZ2MS9 significantly increased the total length of maize roots by about 70% (Fig. 5A), the root projected surface area by about 80% (Fig. 5B), and lateral root length by about 85% (Fig. 5C) when compared to the control and Δ*ipdC* treatments. In addition, RZ2MS9 inoculation increased aerial part dry weight by 75% (Fig. 5D), whereas Δ*ipdC* inoculation had no effect on this plant parameter compared to the control treatment. Although maize roots inoculated with RZ2MS9 showed more vigorous growth (Fig. 5E), the treatments did not significantly affect maize root dry weight.

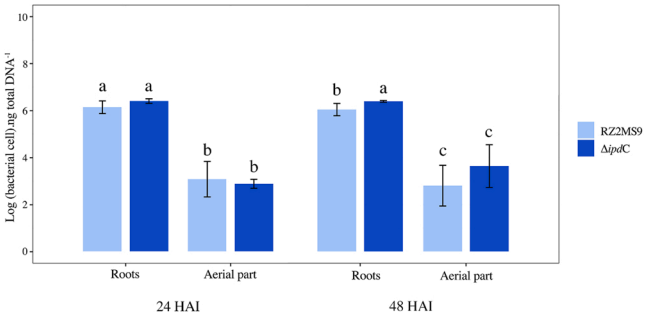

Fig. 3. Maize colonization by *Bacillus thuringiensis* RZ2MS9 and Δ*ipdC* at 24 and 48 h after inoculation (HAI). The bacterial abundance was log-transformed to stabilize the variance. The results are the means of four replicates per sample. The bars represent the standard deviation of each treatment. Values with the same letter in each graph are not significantly different according to Tukey's test ($p > 0.05$).

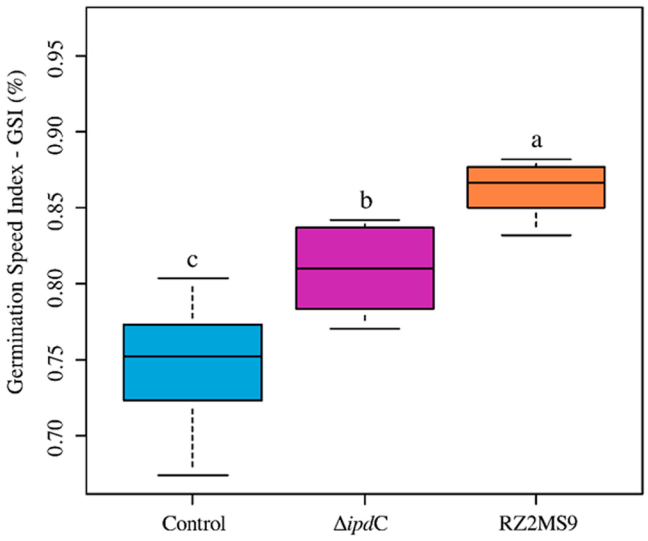

Fig. 4. Germination speed index (GSI) of maize seeds inoculated with RZ2MS9 and Δ*ipdC*. The different letters on the bars indicate statistical differences according to the *Scott Knott* test ($p < 0.05$). Standard deviations are from experiments with four biological replicates. Each biological replication was composed by 25 maize seeds.

4. Discussion

Reverse genetics has contributed to elucidate the molecular mechanisms involved in plant-microbe interactions. One of the main applications of this approach is to deactivate key genes to understand their function (Sander and Joung, 2014; Boettcher and MCmanus, 2015; Shah et al., 2015). In this study, we successfully knocked out the *ipdC* gene from the PGPR *Bt* RZ2MS9 using the CRISPR-Cas9 system to investigate the contribution of the IAA biosynthesis mechanism in the bacterial interaction with maize.

The efficiency of gene knockout mediated by the CRISPR-Cas9 system has been described for different *Bacillus* species, such as *B. mycoides* (Yi et al., 2018), *B. subtilis* (Liu et al., 2019), *B. licheniformis (*Zhou et al., 2019), *B. anthracis* and *B. cereus* (Wang et al., 2019), *B. thuringiensis* (Soonsanga et al., 2020), *B. megaterium*, (Hartz et al., 2021), *B. amyloliquefaciens* (Zhao et al., 2020). All those were transformed using the recombination of donor DNA sequences.

Soosanga et al. (2020) recently used the CRIPSR-Cas9 system to knockout protease genes in *Bt*. The components of the CRISPR-Cas9 system were adapted into the pLPPR9, which is derived from *Lactobacillus planttarum*, and due to its low-copy-number plasmid, reduces the toxicity effect of Cas9 in cells. The number of transformants obtained varied according to the arrangements adapted in pLPPR9-Cas9, ranging from 1×10^3 to 1.6×10^1 per μg of plasmid.

Similarly, the Δ*ipdC* knockout described in our study was obtained by using the plasmid pCas which is characterized as a low-copy-number plasmid. We used the CRISPR-Cas9 system components that were adapted in two distinct plasmids, pCas and $pTarget_{ipdC}$, containing the nuclease Cas9 and the sgRNA, respectively. We obtained 3×10^3 transformants per μg of plasmid in our study. The DNA repair was done by the activation of *λ-Red* recombination machinery contained in the pCas. The total time required to obtain the Δ*ipdC* mutants was approximately 21 days (Supplementary Table 2).

The knockout of genes involved in bacterial biosynthesis of IAA, such as the *ipdC* gene, has resulted in drastic reduction in phytohormone biosynthesis by different bacteria including *Azospirillum, Agrobacterium, Stenotrophomonas, Serratia, Pseudomonas, Pantoea* and others (Remans et al., 2006). Consequently, bacterial interaction with plant hosts may be affected, as well as the bacterial ability to promote plant growth. To date, the molecular techniques commonly employed to obtain bacterial

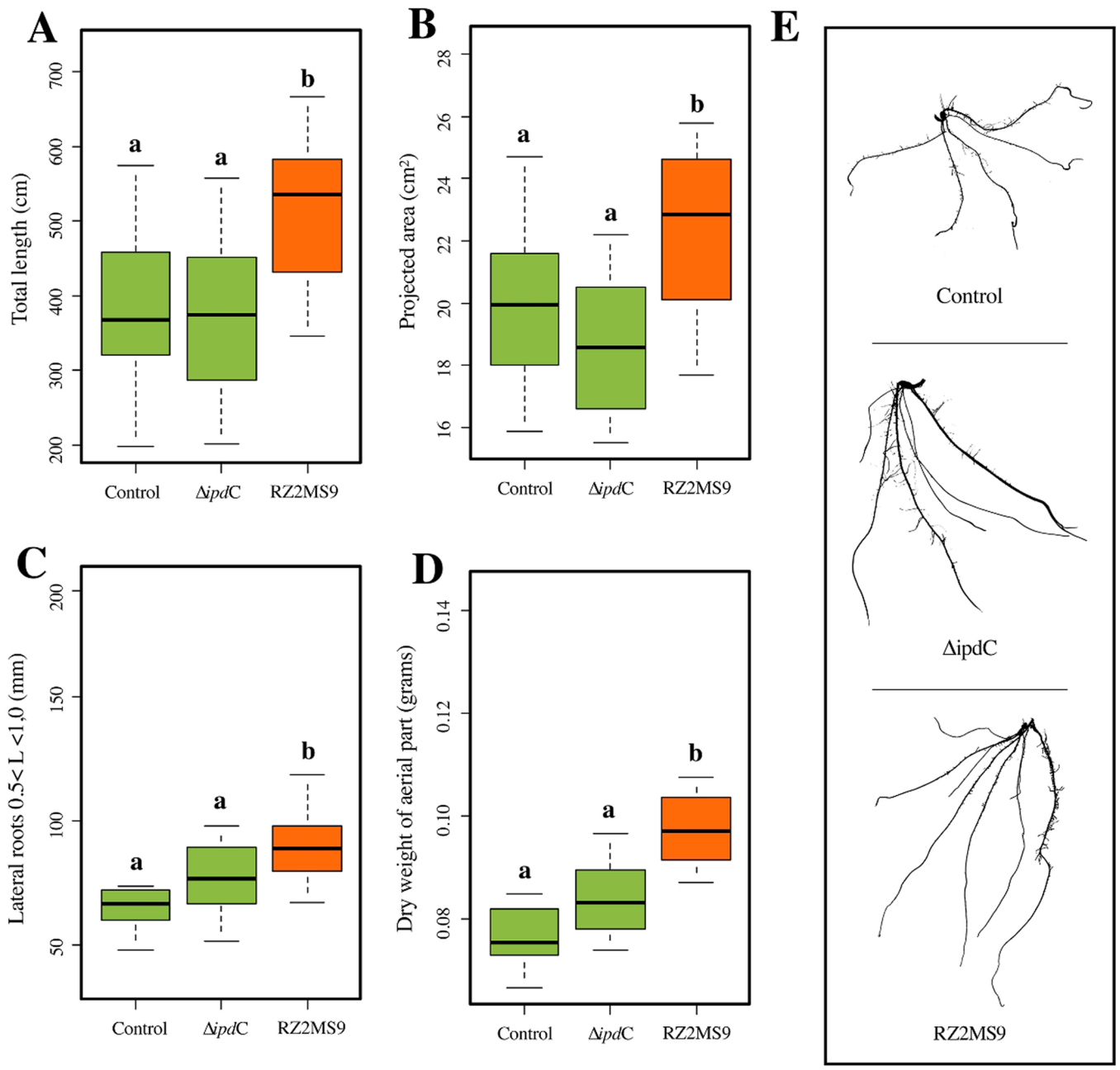

Fig. 5. Maize growth promotion by RZ2MS9 and Δ*ipdC*. **A.** total root length (centimeters). **B.** projected area of the roots (centimeters^2). **C.** length of lateral roots (millimeters). **D.** weight of the dry mass of the aerial part of the plants (grams). **E.** The maize root system images were captured with the Epson® Expression 11000XL scanner. The different letters on the bars indicate statistical differences according to the *Scott Knott* test ($p < 0.05$). Average and standard deviations were obtained from sixteen replicates.

mutants defective in IAA production consist of using plasmids containing transposons, *Targe Tron System*®, and homologous recombination with a suicide vector (Patten and Glick, 2002; Malhotra and Srivastava, 2008; Hassan and Bano, 2016; Ouyang et al., 2017). To the best of our knowledge, this is the first study reporting the generation of an IAA defective *Bt* mutant using the CRISPR-Cas 9 system.

The knockout of the *ipdC* gene in RZ2MS9 significantly decreased the bacterial biosynthesis of IAA, but it did not completely cease its production of auxin. This is probably due to the existence of another IAA biosynthesis pathway in RZ2MS9, the TAM pathway, detected in the bacterial genome by Batista et al. (2021). Thus, further knockouts targeting the genes involved in the TAM pathway of RZ2MS9 should be performed to obtain a better understanding of the bacterial IAA biosynthesis mechanism.

The bacterial biosynthesis of IAA can be induced or suppressed by different endogenous and exogenous factors (Bulgarelli et al., 2013). Exogenous factors include the presence of the precursor tryptophan (trp) as well as the IAA itself either produced by the bacteria or from exogenous sources (Duca et al., 2014). We observed that when trp is added to the medium, auxin production by RZ2MS9 is increased, whereas in the presence of additional IAA in the medium, bacterial phytohormone biosynthesis is suppressed possibly as a result of a feedback response.

In the absence of exogenous trp, IAA biosynthesis was detected by the RZ2MS9 and Δ*ipdC*. However, there was no significant difference when compared between strains. This phenomenon can be attributed to the presence of residual trp in the components of the organic fraction of the LB medium.

During bacterial-plant interaction, root exudates can enhance

bacterial IAA production due to the presence of trp or other inducing molecules (Duca and Glick, 2020). It is known that maize root exudates can affect different processes in bacteria such as chemotaxis, biofilm formation, root colonization, and the expression of genes involved in cell functions and in the interaction with plants (Fan et al., 2012; Mwita et al., 2016; Jin et al., 2019). Additionally, maize exudates can stimulate bacterial IAA biosynthesis by activating IAA metabolic pathways genes. For instance, the transcriptome profile of *B. amyloliquefaciens* FZB42 revealed that *alsS* and *alsD* genes, involved in IAA biosynthesis, were activated in the presence of maize root exudates. This was mainly attributed to the presence of trp in the plant exudates (Zhang et al., 2015). In our study, maize exudates added to LB medium (10%) increased RZ2MS9 biosynthesis of IAA by about 170%, indicating that maize exudates have an inducing effect on the biosynthesis of IAA by the bacterium.

Bacterial colonization of plant tissues is a crucial step for a PGPR to successfully promote plant growth (Reinhold-Hurek and Hurek, 2011; Verbon et al., 2016). For auxin-producing PGPR, the colonization step may be even more important, as the IAA produced by the bacteria can be more easily delivered to the plant when reaching the basipet or acropet flow transport channels, for example (Compant et al., 2010; Cassán et al., 2014; Di et al., 2016). During the colonization process, bacterial IAA can act as a repressor of auxin signalling in plants, making it possible to bypass the plant defense system. Thus, the bacterial IAA biosynthesis may be part of a strategy to suppress host plant resistance leading to a more successful colonization. In addition, the ability of bacteria to synthesize IAA, as well as the amount of IAA synthesized, can determine the success of colonization and the bacterial beneficial or deleterious effect on plant development (Partida-Martinez and Heil, 2011; Hussain et al., 2015; Tsukanova et al., 2017). Likewise, the amount of IAA synthesized by RZ2MS9 in our study appears to influence maize colonization. At 48 HAI, the Δ*ipdC* was more abundant in maize roots than RZ2MS9, this may be related to the reduced amount of IAA produced by the mutant due to the knockout of the *ipdC* gene. The mutant's reduced auxin production may have allowed it to colonize maize roots whithout being detected and suppressed by the plant defense systems.

It is noteworthy that there are only few studies evaluating the role of genes involved in bacterial IAA biosynthesis in plant colonization by PGPR. Our study is a pioneer in obtaining a *Bt* mutant impaired in the *ipdC* gene through the CRISPR-Cas9 system to investigate the contribution of this gene and of the bacterial IAA biosynthesis mechanism in plant-bacteria interaction.

In this study, we demonstrated that the *ipdC* gene knockout affected the ability of *Bt* RZ2MS9 to colonize maize and promote plant growth and that bacterial IAA biosynthesis is a major mechanism involved in the beneficial RZ2MS9-maize interaction. We found that the application of RZ2MS9 significantly increased all maize growth parameters evaluated compared to the non-inoculated control, whereas the mutant Δ*ipdC* had no significant effect on maize growth. Interestingly, germination speed was increased by applying both RZ2MS9 and Δ*ipdC* compared to the control, but it is known that IAA is a phytohormone that is not as active as abscisic acid (ABA) and gibberellin (GA) during seed germination. Interactions between bacterial IAA and these phytohormones, as well as between IAA and ethylene and brassinosteroids can indirectly affect the plant cellular processes involved in seed germination induction and plant establishment (Miransari and Smith, 2014). All of these complex interactions will be considered for further investigation.

Understanding the molecular mechanisms used by a PGPR during its interaction with the plant host is essential for the development of optimized microbial products for agricultural purposes. Here, we have demonstrated the key role of IAA biosynthesis by the PGPR *Bt* RZ2MS9 during maize interaction. New knowledge obtained in our study allow mapping the interrelationship between regulatory pathways of bacterial phytohormone synthesis and the effect of bacteria on plant colonization and growth.

5. Conclusions

In this study, we successfully obtained a mutant of the auxin-producing PGPR *Bt* RZ2MS9 that is defective in the *ipdC* gene, a gene involved in the bacterial production of IAA via the trp-dependent IPyA pathway. The mutant, named Δ*ipdC*, was obtained by using the CRISPR-Cas9 system and produced 57% less IAA in the presence of trp than the wild-type strain. The gene knockout had no effect on the bacterial growth pattern, but it affected the bacterium's ability to colonize maize roots. In addition, the mutant lost its ability to promote maize growth. Therefore, we demonstrated that the RZ2MS9 *ipd*C gene and the bacterial IAA biosynthesis mechanism are key contributors to the beneficial effect of the PGPR RZ2MS9 on maize growth.

CRediT authorship contribution statement

Everthon Fernandes Figueredo: Conceptualization, Methodology, Validation, Writing the original draft. **Thiago Angelo da Cruz:** Methodology, Investigation. **Jaqueline Raquel de Almeida:** Methodology. **Bruna Durante Batista:** Writing – review & editing. **Joelma Marcon:** Investigation. **Pedro Avelino Maia de Andrade:** Formal analysis. **Carolina Alessandra de Almeida Hayashibara:** Investigation, Writing – review & editing. **Mauricio Santos Rosa:** Investigation. **João Lúcio de Azevedo:** Writing – review & editing. **Maria Carolina Quecine:** Conceptualization, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2022.127218.

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