Zea mays L. plant growth promotion by Tsukamurella paurometabola *strain C-924*

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RESEARCH

ABSTRACT

Plant growth-promoting bacteria is a group in rhizosphere colonizing bacteria, which produces substances that enhance plant growth and/or protect them against pathogens. *Tsukamurella paurometabola* C-924 is a strain with nematocidal activity isolated from the rhizospheric soil of a banana plantation in Camagüey, Cuba. The aim of this work was to determine plant growth promoting traits of *T. paurometabola* C-924 as well as to assess the effect of this strain on the growth of *Zea mays* L. in the absence of nematodes. Results shown that *T. paurometabola* C-924 produced indole acetic acid, proteases, chitinases and had the capability to solubilize calcium triphosphate. *T. paurometabola* C-924 also showed antagonistic activity versus the phytopathogenic fungi tested (*Sarocladium oryzae*, *Alternaria longipes*, *Pestalotia palmarum* and *Pythium debaryanum*) and it improved the development of *Z. mays* plants in greenhouse conditions. *T. paurometabola* C-924 might have potential in future field application not only as a nematocidic agent, but also as plant growth promoter. This is the first report of an isolate of *T. paurometabola* with plant growth promoting activity.

Keywords: *Tsukamurella*, plant growth promoting rhizobacteria, indole acetic acid, phosphate solubilization, antifungal activity, *Zea mays*

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RESUMEN

Promoción del crecimiento de plantas de *Zea mays* L. por *Tsukamurella paurometabola* cepa C-924. Las bacterias promotoras del crecimiento vegetal son colonizadoras de la rizosfera y producen sustancias que estimulan el crecimiento de las plantas y/o las protegen contra organismos patógenos. *Tsukamurella paurometabola* C-924 es una cepa con actividad nematicida aislada de suelo rizosférico en una plantación de banana en Camagüey, Cuba. El objetivo de este trabajo fue determinar algunos de los caracteres promotores del crecimiento vegetal de *T. paurometabola* C-924 y evaluar el efecto de esta cepa en el crecimiento de *Zea mays* L. en ausencia de nematodos. Los resultados mostraron que *T. paurometabola* C-924 produce ácido indolacético, proteasas y quitinasas y solubiliza fosfato tricálcico. También mostró actividad antagónica contra los hongos fitopatógenos ensayados (*Sarocladium oryzae*, *Alternaria longipes*, *Pestalotia palmarum* and *Pythium debaryanum*) y estimuló el desarrollo de las plantas de *Z. mays* L. en condiciones de invernadero. *T. paurometabola* C-924 es potencialmente aplicable en el campo no solo como agente nematicida, sino también como promotor del crecimiento vegetal.

Palabras clave: *Tsukamurella*, bacterias promotoras del crecimiento vegetal, ácido indolacético, solubilización de fosfatos, actividad antifúngica, *Zea mays*

*I*ntroduction

Modern agriculture is heavily dependent on the application of chemical inputs, particularly fertilizers and pesticides. However, overuse of fertilizers can cause unanticipated environmental impacts. Many concerns regarding human health and environmental protection with agriculture are focused on reduction in the use of chemical pesticides and inorganic fertilizers, which compelling the search for alternatives that enhance soil and environmental quality [1]. One potential way to decrease negative environmental impacts resulting from continued use of chemical fertilizers is inoculation with plant growth promoting rhizobacteria (PGPR) [2].

The PGPR is a group of rhizosphere colonizing bacteria, which produce substances that increase the growth of plants and/or protect them against pathogens [3]. One of the important mechanisms for these beneficial effects is PGPR-elicited enhanced nutrient availability and nutrient use efficiency. Recently, Glick *et al*. [4] reviewed that some PGPR may influence plant growth by synthesizing plant hormones or facilitating uptake of nutrients from the soil through different mechanisms, such as: synthesis of phytohormones, atmospheric dinitrogen fixation and solubilization of inorganic phosphate.

In addition to their primary effects on plant productivity and health, recent work has shown that these beneficial microorganisms possess secondary effects that may bestow them increased interest for plant growers [5]. More specifically, PGPR have shown activities related to biocontrol of soilborne pathogens. Conversely, biocontrol agents have demonstrated properties that directly promote plant growth [6].

Most approaches for biological control of plant diseases have used single biocontrol agent as antagonist against a single pathogen, which may partially account for the reported inconsistent performance by biocontrol preparations [7]. The soil microorganisms that suppress plant diseases have evolved with plants and are primary factors determining plant health. However, the importance of these bacteria has not been generally recognized and hence, they have not

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been a focus of interest for developing them as biofertilizers and exploitation. The relatively narrow spectrum activity of biocontrol agents against plant pathogens is a major constraint limiting their commercial use. To transcend this difficulty, strains with superior biocontrol and plant growth promotion activities must be developed, with multifaceted approaches in field conditions [6].

In this sense, the use of *Tsukamurella paurometabola* C-924 was explored. It is a bacterial strain isolated from rhizospheric soil in Camagüey, Cuba, which was obtained by screening for isolating strains with potential biological control activity on plant parasitic nematodes. Later on, in field experiments, *T. paurometabola* C-924 showed strong activity against *Meloidogyne incognita*, *Radopholus similis* and *Pratylenchus* spp. [8]. However, some features observed in plant development indicated that the strain also increased plant growth in the absence of these pathogens. For this reason, the aim of this work was to determine some of the plant growth promotion traits of *T. paurometabola* C-924 and to assess the effect of this strain on the growth of *Zea mays* L. in the absence of nematodes.

Materials and methods

Assays for growth promotion abilities of *T. paurometabola* C-924

Bacterial strain and culture conditions

T. paurometabola C-924 was obtained from the collection of the Centre for Genetic Engineering and Biotechnology of Camagüey, Cuba. The bacterium was cultured in Tryptone Soy Agar (15.0 g/L enzymatic digest of casein, 5.0 g/L soy peptone and 5.0 g/L NaCl; Sigma, USA) at 37 ºC for 48 h.

Indole acetic acid production

T. paurometabola C-924 was grown at 37 ºC in the dark with gently shaking in 250 mL of Tryptone Soy Broth (TSB). At different times (0, 3, 6, 9, 12, 24, 48, 72, 96, 120 and 144 h) 2.5 mL samples were withdrawn. One milliliter was taken to determine the optical density of the culture at 600 nm using a spectrophotometer Spectronic 20 D (Thermo Scientific, USA). The rest was centrifuged at 7500 × *g* for 10 min and the supernatants were filtered through a 0.2 µm membrane. One milliliter of each filtrate was mixed with 1 mL of Salkowski reagent [9]. The mixture was incubated at room temperature for 30 min and the absorbance was measured at 530 nm. Concentration of indole acetic acid (IAA) produced was measured with the help of standard graph of IAA (Sigma, USA) obtained in the range of 2-50 µg/mL. Experiments were done in triplicate.

Mineral phosphate solubilization

Quantitative estimation of phosphate solubilization was carried out in NBRIP medium (10.0 g/L glucose, 5.0 g/L $Ca_3(PO_4)_2$, 5.0 g/L $MgCl_2 \cdot 6\ H_2O$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L KCl and 0.1 g/L $(NH_4)_2SO_4$) [10]. Autoclaved uninoculated medium served as control. Isolated colonies of *T. paurometabola* C-924 were inoculated in flasks containing 150 mL of medium and incubated for 5 days at 30 ºC with shaking. Samples were taken every 24 h. The cultures were harvested by centrifugation at 7500 × *g* for 10 min. Phosphate in culture supernatant was estimated using the Fiske and Subbarow method [11]. *Pseudomonas aeruginosa* ATCC 25922 was used as a positive control. Experiments were done in triplicate, with mean values calculated for statistical analysis.

Antifungal assay

The target strains used in this study were kindly donated by the Plant Health Provincial Laboratory from Camagüey, Cuba. The phytopathogenic fungi were routinely cultured in Potato Dextrose Agar (PDA) at 30 ºC. The species used in the antifungal assay were *Sarocladium oryzae, Alternaria longipes*, *Pestalotia palmarum* and *Pythium debaryanum.*

For each fungus, the procedure was the following: The bacterial culture grown in TSB was sprayed on a PDA plate. An 8 mm diameter agar plug containing fungus mycelium was placed in the middle of the plate and incubated at 30 ºC. The diameter of the inhibition zones was registered for seven days and the percentage of inhibition relative to the control (without bacteria) was evaluated [12].

Lytic enzymes evaluation

Chitinases

Bacterial cultures were grown on TSB medium (50 mL) supplemented with colloidal 0.01 % (w/v) chitin, with shaking at 37 ºC for 24 h, further centrifuged at 7500 × *g* and filtered through a 0.2 µm membrane. Filtrates were assayed for chitinolytic activity in Petri dishes prepared with 0.5 % colloidal chitin and 0.8 % agarose. Wells of 8 mm diameter were punched into the agarose and filled with 100 µL of each filtrate. Plates were incubated for 72 h at 30 ºC. The chitinolytic activity was evaluated by measuring the diameter of the hydrolyzed halo in the medium. *Serratia marcescens* ATCC 13880 was used as a positive control for chitinases production. Experiments were done in triplicate.

Proteases

T. paurometabola C-924 was grown on TSB (250 mL cultures) at 37 ºC with shaking for 48 h. Samples of 2 mL were taken at different times (11, 24 and 48 h), centrifuged at 7 500 × *g* for 10 min and filtered through a 0.2 µm membrane. Filtrates were assayed for proteolytic activities in plates prepared with 50 mmol/L Tris HCl buffer, pH 7.5, 1 % gelatin and 0.8 % agarose. Wells of 6 mm diameter were punched into the medium and filled with 100 µL of each filtrate. Plates were incubated for 18 h at 30 ºC, and, subsequently, the surface of the medium was flooded with 1.0 mL of Frazier solution [13]. Once added the solution, gelatin was precipitated and the medium became cloudy. The proteolytic activity was qualitatively evaluated by observing the clear halo in the medium. Collagenase IA (0.04 mg/mL; Sigma, USA) was used as a positive control. Experiments were done in triplicate.

Plant growth promotion assay in *Zea mays* L.

Bacterial strains and growth conditions

T. paurometabola C-924 inoculum was produced by growing the strain 18 h in a culture shaken at 37 ºC in 250 mL of TSB. At the log phase of growth, bacterial

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suspension was centrifuged (7500 × *g* for 10 min at 4 ºC) and washed twice with saline solution (NaCl 0.85 %). Bacterial concentration was adjusted to 10^8 c.f.u./mL, according to the previously established correlation between optical density and c.f.u. number. It was applied at a rate of 10 mL per pot (10^8 c.f.u./mL) at the sowing time.

Pseudomonas fluorescens C 16, a strain with growth promotion activity, from the collection of the Cuban National Institute for Basic Research in Tropical Agriculture (INIFAT), was used as a positive control for PGPR activity. It was in a solid formulation mixed with humus, it was applied in a rate of 10 g per pot (10^8 c.f.u./g) at the sowing time.

Plant growth conditions and soil

Zea mays L. variety DC 1 plants were grown for 36 days in a greenhouse under controlled conditions at 30 ºC during the day, and 22 ºC during the night, with a 14 h photoperiod on pots of 1 dm^3. Pots were daily weighed throughout the experiment, and water loss replaced daily by top watering to maintain soil moisture close to 100 % field capacity during the period of plant growth.

Studies were carried out on a brown soil with carbonates, with 3.74 % of organic matter and 19.14 mg of soil soluble phosphorus (P_2O_5/100 g). Before the experiment, to confirm the absence of root-knot nematodes, soil samples were bioassayed with indicator plants at the Plant Health Provincial Laboratory of Camagüey, Cuba.

Experimental design and plant growth parameters

The plant growth promotion experiment followed a design with 3 treatments (plants inoculated with *T. paurometabola* C-924, plants inoculated with *P. fluorescens* C 16 and not inoculated) and each one was replicated 5 times.

To evaluate the plant response to bacterial inoculation the following growth parameters were assessed: shoot height, shoot diameter, number of leaves and dry weight. The dry weight of the plants was determined after shoots and roots were dried in an oven at 70 ºC for 48 h.

Statistical analyses

Statistical analyses were performed using the SPSS program (Version 15.0). The data were evaluated through analysis of variance (Anova). To detect the statistical significance of differences ($p < 0.05$) between means, the Tuckey test was performed.

Results

In vitro assay of plant growth promoting abilities of *Tsukamurella paurometabola* C-924

The capacity to synthesize IAA is an important feature for a strain to be considered a PGPR. It is well known that this hormone participates in the promotion of plant growth by increasing the radical surface of the inoculated plants. Figure 1 shows that the strain *T. paurometabola* C-924 synthesizes IAA since the first stages of the culture, this hormone levels stable until approximately the initial 48 h, when its concentration

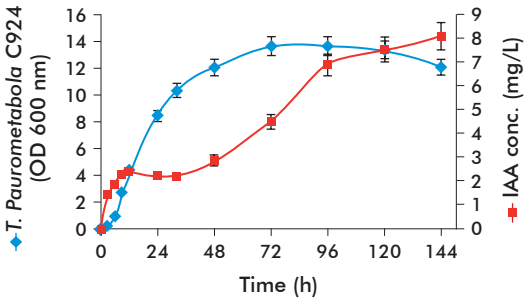

Figure 1. Bacterial growth curve and indole acetic acid (IAA) production by *Tsukamurella paurometabola* C-924. This strain was grown as described in material and methods and IAA accumulation was measured at the indicated times. Vertical bars indicate standard deviations of three replicates.

increased at the stationary phase of the culture. The highest levels of IAA were obtained at the end of this phase, reaching 8.06 mg/L.

Additionally, *T. paurometabola* C-924 was tested for its ability to solubilize the precipitated tricalcium phosphate [$Ca_3(PO_4)_2$] present in the NBRIP medium, at levels of approximately 95 mg/L (Table). Since the third day of the experiment, the concentration of phosphate solubilized by *T. paurometabola* C-924 was statistically significant, compared to that solubilized by the bacterium *P. aeruginosa* ATCC 25922 used as positive control.

T. paurometabola C-924 also secretes chitinases during growth in the presence of colloidal chitin. The diameter of the hydrolyzed halo in the wells inoculated with *T. paurometabola* C-924 culture supernatant (6.2 mm) was statistically significant, similar to the halos of *Ser. marcescens* ATCC 13880 (6.6 mm). Proteases were also detected for *T. paurometabola* C-924 in all the culture samples (Figure 2).

Mycelial growth of tested fungi was inhibited by *T. paurometabola* C-924. Inhibition was evident from day 3 onwards, reaching a maximum value at day 7. The highest inhibition percent were found either in the interactions with *P. palmarum* (52 %) and *A. longipes* (45.2 %). *S. oryzae* was inhibited 21.5 % and the lowest inhibition rate was found with *P. debaryanum* (8.0 %).

Growth promotion of maize plants inoculated with *Tsukamurella paurometabola* C-924 under greenhouse conditions

Shoot elongation, shoot diameter, number of leaves and dry matter in maize plants inoculated with *T. paurometabola* C-924 were assessed 36 days after

Table. Phosphate solubilization by *Tsukamurella paurometabola* C-924 strain and *Pseudomonas aeruginosa* on NBRIP medium

Time (days)	*T. paurometabola* C-924 (mg/L)*	*P. aeruginosa* ATCC 25922 (mg/L)*
0	0	0
1	26.52 ± 3.22	25.57 ± 2.48
2	39.34 ± 5.47	33.22 ± 4.86
3	75.16 ± 6.88 a	38.20 ± 5.31 b
5	95.19 ± 8.13 a	44.40 ± 5.02 b

* Means ± SD within different letters denote a statistically significant difference according to the Tukey test ($p < 0.05$).

Figure 2. Proteases produced by *Tsukamurella paurometabola* C-924 on agarose gelatin at different times. Collagenase IA was used as a positive control.

seeds sowing under greenhouse conditions. Figure 3A shows a statistically significant increase in shoot elongation in plants inoculated with *T. paurometabola* C-924 or *Pse. fluorescens* C 16. Both strains significantly promoted shoot growth ($p < 0.05$) compared to control noninoculated plants.

The effects of *T. paurometabola* C-924 and *P. fluorescens* C 16 on shoot diameter and number of leaves were different for both strains (Figure 3B and C). While *T. paurometabola* C-924 increased significantly these parameters, plants inoculated with *P. fluorescens* C 16 showed no differences in comparison to non inoculated plants. Nevertheless, both strains improved significantly the dry matter compared to the control (Figure 3D).

Discussion

The plant rhizosphere is an environment of intense microbe-plant interactions for micro- and macronutrients [2]. PGPR are regarded as alternative to the use of chemicals in agriculture [3]. An important factor to be considered when screening new isolates is the presence of multiple mechanism of action on the strains to influence plant growth.

The production of phytohormones by PGPR has been shown to be one of the most important mechanisms by which many rhizobacteria promote plant growth [14]. About 80 % of bacteria isolated from plant rhizosphere are able to produce IAA [15]. *T. paurometabola* C-924 produced IAA at levels comparable to those described in previous reports. Ahmad *et al.* [3] described levels of 2.13 and 3.6 mg/L for *Azotobacter*

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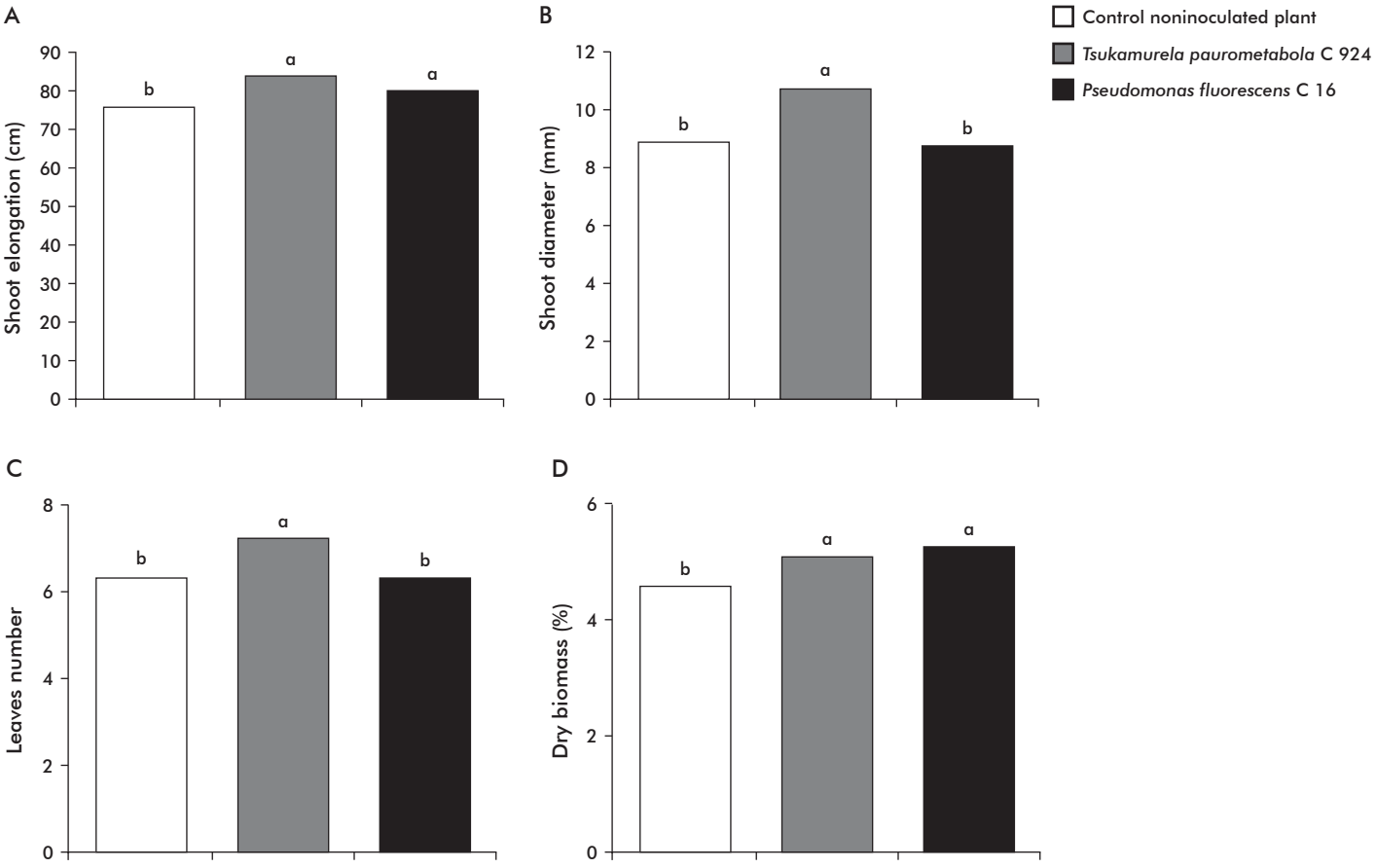

Figure 3. Effect of inoculation with *Tsukamurella paurometabola* C-924 or *Pseudomonas fluorescens* C 16 on the growth of *Zea mays* L. A) Elongation of shoots. B) Diameter of shoots. C) Leaves number. D) Dry biomass. Different letters represent significantly different values according to Tukey test ($p < 0.05$).

and *Pseudomonas* species, whereas Gravel *et al*. [16] reported levels of 3.3 and 6.2 mg/L for *P. putida* and *Trichoderma atroviride*. However, *T. paurometabola* C-924 showed higher IAA production levels at the stationary phase of growth. Production of IAA has been reported for several PGPR belonging to the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Pontibacter* and many others, but to our knowledge there are no reports of IAA production by a *Tsukamurella* strain.

To assess whether this strain has the capability to act as an antifungal biocontrol agent, antagonism towards phytopathogenic fungi was investigated. *T. paurometabola* C-924 strain displayed strong fungicidal activity on *P. palmarum* or *A. longipes* and a lower inhibition rate on *S. oryzae* or *P. debaryanum*. This behavior suggests that this bacterium produces some compounds that could be responsible for the fungal growth inhibition. The results indicate that *T. paurometabola* C-924 is a fungal antagonist at least towards these fungi species. Since *P. palmarum* and *A. longipes* are the causative agents of serious diseases in a variety of plants of economic importance, it would be interesting to further investigate the capability of *T. paurometabola* C-924 to antagonize fungi and to extend the study to other genera and species.

A variety of microorganisms with antifungal activity attacks pathogens by excreting cell wall hydrolases [17]. Chitinases, β-glucanases, and proteases can degrade cell walls of plant pathogenic fungi and oomycetes [18]. In our study, the production of extracellular chitinases and proteases by *T. paurometabola* C-924 could be closely related with the *in vitro* antifungal activity showed by the strain. According to Ordentlich *et al*. [19], the ability to produce extracellular chitinases is considered crucial for *S. marcescens* to act as antagonist against *Sclerotium rolfsii*, and it has been also demonstrated that these enzymes synthesized by *Pseudomonas stutzeri* digest and lyse mycelia of *Fusarium solani* [20]. The cell wall of *Pyt. debaryanum* does not contain chitin, which may explain the lower inhibitory effect caused by *T. paurometabola* C-924 on this specie. Besides, chitin and glucan oligomers released during degradation of fungal cell wall could act as elicitors of defence mechanisms in plants. The induced protection by selected strains of non-pathogenic, root-colonizing PGPR have been shown to be capable of induce disease resistance, promoting plant growth [21].

Proteases are important beyond the biocontrol point of view. There is increasing evidence that plants are able to take up nitrogen not only in mineral form, but also as simple organic molecules. Due to the abundance of protein in organic residues, protease plays a central role in providing bioavailable nitrogen to plants [22]. In this sense, proteases from *T. paurometabola* C-924 could also play a role in the growth promotion of *Z. mays*.

T. paurometabola C-924 inoculation promoted statistically significant rise in shoot elongation, shoot diameter, number of leaves and dry biomass of *Z. mays* when compared to control. This effect was not observed at all in shoot diameter and leaves number in plants inoculated with *Pse. fluorescens* C 16. The capability of *T. paurometabola* C-924 to enhance solubilization of mineral phosphate could play an important role to improve plant growth depending on the nutritional resources of the soil. Some other traits such as IAA production or other phytohormones not determined in this work could also make possible that *Z. mays* growth response to *T. paurometabola* C-924 inoculation. Related studies have reported strains from different species which effectively promoted *Z. mays* growth, such as *Bacillus subtilis* [23], *Pseudomonas auriantica* SR 1 [24], *Serratia* sp. SY 5 [25], *P. putida* strain R-168, *P. fluorescens* strain R-93, *Azospirillum lipoferum* DSM 1691 and *Azospirillum brasilense* DSM 1690 [26] with different mechanism of action for each strain.

Although there have been a few reported *T. paurometabola* isolates from the endophytic bacterial community of marigold [27] and soybean seeds [28], their ability to promote plant growth have not been studied. Therefore, this is the first report of a strain of this specie with PGPR abilities. Belimov *et al.* [29] isolated a *Rhodococcus* sp. strain capable of stimulating root elongation of *Brassica juncea* seedlings and to produce IAA. Coincidently, *Rhodococcus* and *Tsukamurella* are related genera. *T. paurometabola* has had a long taxonomical history, with names including *Corynebacterium paurometabolum*, *Gordona aurantiaca*, and *Rhodococcus aurantiacus*. This taxonomical puzzle was finally solved by Collins *et al*. [30] in 1988, showing that 16 S RNAs of *Rod. aurantiacus* and *C. paurometabolum* were 99 % homologous. Then, they proposed to reclassify and rename this microorganism as *T. paurometabola*.

Conclusions

Soil inoculation with *T. paurometabola* C-924 increased shoot elongation, shoot diameter and leaves number in maize plants grown under greenhouse conditions. *In vitro* assays showed this bacterium is able to produce IAA, solubilize phosphate, secrete lytic enzymes, and inhibit the growth of phytopathogenic fungi. In this sense, *T. paurometabola* C-924 appears to have potential to be used not only as a nematocidic agent, but also as plant growth promoter in future field applications.

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