Characterization of the vegetal growth promoting capacity of Tsukamurella paurometabola *C-924 and the main mechanisms involved*

REPORT

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ABSTRACT

The *Tsukamurella paurometabola* C-924 strain, previously isolated from banana rhizosphere as antagonist of phytoparasitic nematodes, was shown to display other beneficial effects in plants. This work was aimed at determining its growth-promoting activity, and characterizing the main mechanism involved in this process. It was confirmed that *T. paurometabola* C-924 displays growth-promoting activity in plants through various mechanisms. Therefore, under the tested conditions, *T. paurometabola* C-924 was able to release indoleacetic acid, solubilize phosphate, produce ammonia from organic matter and release lytic exoenzymes, which may protect plants during the attack of phytopathogens. Its compatibility with other soil microorganisms used as biofertilizers (*Rhizobium leguminosarum*, *Pseudomonas fluorescens* and *Azotobacter chrococcum*) was also tested, favoring the colonization process of mycorrhizogen fungi and stimulating the formation of arbuscular mycorrhizae. It was further determined that *T. paurometabola* C-924 stimulates the development of economically relevant crops, such as: beans, corn, banana and lettuce, by increasing height, structuring of the radicular system, foliage and dry weight. This was the very first report ever on the growth promoting activity of *T. paurometabola*, and also in the *Tsukamurella* genera, describing the processes involved. That strain bears potentialities for application as biofertilizer in different agricultural systems. This research granted the 2013 Award of the Cuban National Academy of Sciences.

Keywords: *Tsukamurella*, plant grow promotion, indoleacetic acid, phosphate, ammonia, proteases, mycorrhizae

Biotecnología Aplicada 2014;31:168-171

RESUMEN

Determinación de la capacidad promotora del crecimiento vegetal de *Tsukamurella paurometabola* C-924 y caracterización de los principales mecanismos involucrados en el proceso. La cepa *Tsukamurella paurometabola* C-924 aislada a partir de rizosfera de plátano como antagonista de nematodos fitoparásitos; en experimentos previos se observó que además de su actividad nematicida ejercía otros efectos sobre las plantas. El objetivo del trabajo fue determinar la capacidad promotora del crecimiento vegetal de *T. paurometabola* C-924 y caracterizar los principales mecanismos involucrados en el proceso. Se confirmó que la cepa posee actividad estimuladora sobre los cultivos a través de diferentes mecanismos de acción. Bajo las condiciones ensayadas produce ácido indolacético, es capaz de solubilizar fosfatos y produce amoniaco a partir de materia orgánica. Se comprobó que produce exoenzimas líticas que pueden proteger las plantas contra el ataque de patógenos. Se estudió su compatibilidad con otros microorganismos del suelo empleados como biofertilizantes (*Rhizobium leguminosarum*, *Pseudomonas fluorescens* y *Azotobacter chrococcum*) y se observó que favorece el proceso de colonización de hongos micorrizógenos, estimulando la formación de micorrizas arbusculares. Se determinó además que estimula el desarrollo de cultivos de interés económico como: frijol, maíz, plátano y lechuga. Se logró con su aplicación plantas de mayor altura, mayor desarrollo del sistema radical, mayor follaje y peso seco. Este trabajo informó por primera vez para la ciencia la actividad promotora del crecimiento vegetal para la especie *T. paurometabola* y los mecanismos involucrados en este proceso. *T. paurometabola* C-924 tiene potencialidades para su empleo como biofertilizante en diferentes sistemas agrícolas. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2013.

Palabras clave: *Tsukamurella*, promoción del crecimiento vegetal, ácido indolacético, fosfatos, amoniaco, proteasas, micorrizas

Introduction

The increased concerns on the use of agrochemicals due to its aggressiveness to the human health and the environment have raised the interest to develop beneficial microorganisms, to enhance crops health and increase production yields. In this context, several soil microorganisms has shown to be potentially useful candidates, some of them been already considered for different integral pest management practices and yield improvement strategies [1].

For such a purpose, rhizospheric beneficial microorganisms are generally classified in two large groups according to their best studied primary or beneficial

1. Compant S, Clément C, Sessitsch A. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol Biochem. 2010;42(5):669-78.

effects on plants: 1) those directly promoting vegetal growth, and 2) the ones contributing to crops productivity through pathogen control. Several works have unraveled other relevant secondary effects these microbial groups may contribute for agriculture. For example, the growth promoters have shown to display pathogen control activity. Conversely, biological control agents have also shown certain properties directly promoting vegetal growth [2].

In this line of research, the gram-positive *Tsukamurella paurometabola* strain C-924 was isolated at the Center for Genetic Engineering and Biotechnology (CIGB), displaying an efficacious control over plant nematodes and being used as the active ingredient to develop the bionematicidal product HeberNem® [3]. Noteworthy, detailed observation of plant growth and development gave some clues that may indicate this bacterium could also have plant growth-promoting activity even in the absence of nematodes.

For that purpose, a strategy was carried out to determine, if present, the vegetal growth promoting activity of *T. paurometabola* C-924 and to get insight into the main underlying mechanisms. This research granted the 2013 Award of the Cuban National Academy of Sciences.

Results and discussion

Phytohormone production is regarded as the major direct vegetal growth promoting mechanism used by any bacterium to stimulate plant physiology. Particularly, the indoleacetic acid (IAA) is the best characterized phytohormone of those produced by rizhobacteria, which regulates key vegetal processes. Therefore, the production of IAA was evaluated in cultures of *T. paurometabola* C-924 in Tryptone soy broth (TSB). The bacterium was able to produce IAA at levels comparable to those reported by other groups for bacterial strains well characterized as vegetal growth promoters. Up to 8.06 mg/L IAA concentrations were produced at stationary state [4], such levels previously reported by Mehnaz *et al.* [5] for *Pseudomonas putida* CR7 and *Sphingobacterium canadense* CR 11 isolates in corn. This is the very first report of such activity for *T. paurometabola*, and also in the *Tsukamurella* genera.

Furthermore, phytorhormone production is not the single mechanism by which a plant-growth-promoting rhizobacteria (PGPR) can stimulate plants. Such direct effect can be also promoted by increasing the availability of nutrients in the rhizosphere. Particular attention has been put on phosphate solubilization from either organic or inorganic sources, due to phosphate is usually found at very low concentrations in many cultivable soils [1]. Seeking for evidence on this matter, the phosphate solubilizing activity of *T. paurometabola* C-924 was analyzed in NBRIP solid and liquid media. *Pseudomonas aeruginosa* ATCC 25922 was used as positive control. After ten-day incubation at 30 ºC, phosphate solubilization halos were detected, surrounding the colonies of both microorganisms in solid culture (Figure 1). Both strains also showed phosphate solubilizing capacity in liquid cultures under the assayed conditions. Remarkably, this activity was significantly higher for *T. paurometabola* C-924 compared to that of *P. aeruginosa* ATCC 25922 after 5 days of culture [4].

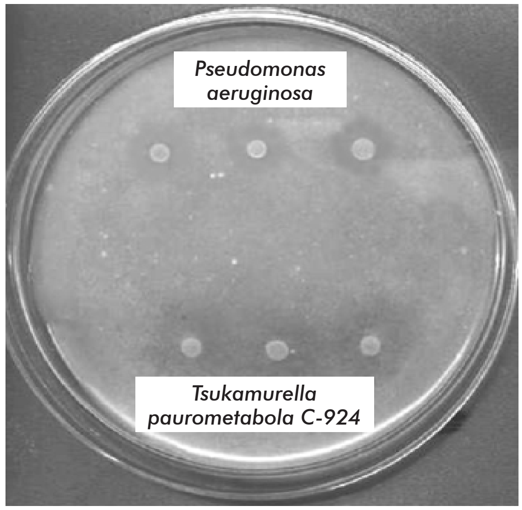

Figure 1. Phosphate solubilization activity of *Tsukamurella paurometabola* C-924 and *Pseudomonas aeruginosa* ATCC 25853 in solid NBRIP medium, ten days after incubation.

Moreover, it has also been shown that bacterial phytohormones responsible for stimulating root development would also be indirectly influencing a more efficient capacity of the plant for phosphate extraction from the soil. That is the reason behind the dual effect of the so-called 'biofertilizers' by simultaneous involvement in the direct solubilization of inorganic phosphorous and the mineralization of the organic one together with the sustained stimulatory effect on the plant root system, or even for mycorrhizal formation [6]. Since that point of view, the phosphate solubilization capacity of the *T. paurometabola* C-924 strain from an inorganic source together with its potential to produce IAA, could generate a synergic positive effect on plant development.

Another of the plant nutrients scarce in soil is nitrogen. Since the beginning of the twenty century, one of the preferred agriculture fertilization techniques comprised the injection of ammonia into soil. Significantly, it was found that *T. paurometabola* C-924 was also a high ammonia producer when cultured in amino acids-rich broth as TSB. This was caused by the oxidative deamidation process that occurs during the metabolism of amino acids present in the medium. It is also a very favorable event, because the ammonia produced by the strain becomes a nitrogen source for plants when transformed to ammonium in the soil.

In a different scenario, pathogenic microorganisms in the soil are one of the major factors limiting agricultural yields and crop quality. The huge amounts of agrochemicals used for its control and its associated damage to soil microbiota and to the functionality of soil microbial ecosystems, has open opportunities to develop biological control agents increasingly relevant for sustainable agriculture. A wide range of microbial compounds have been identified as phytopathogen suppressors, with the subsequent reduction of plant damage. Defense-related enzymes as chitinases, peroxidases and lipases are among them [7].

It has been characterized the attack of microorganisms regarded as antagonists to pathogens by

2. Siddiqui ZA. PGPR: Prospective biocontrol agents of plant pathogens. In: Siddiqui ZA, editor. PGPR: Biocontrol and Biofertilization. Dordrecht: Springer; 2006. p. 111-42.

3. Compte O, Leon L, Marín M, Mena J, Pujol M, Hernández AM, *et al.*, inventors; Mena J, Pimentel E, Hernández AT, Veloz L, Marín M, Compte O, *et al.*, assignee. Pesticidal and Antiparasitic Compositions. United States patent US 20040071663 A1. 2004 April 15.

4. Marín M, Wong I, Mena J, Morán R, Pimentel E, Sánchez I, *et al*. *Zea mays* L. plant growth promotion by *Tsukamurella paurometabola* strain C-924. Biotecnol Apl. 2013;30(2):105-10.

5. Mehnaz S, Kowalik T, Reynolds B, Lazarovits G. Growth promoting effects of corn (*Zea mays*) bacterial isolates under greenhouse and field conditions. Soil Biol Biochem. 2010;42(10):1848-56.

6. Martínez-Viveros O, Jorquera MA, Crowley DE, Gajardo G, Mora ML. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. J Soil Sci Plant Nutr. 2010; 10(3): 293-319.

7. Myresiotis CK, Karaoglanidis GS, Vryzas Z, Papadopoulou-Mourkidou E. Evaluation of plant-growth-promoting rhizobacteria, acibenzolar-S-methyl and hymexazol for integrated control of *Fusarium* crown and root rot on tomato. Pest Manag Sci. 2012;68(3):404-11.

releasing enzymes which hydrolyze the pathogens cell walls [8]. Some studies are contradictory, the evidences suggesting a relevant role but not in all the cases. Nevertheless, the production of lytic enzymes has been traditionally considered as a biological control mechanism [2]. The reason backing up this assumption is that chitinases, β-glucanases and proteases can actually degrade the cell wall of pathogenic fungi and oomycetes. Additionally, chitin and glycan oligomers released by such degradation process could act as activators of the different plant defense mechanisms [9].

In this line of research, it was determined that *T. paurometabola* C-924 secretes chitinases while cultured in the presence of colloidal chitin. The mean hydrolysis halo diameter in the wells inoculated with *T. paurometabola* C-924 culture supernatants (6.2 mm) was significantly similar to the mean diameter seen in wells inoculated with *Serratia marcescens* ATCC 13880 (6.6 mm) as a positive control.

It was similarly investigated the production of proteases by *T. paurometabola* C-924 in TSB; halos were detected in gelatin hydrolysis assays. Significantly, it was possible to correlate the excretion of these proteins into the medium as related to the microorganism cellular growth. The enzyme activity was corroborated, and the proteases molecular weight characterized by electrophoresis of semi and fully denatured samples of *T. paurometabola* C-924 culture supernatants. Gelatin hydrolysis bands were detected in both types of samples near 43kDa, which matched with controls of collagenase IA (Figure 2). Proteases are not relevant just for antagonist effect on microorganisms. They also play a key role in the supply of nitrogen in a metabolic compatible form for plants absorption and nutrition. In this sense, the proteases excreted by *T. paurometabola* C-924 could further promote the growth of the crops analyzed [4].

The persistence of *T. paurometabola* C-924 in the soils is paramount to exert all the effects given above. Hence, it was studied, with the strain steadily persisting in soil over time, with slight crop-dependent variations. In banana, the strain was found during plantation at an approximate concentration of 10^8 colony-forming units (c.f.u.) per gram of soil. After 30 days, its viability was found closely the same, starting to decline afterwards to 10^6 c.f.u./g at the end of the experiment. A different pattern was observed in beans and corn, with 10^7 c.f.u./g of soil at plantation after the second inoculation, which increased until day 14 up to approximately 10^9 c.f.u./g of soil, further declining to near 10^9 c.f.u./g of soil at the end of the study. This behavior may have been related to soil drainage by irrigation, as well as to other biotic or abiotic factors influencing bacterial survival in the inoculated soils. Noteworthy, it was demonstrated that this strain is capable to persist once inoculated at populations and for periods large enough to exert their plant growth-promoting effect.

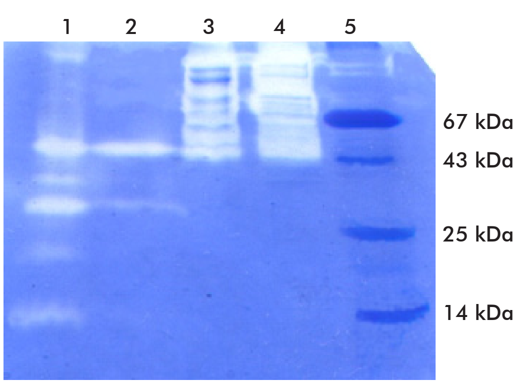

Figure 2. Analysis of *Tsukamurella paurometabola* C-924 culture supernatant samples by 10 % SDS-PAGE and 0.1 % gelatin (w/v). Lanes: 1, semidenatured samples; 2, denatured samples; 3, semidenatured type IA collagenase (Sigma); 4, semidenatured type IA collagenase; 5, standard molecular weight markers (14; 25; 43 and 67 kDa).

Crop variety and species also influence the effectiveness of biofertilizer strains for plant growth. For this reason, experiments were run to corroborate the activity of *T. paurometabola* C-924 in banana, and in economically relevant species other than the original one (beans and corn). These are highly demanded crops requiring harvest yield improvement and substitution of consumables such as fertilizers. Interaction experiments in beans and corn showed that *T. paurometabola* C-924 significantly stimulated seed germination after inoculation, compared to non-inoculated controls. This effect could be derived from its ability to produce IAA, which stimulates seed germination and general development of plants, among other functions [10]. The inoculation with PGPRs producing IAA was previously used to stimulate seed germination, accelerate root growth, modify the radical system architecture and increase root biomass [11].

In general, the inoculation of soils with *T. paurometabola* C-924 significantly influenced the development of crops under study. During its interaction with banana plants it significantly raised the plant fresh weight. Similarly, endophytic *Rhizobium* strains isolated from banana roots increased plant development in height and yields [12].

It was possible to determine that the inoculation of soils with *T. paurometabola* C-924 significantly affected the nodulation process of *Rhizobium leguminosarum* biovar *phaseoli* CFH, since higher nodule formation was seen in treatments lacking *T. paurometabola* C-924, including the control. This fact suggested the presence of native strains in the soil. On the other hand, *T. paurometabola* C-924 releases large amounts of ammonia during the degradation of organic matter. Those high levels of nitrogen in the soil could inhibit *R. leguminosarum* nodule formation in the plant radicular system, by decreasing the plant needs for that interaction to suffice its nitrogen requirements to grow. Nevertheless, in our experiments, the changes in nodulation did not negatively impact plant development. By the contrary, plants receiving *T. paurometabola* C-924 grew faster and developed more leaves than for the other treatments. This is possibly due to the intrinsic limitations of the pot plantation system used, which tends to restrict plant development to some extent. That is why, it is recommended to step forward to field tests, to ascertain the effect of this strain not only on phonological variants, but also on crop yields under field conditions [12].

8. Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JH, *et al*. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science. 2011;332(6033):1097-100.

9. Karthikeyan M, Jayakumar V, Radhika K, Bhaskaran R, Velazhahan R, Alice D. Induction of resistance in host against the infection of leaf blight pathogen (*Alternaria palandui*) in onion (*Allium* cepa var. *aggregatum*). Indian J Biochem Biophys. 2005; 42(6):371-7.

10. Kovaleva LV, Zakfiarova EV, Minkina YV, Voronkov AS. Effects of flavonols and phytohormones on germination and growth of petunia male gametophyte. Allelopathy J. 2009;23(1):51-61.

11. Tsavkelova EA, Cherdyntseva TA, Botina SG, Netrusov AI. Bacteria associated with orchid roots and microbial production of auxin. Microbiol Res. 2007;162(1):69-76.

12. Martínez-Romero, E. Diversity of *Rhizobium-Phaseolus vulgaris* symbiosis: overview and perspectives. Plant Soil. 2003;252(1):11-23.

Particularly in corn, the inoculation of *T. paurometabola* C-294 improved plant development attending to germination, height, stem diameter and number of leaves, compared to the uninoculated control (Table) [4]. Similar effects have been reported for *Azotobacter chroococcum*, *Azospirillum brasilense*, *P. putida* and *Bacillus lentus* strains, significantly promoting corn development and production yields [13]. There were also reports for *Enterobacter cloacae* CR1, *Stenotrophomonas maltophilia* CR3, *P. putida* CR7, *Sphingobacterium canadense* CR11 and *Burkholderia phytofirmans* E24, all of them increasing the stem length in corn plants cultured under greenhouse conditions [5].

Overall, the results on the interaction of *T. paurometabola* C-924 with other plant growth stimulating microorganisms did not show any antagonism with the PGPRs *Pseudomonas fluorescens* C 16, *A. chroococcum* INIFAT 12 and *R. leguminosarum* biovar *phaseoli* CFH. Similarly, crop inoculation with *T. paurometabola* C-924 rendered similar or better results than those achieved with the assayed strains [14].

The internal colonization of roots by mycorrhizogen fungi, together with water and nutrient supply, significantly impacts plant physiology. When present, the changes promoted by mycorrhization provide favorable conditions for plants to healthy grow and develop, with higher plasticity in their adaptive responses against environmental changes. In fact, mycorrhization increases their stress- and drought- tolerance and make them more resistant to the attack of root-targeting pathogens. A synergic interaction was found in experiments with arbuscular mycorrhizal fungi, among *T. paurometabola* C-924, *Glomus clarum* and *Glomus fasciculatum* in lettuce crops. Plants simultaneously treated with *T. paurometabola* C-924 and both fungi strains showed a significantly improved growth compared to independent treatments [15].

Relevance of the study

This was the first report ever on the vegetal growth-promoting activity for the *T. paurometabola* species, together with demonstration on its ability for phosphate solubilization and to produce IAA, ammonia and lytic exoenzymes. These activities pave the way for the use of *T. paurometabola* C-924 as vegetal stimulator in the form of biofertilizer for different agricultural systems and expand the spectrum of use of the HeberNem bionematicide. This scientific result was the base for a patent granted in Cuba, the European Union, South Africa and other countries.

Table. Growth of corn plants inoculated with *Tsukamurella paurometabola* C-924, *Azotobacter chroococcum* INIFAT 12 or *Pseudomonas fluorescens* C16 strains

Time of culture (d)	Plant height (cm)				Standard error
	Control	*T. paurometabola* C-924	*A. chroococcum* INIFAT 12	*P. fluorescens* C16	
7	11.73 b	14.15 a	11.82 b	13.03 a	0.667*
14	30.50 c	35.53 a	30.58 c	32.9 b	1.471*
21	43.03 c	51.71 a	45.44 bc	48.5 b	2.314*
28	50.60 c	59.17 a	56.17 b	58.66 ab	1.983*
35	69.79 c	77.70 a	71.70 c	75.15 b	1.907*
42	72.41 c	83.40 a	76.53 b	78.64 b	1.825*

[a, b, c] Different letters and asterisks (standard error) represent significant differences ($p < 0.05$, Tukey's multiple comparison test).

Conclusions

T. paurometabola C-924 has the ability to produce IAA, to solubilize phosphates, excrete chitinases and proteases, and to produce ammonia from amino acids, altogether enriching the rizosphere with nutrients required for plant growth. This strain was capable of stimulating germination and growth of banana, beans and corn crops under greenhouse conditions, while stimulating the growth of arbuscular mycorrizae. In summary, this bacterium certainly has vegetal growth-promoting activity, besides its nematicidal effect, further expanding its spectrum of use as biofertilizer in different systems of economically relevant crops.

Acknowledgements

The authors are grateful for the support provided by the Soil Experimental Station and the Province Laboratory of Vegetal Health in Camagüey, Cuba, while conducting this research.

13. Yazdani M, Bahmanyar MA, Pirdashti H, Esmaili MA. Effect of phosphate solubilization microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and yield components of corn (*Zea mays* L.). Proc World Acad Sci Engineer Technol. 2009;49:90-2.

14. Marín M, Mena J, Chaveli P, Morán R, Pimentel E. Interacción de *Tsukamurella paurometabola* C-924 con *Rhizobium leguminosarum* biovar *phaseoli* CFH en el cultivo de frijol. Acta Agronómica. 2013; 62(1):52-8.

15. Marín M, Mena J, Franco R, Pimentel E, Sánchez I. Effects of the bacterial-fungal interaction between *Tsukamurella paurometabola* C-924 and *Glomus fasciculatum* and *Glomus clarum* fungi on lettuce microrrizal colonization and foliar weight. Biotecnol Apl. 2010;27(1):48-51.