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Original article

Growth promotion in chard, carrot and radish plants by *Pseudoxanthomonas indica* named H32

Promoción del crecimiento en plantas de acelga, zanahoria y rábano por *Pseudoxanthomonas indica* nombrada H32

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ABSTRACT: One of the main problems in current agriculture is the reduction of chemical fertilizers by replacing them with biofertilizers composed of beneficial microorganisms for soils and plants. Therefore, the present work aims to evaluate the effect of *Pseudoxanthomonas indica* named H32, previously isolated from healthy tomato plants rhizosphere, on the growth, development and yield of chard, carrot and radish crops. To this end, its ability to produce indoleacetic acid (IAA) was determined. In addition, its property to solubilizing insoluble phosphate in Pikovskaya-agar medium. The seeds were inoculated by soaking in a H32 cells suspension with 10^5 UFC/mL in 0.1 % carboxymetyl cellulose solution, for 3 h. A similar procedure using only carboxymetyl cellulose solution was done for non-inoculated seeds. All Seeds were then sown in 1 x 10 m plots. H32 was inoculated around the root of the seedlings of inoculated seeds, seven days after germination. The results showed that plants from inoculated seeds had greater humid weight, greater height, and roots with greater length and weight than plants from non-inoculated seeds ($P < 0.01$). The inoculation of the seeds and soil with H32 increased the yield by 19 % for radish, 88.7 % and 68.5 % for chard and carrot, respectively. These results show that *Pseudoxanthomonas indica* H32 has potential for use as a growth promoter in the crops studied.

Key words: Indoleacetic acid, germination, soil bacteria, phosphates, biofertilizers.

RESUMEN : Uno de los principales problemas en la agricultura actual es la reducción de los fertilizantes químicos mediante su reemplazo por los bioferlizantes compuestos por microorganismos benéficos para el suelo y las plantas. Por tanto, el presente trabajo tiene como objetivo evaluar el efecto de la *Pseudoxanthomonas indica* nombrada H32, aislada previamente de la rizosfera de plantas sanas de tomate, en el crecimiento, desarrollo y rendimiento de los cultivos de acelga, zanahoria y rábano. Para ello se determinó su capacidad de producir ácido indolacético (AIA). Además, su propiedad de solubilizar fosfato insoluble en medio Pikovskaya-agar. Las semillas se inocularon mediante su remojo en una suspensión de células de H32 con 10^5 UFC/mL en solución de carboximetil celulosa al 0,1 %, por 3 h. Se realizó un procedimiento similar usando solo solución de carboximetil celulosa para las semillas no inoculadas. Todas las semillas se sembraron en parcelas de 1 x 10 m. Se inoculó H32 alrededor de la raíz de las plántulas de semillas inoculadas, siete días después de germinadas. Los resultados mostraron que las plantas de semillas inoculadas tuvieron mayor masa húmeda, mayor altura y raíces con mayor longitud y masa que las plantas de semillas no inoculadas ($P < 0,01$). La inoculación de semillas y suelo con H32, incrementó el rendimiento en 19 % para el rábano, en 88,70 % y 68,5 % para la acelga y zanahoria respectivamente. Estos resultados evidencian que *Pseudoxanthomonas indica* H32 tiene potencial para su uso como promotor de crecimiento en los cultivos estudiados.

Palabras clave: Acido indolacético, germinación, bacterias del suelo, fosfatos, biofertilizantes.

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INTRODUCTION

One of the challenges of modern agriculture is to increase crop yields to feed the world's growing population with the least impact on the environment. To improve crop production without chemical fertilizers that damage health and cause soil fertility losses, research has focused on the development of new bioproduct technologies based on rhizobacteria as plant growth promoters (PGRP). Different studies have been conducted to describe the stimulating effect of PGRP on different agricultural crops. The results include an increase in germination rates, root or stem size, yields, leaf number, leaf area mass, dry matter and resistance to drought and other stress factors. This has been published in related books and several journal articles, demonstrating the current interest in microorganisms with these characteristics (1,2). PGRPs can act by direct mechanisms including phosphate solubilization, nitrogen fixation, and productionn of growth-regulating phytohormones (3,4). Among these, auxins are the major ones responsible for plant growth. Auxins in general and specifically indoleacetic acid (IAA) are also involved in the regulation of dormancy and seed germination. Bacterial IAA synthesis is generally dependent on the amino acid tryptophan. Recently, (5) reported the use of *Pseudomonas* spp. strain VSMKU 4050 on tomato seedlings. It significantly increased their growth compared to other treatments. It is estimated that each radish seedling exudes 290-390 ng of tryptophan per day to the soil. Inoculation of radish plants with *Pseudomonas* spp. rhizobacteria increased root biomass 1.4-fold. The benefits of the effect of bacterial inoculation on radish plants can be explained by the fact that the introduced rhizobacteria produce the plant growth-stimulating hormone, IAA from tryptophan contained in the plant exudate and which has been identified as the main precursor of the biosynthesis pathway of indole compounds in bacteria (6). In addition, PGRPs with the characteristic of solubilizing phosphates are also being studied for their potential to be formulated and applied to crops as biofertilizers. This characteristic constitutes an extremely important process for croplands, since these soils have accumulated large amounts of non-soluble phosphorus over the years, due to the massive application of chemical fertilizers, which can only be recovered through the action of solubilizing microorganisms. Currently, it is known that most PGRP act on plants through more than one mechanism of action (7). Recently (8) shows that the isolated strain *Pseudoxanthomonas indica* RSA-23 has the potential to act against pathogenic microorganisms. The objective of this work is to evaluate the effect of some characteristics of the bacterium H32, such as the production of IAA and its property to solubilize phosphate, on the growth and development of economically important crops, such as radish (*Raphanus sativus*), chard (*Beta vulgaris*) and carrot (*Daucus carota*).

MATERIALS AND METHODS

Bacteria

The bacterium H32 was isolated from the rhizosphere of healthy tomato plants, as described by (9). Its characterization by sequencing a fragment of the gene coding for 16S rRNA showed that it belongs to the species *Pseudoxanthomonas indica*. The results of its biochemical tests correspond to the main characteristics described for this bacterial species.

Determination of indoleacetic acid (IAA) produced by H32

Pseudoxanthomonas indica H32 was grown in Luria Bertani broth (LB) culture medium; 10.0 g L-1 sodium chloride; 5.0 g L^{-1} yeast extract and 5.0 g L^{-1} Tryptone, based on a colony previously grown on the same medium with agar; 15.0 g L^{-1} (LBA), and pH = 7.2. The culture (0.1 mL) was transferred to two 250 mL erlenmeyers containing 50 mL of LB broth with and without tryptophan (100 mg L^{-1}). Both were incubated at 37 °C and 200 rpm in Shaker Incubator (New Brunswick G 25). For the kinetics of IAA production, the experiment was performed in triplicate and the colorimetric method was used, with Salkowski's reagent (2 % v/v 0.5 M FeCl3 in 35 % $HClO_4$ solution) (10). The method was applied to supernatant samples. AIA standard (SIGMA) was prepared at concentrations (between 0 and 50 mg L^{-1}) to achieve the standard curve and was reacted at a ratio of 2 mL of Salkowski's reagent to 1 mL of the standard. It was incubated in the dark for 30 min at room temperature and then the absorbance was measured in spectrophotometer (Biochrom Libra S80) at 530 nm. For cell separation, 3 mL culture samples were centrifuged and membrane filtered (0.2 µm). Subsequently, 1 mL was used for reaction with Salkowski reagent. The culture medium without H32 was used as a control. The concentration of AIA was expressed in µg mL^{-1}.

Phosphate solubilization *in vitro*

The insoluble phosphate solubilization characteristic of H32 bacteria was determined in Pikovskaya-agar culture medium (11). After sterilization, the medium was added to Petri dishes, forming a thin layer. To seed the bacteria, 0.01 mL of a cell suspension (10^5 CFU mL^{-1}) of *Pseudoxanthomonas indica* H32 and *Pseudomonas aeruginosa* ATCC 27853 (positive control) was inoculated. LB broth (0.1 mL) was used as a negative control. The plates were incubated at 28 °C for seven days in an incubator (RETOMED IF 3B). Subsequently, the diameter (mm) of the translucent halo around the colony was measured, which indicates phosphate solubilization. In addition, the solubilization index (SI) was calculated with the formula: SI = colony diameter + solubilization halo/colony diameter (12).

In vitro germination test

This assay was performed with seeds of radish (*Raphanus sativus*) variety Early Scarlet globe, carrot (*Daucus carota*) variety New Kuroda, and chard (*Beta vulgaris*) variety PK-7. Each type of seeds was divided into two groups (inoculated with H32 and the non-inoculated group). They were disinfected in 70 % ethanol solution for 5 min. They were washed three times with sterile distilled water, disinfected for 10 min with 1 % sodium hypochlorite solution. They were drained and were rinsed abundantly with sterile water. Seeds were immersed in a cell suspension (10^5 CFU mL^{-1}) in sterile 0.1 % carboxymethylcellulose solution for seed inoculation. They were prepared from a culture of H32 in LB broth, for 48 h. Seeds of the non-inoculated group were immersed in a cell suspension (10^5 CFU mL^{-1}) in sterile 0.1% carboxymethylcellulose solution, were prepared from H32 culture in LB broth, for 48 h. Seeds of the non-inoculated group were soaked only in sterile 0.1% carboxymethylcellulose solution. All seeds were swollen for 3 h. They were distributed in three groups of 30 seeds per Petri dish (14 mm diameter) and they were placed on sterile filter paper previously moistened with sterile distilled water. The plates were placed in an incubator (RETOMED IF 3B) at the optimum temperature for each type of seed. Seeds were observed daily, and the number of germinated seeds per day was recorded, considering germination as a criterion when the seed had an exposed radicle greater than or equal to 2 mm. After germination, the germination percentage (G %) and the mean daily germination (GMD) were determined for each plate, according to (13). (G %) = (Gf / N) x100, Gf: total seeds germinated at the end of the trial; N: total seeds, (GMD) = G % / Tf, Tf: days until the end of germination.

Evaluation of plant growth promotion in greenhouse trials

Trials were conducted inside a greenhouse measuring 14 m wide by 24 m long, with 10 x 1 m plots and were fertilized with 15 % earthworm humus. Radish, chard and carrot seeds were used and were divided into two groups. They were disinfected, and swollen as described in the previous in vitro germination assay; then placed in sterile Petri dishes, inside a Laminar flow cabinet (FASTER Bio48) for drying overnight, before sowing. Each culture was sown in two blocks (100 seeds/block); one of seeds inoculated with H32 and the other of non-inoculated seeds. All seeds were sown 2 cm deep. Radish and chard seeds were sown at a distance of 20 cm, and carrot seeds were sown at a distance of 12 cm. A drip irrigation system was applied at the appropriate frequency according to soil moisture.

Soil inoculation with *Pseudoxanthomona indica* H32

Soil inoculation of H32 was performed seven days after seedling germination, and only where H32-inoculated seeds were sown. A fresh culture of H32 was diluted to 10^6 CFU mL^{-1} in soft water, each plant was inoculated with 100 mL over the root area.

Detection of *P. indica* H32 in the soil

Soil samples containing 10 g of the rhizosphere of the plants in the plots inoculated with H32 were taken weekly in duplicate and mixed in a flask with 90 mL of sterile peptone water with 0.1 % tween 80 (SSPT). The flasks were shaken 20 min on an orbital shaker (WIGGENS WS-100D) at 150 rpm and laboratory temperature. After 5 min rest, 1mL of the liquid phase was taken for 1:10 serial dilutions in sterile SSPT to 10-6 and 10-7 dilutions. Then, 0.1 mL was seeded on selective MacConkey agar medium with 50 mg L^{-1} Kanamycin. After 48 h of incubation at 37 °C, characteristic H32 colonies (red, shiny, round colonies with distinct borders and a pink halo around) were counted using the Stereoscopic Microscope (Motic, RS). The concentration of H32 was expressed as CFU g^{-1}.

Parameters evaluated in the greenhouse trial

Radish and chard plants were harvested five weeks after sowing and the following parameters were evaluated to a sample of 40 plants per treatment; Wet mass (g), mass (g) and bulb diameter (cm), in radish plants. In addition to height (cm), wet mass (g), number of leaves, leaf length and leaf width (cm) in chard plants. Carrot plants were harvested 90 days after sowing. A sample of 20 plants per plot was evaluated for wet mass (g), root mass (g), main root length (cm), and leaf and stem mass (g). Weighing was done on an electronic balance (Sartorius), bulb diameter was measured with a caliper (Mitutoyo) and the other measurements with a graduated tape measure (Facom). In addition, the yield (R) of the plots was determined and expressed in kilograms. R (kg) = P x G/1000; P, average wet mass of plants (g) and G, percentage of germinated plants in the plot.

Statistical analysis

A comparative statistical analysis was performed using the Student's t-test of the STATGRAPHICS Centurion XVI software to determine the parameters with statistically significant differences with respect to the control.

RESULTS AND DISCUSSION

The selection of microorganisms with specific effects on certain plant species of economic interest has led to the creation of bioproducts called biofertilizers (14, 15), making the search for promising candidate strains for the industrial production of these bioproducts a necessity. In this sense, we isolated beneficial bacteria from the risosphere of agricultural plant species and determined their mechanisms of stimulation in germination and growth. One of these mechanisms is involved in the production of phytohormones, as the most important direct growth-promoting mechanism through which a bacterium can

influence plant physiology (16). This is because in extremely small concentrations they are able to influence the biochemical, physiological and morphological processes of plants (17). *Pseudoxanthomona indica* H32 used in this study for seed and soil treatment could contribute to these purposes. As described, IAA is the best-studied phytohormone produced by rhizobacteria. This auxin regulates important processes in plants such as cell division, elongation and differentiation, apical dominance, increase of root hairs, tropic responses, flowering and senescence (4, 18). *P. indica* H32 on liquid LB medium was cultured and were determined the presence of indole compounds in the medium at levels of 3.73 mg L^{-1} in the stationary phase of the culture solely from the amino acid tryptophan present in LB medium. This medium was supplemented with tryptophan the IAA concentration produced rose to values of 12 mg L_{-1}. It was observed that the production of IAA by *P. indica* H32 was not associated with growth, since it is produced in low amounts during all stages of the culture, but the highest concentrations were obtained in the stationary phase at six days of culture. This is because the factors that modify IAA synthesis are diverse, including acidification of the medium, osmotic stress, and carbon source limitation (19), characteristics related to the physiological state of the bacteria that reach the stationary phase of the culture, Figure 1.

Pseudoxanthomonas indica H32 and Pseudomonas aeruginosa ATCC were seeded on Pikovskaya-agar culture medium. In addition, after 72 hours, a translucent area was observed around the colonies, a fact that qualitatively indicated phosphate solubilization. *P. indica* H32 had a mean solubilization index (SI) value of 2.71, similar to the SI=2.63 value of *P. aeruginosa* ATCC 27853, according to the t-Student test, (P > 0.05). In general, *P. indica* H32 stimulates plant growth from its potentialities to produce IAA. In addition, because of its property to solubilize phosphates and as a consequence facilitate the plant to extract nutrients from the soil in a more efficient way (20, 21). This coincides with the dual effect of some biofertilizers, mediated by the solubilization of inorganic phosphorus, the mineralization of organic phosphorus and the positive effect on the stimulation of the development of the plant's root system or the formation of mycorrhizae (2). On the other hand, the concentration of inoculated *P. indica* H32 (105 CFU mL^{-1}) (22) stimulated the germination of crops studied. The highest values were observed in inoculated seeds. These results infer that H32 could also synthesize active gibberellins, phytohormones that stimulate the activity of α-amylases and other germination-specific enzymes such as proteases and nucleases involved in the hydrolysis and assimilation of accumulated starch in seeds, Table 1.

In the greenhouse trial, a greater number of seedlings emerged in the plots where seeds inoculated with H32 were sown. The following tables summarize the results of all parameters evaluated and their statistical analysis. Inoculation of radish seeds with strain H32 produced plants with higher wet mass and bulbs of greater development. A

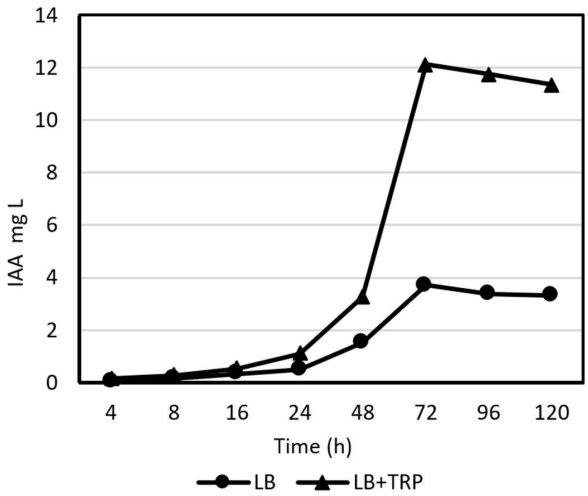

Figure 1. Mean values of IAA production by *P. indica* during culture at 37 °C and 200 rpm in LB liquid medium with and without tryptophan (n = 3)

Table 1. Effect of *P. indica* H32 on *in vitro* mean daily germination (MDG)

Seeds	MDG (% G day^{-1}). N = 90		Test -t
	non-inoculated	H32- inoculated	Values of P and α
radish	15.84 ± 0.835 (a)	24.59 ± 0.415 (b)	P = 0.0111662 α = 5 %
carrot	8.223 ± 0.223 (a)	9.63 ± 0.2136 (b)	P = 0.0104048 α = 5 %
chard	11.39 ± 0.14 (a)	12.36 ± 0.14 (b)	P = 0.00804829 α = 1 %

Mean values ± standard error. Comparison with the non-inoculated seed group by Student's t-test.
Unequal letters indicate significant differences

similar result was obtained in the carrot plot inoculated with H32, where the main roots had significantly higher mass compared to the controls. In the chard seed plot inoculated with H32, when calculating plant wet mass, height, number of leaves and leaf width and length, it was observed that also the mean values of all these parameters were significantly higher than the mean values achieved in non-inoculated plants. The yields of all the plots inoculated with H32 showed an increase with respect to the non-inoculated plots, especially the chard and carrot plots, which increased to values above 50 %, Tables 2 and 3.

The application of H32 in the plots showed its influence on root formation and development and plant growth. In addition, H32 remained in the soil at concentration values of $3x10^4$ CFU g^{-1}, even five weeks after its application. This fact can be attributed to the ability of H32 to colonize the root, produce the auxin IAA and cause phosphate solubilization, coinciding with what was previously reported (23,24),. They argue that soil inoculation with beneficial rhizobacteria that have this characteristic, improves the development of the root system and the assimilation of nutrients, such as Ca, K, Fe, Cu, Mn and Zn, by the plant.

Table 2. Mean values and standard error of germination percentage and parameters evaluated in radish, chard and carrot plants grown in greenhouses

Parameters	Non-inoculated	H32-inoculated	Increasing (%)	Test -t Values P. and α
Radish plants				
Germination (%) (N=100)	90.3 ± 1.4224 (a)	98.1 ± 0.4932 (b)	8.64	P= 0.00660239 α = 1 %
Wet mass (g)	166.68 ± 4.907 (a)	181.72 ± 5.526 (b)	9.03	P= 0.045164 α = 5 %
Bulb mass (g)	101.77 ± 3.593 (a)	120.18 ± 4.097 (b)	18.09	P= 0.0011404 α = 1 %
Bulb diameter (cm)	5.133 ± 0.096 (a)	5.629 ± 0.098 (b)	9.7	P= 0.000536 α = 0.1 %
Chard plants				
Germination (%) (N=100)	75.0 ± 6.3430 (a)	90 ± 3.5008 (b)	20.01	P= 0.0451243 α = 5 %
Wet mass (g)	238.44±24.5851 (a)	374.95 ± 22.001 (b)	57.25	P= 0.0001873 α = 0.01 %
Height (cm)	31.02 ± 0.789 (a)	36.31 ± 0.873 (b)	18.28	P= 0.0000636 α = 0.01 %
Leaf number	16.6 ± 0.426 (a)	20.85 ± 0.612 (b)	25.60	P= 0.00000147 α = 0.01 %
Leaf length (cm)	18.84 ± 0.655 (a)	23.129 ± 0.351 (b)	22.77	P= 0.00000400 α = 0.01 %
Leaf width (cm)	14.57 ± 0.519 (a)	19.05 ± 0.586 (b)	30.75	P= 0.00000137 α = 0.01 %
Carrot plants				
Germination (%) (N=100)	81.23 ± 1.2333 (a)	87.32 ± 1.3955 (b)	7.5	P= 0.030791 α = 5.0 %
Wet mass (g)	87.34 ± 5.885 (a)	137. 01 ± 6.837 (b)	56.86	P= 0.00000271 α = 0.01 %
Root mass (g)	46.0 ± 4.121 (a)	70.11 ± 4.630 (b)	52.41	P= 0.0003911 α = 0.1 %
Main root length (cm)	10.17 ± 0.322 (a)	12.15 ± 0.332 (b)	19.42	P= 0.0001249 α = 0.1 %
Leaf and stem mass (g)	41.34 ± 2.926 (a)	66.90 ± 4.455 (b)	61.83	P= 0.0000250 α = 0.01 %

N=40, radish and chard plants. N=20, for carrot plants. Increase of the parameters in plants inoculated with *P. indica* H32, in relation to non-inoculated plants. Unequal letters indicate significant differences between means

Table 3. Yield in plots of plants inoculated and non-inoculated with *P. indica* H32. Yield increase in inoculated plots compared to non-inoculated plots

Crops	Yield (kg) (non-inoculated)	Yield (kg) (inoculated)	Increasing (%)
Radish	15.0	17.8	19.0
Chard	17.883	33.745	88.70
carrot	7.07	11.92	68.5

CONCLUSIONS

The results of this research with *Pseudoxanthomona indica* H32 contribute to the knowledge of new autochthonous microorganisms with potential in plant growth promotion. Thanks to its characteristics, it can be used in the formulation of biofertilizer products with the objective of improving the quality and yield of crops, substituting or minimizing the use of chemical products according to the needs of farmers. In general, its use would bring as benefit improvements in the economy, contributing to increase crop productivity and preserving the environment.

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