Article

Effect of microcapsules of *Pseudomonas putida* on growth and yield of red pepper

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Abstract

The use of beneficial microorganisms such as rhizobacteria can be an alternative to promote plant growth, plant productivity and improve soil fertility; in addition, not to contaminate the environment, be easy to apply and low cost. The objective of the present work was to evaluate the effect of the inoculation of microcapsules and liquid culture of three strains of *Pseudomonas putida* on the growth and yield of red pepper in greenhouse. Strains FA-8, FA-56 and FA-60 of *P. putida* were used individually and in combination, which were inoculated directly onto the root by means of microcapsules and liquid suspension. The experiment was carried out in a greenhouse with a randomized block arrangement with nine treatments and four repetitions. The results indicate significant differences between the incorporation of microcapsules and liquid culture of rhizobacteria in the plants of red pepper var. California Wonder, highlighting the microcapsules of strain FA-56 with significant increases in height, root volume, dry biomass, fruit yield, soluble solids content (°Brix) and CFU. The immobilization of *P. putida* cells by means of microcapsules gives them protection and gradual release, improving the adhesion and colonization of the rhizobacteria on the roots, inducing better effect in the promotion of the growth and productivity of the red pepper plants, which can be a viable alternative as biofertilizer for the sustainable production of the crop.

Keywords: *Capsicum annuum* L., rhizobacteria, alginate microcapsules, UFC.

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Introduction

The production of vegetables is a very important activity for the global and national agricultural sector, among the horticultural crops of economic and nutritional interest for its fresh or processed consumption. The red pepper (*Capsicum annuum* L.) is in seventh place worldwide (FAO, 2015, Acevedo-Chávez and Sánchez-Chávez, 2017). In Mexico, the production of red pepper has shown an increase due mainly to its export to the United States and Canada, which generates a significant amount of foreign currency, nationally the cultivated area of this vegetable is 2 641.43 ha with a yield average of 42.08 t ha^{-1}, which represents an economic value of 519 321.21 million pesos (SIAP-SAGARPA, 2015, Acevedo-Chávez and Sánchez-Chávez, 2017).

Due to the implementation of intensive agricultural production schemes, such as that used in the cultivation of red pepper to obtain greater volumes of fruit, the constant and excessive use of chemical fertilizers has been promoted, which besides being costly are highly polluting for soil, water and human health (Sánchez *et al*., 2012; Schulz and Glaser, 2012).

Faced with this scenario of intensive agriculture based on chemical and expensive nutritional inputs, the growing use of growth promoting rhizobacteria in plants (PGPR), is promoted by its ability to directly or indirectly induce root health and growth and foliage of plants through the production of growth regulators such as auxins, gibberellins and cytosines (Lugtenberg and Kamilova, 2009; Belimov *et al*., 2015), fixation of atmospheric N_2, solubilization of insoluble phosphorus (Naz and Bano, 2010; Zaidi *et al*., 2015; Pankievicz *et al*., 2015), synthesis of siderophores, antibiotics, antifungal metabolites, salicylic acid, cyanide (Adhikari *et al*., 2013; Sunar *et al*., 2013; Kamou *et al*., 2015) and induction of resistance in plants (Bakker *et al*., 2013; Vacheron *et al*., 2013). Among the diversity of PGPR are a large number of species grouped in the genera *Pseudomonas*, *Azospirillum*, *Burkholderia*, *Serratia*, *Azotobacter*, *Enterobacter*, *Streptomyces*, *Bacillus*, *Rhizobium*, among others (Berg, 2009, Adhikari *et al*., 2013, Kamou *et al*., 2015).

The form of inoculation of rhizobacteria on plants is versatile and simple, since they can be applied to the seed, root or soil (Shen *et al*., 2013; Cordero-Ramírez *et al*., 2013; Zaidi *et al*., 2015). The response of plants to inoculation varies considerably and is a function of the species of rhizobacteria, the host, soil type, environmental conditions, inoculum concentration and mode of inoculation (Becerra-Castro *et al*., 2011; Conejo *et al*., 2014). The methods of inoculation of the PGPR are determinant in the process of colonization, activity and permanence of the bacterial cells in the rhizosphere of the plants, besides indirectly influencing the mechanisms of action that promote growth in plants (Krzyzanowska *et al*., 2012; He *et al*., 2016).

Among the methods of inoculation of the rhizobacteria, the microcapsules stand out, which have proven to be more efficient than the liquid form, due to their quality of providing protection and stability to the bacterial cells, allowing them to survive for longer in the rhizosphere of the cells. plants (Bashan *et al*., 2014; Schoebitz Belchí, 2016). In this regard, Sivakumar *et al*. (2014) when

evaluating the effect of *Bacillus megaterium* microcapsules supplemented with humic acids in rice plants, report outstanding results in the growth of plants, highlighting the action of humic acids in microcapsules by providing chemical stability and availability of C and N, generating a greater number of bacterial cells.

While Abo-Kora *et al*. (2016) when inoculating tomato plants with microcapsules and bacterial suspension of *Pseudomonas fluorescens*, *Azotobacter chroorcoccum*, *Bacillus polymyxa* and *Azospirillum brasilense*, found that both forms of inoculation generated favorable effects on the growth of plants, with the standing out microcapsules of *P. fluorescens* and *A. Brazilian* in plant height and root length, respectively. While Schoebitz *et al*. (2013) when evaluating in wheat plants inoculated with microcapsules based on sodium alginate and a combination of potato starch with sodium alginate, containing in both formulations the rhizobacterial strains *P. fluorescens* and *Serratia* sp. report that the rhizobacteria in both presentations promoted plant height, dry biomass and P foliar content, so they point out that the microcapsule method improves the effect of rhizobacteria by acting as mini-reactors that give bacterial cells stabilization, protection, population increase and progressive release around the rhizosphere of the plants where they are applied (Malusa *et al*., 2012; Schoebitz *et al*., 2013).

Therefore, knowing the effects of different methods of inoculation of rhizobacterial species, can help in the development of efficient biofertilization programs that facilitate rhizobacterial cells expression of their qualities as growth promoters and productivity in plants. Therefore, the objective of the present work was to evaluate the effect of the inoculation of microcapsules and liquid culture of three strains of *Pseudomonas putida* on the growth and yield of red pepper in greenhouse.

Materials and methods

Study site

The study was conducted from june to october 2015, in a 160 m^2 greenhouse type tunnel with lateral ventilation and low level of technology, located in the Faculty of Agricultural Sciences Campus Xalapa of the University Veracruzana at coordinates 19° 30’ north latitude and 96° 55’ west latitude, at an altitude of 1 450 meters above sea level, in Xalapa, Veracruz, Mexico.

According to the Köeppen classification modified by Garcia (1981), the climate is temperate-humid with year-round rainfall C(fm)w”b(i’)g. The average annual temperature is 18 °C and rainfall of 1 509 mm, with abundant rainfall in summer and early autumn.

Rhizobacteria and culture medium

The strains of *Pseudomonas putida* used in this study are cataloged as FA-8, FA-56 and FA-60, which were provided by the Agricultural Chemistry Laboratory of the Campus Xalapa Agricultural Sciences Faculty of the University Veracruzana. Strains were cultured in B-King

liquid medium (glycerol 10 ml L^{-1}, peptone 15 g L^{-1}, magnesium sulfate 1.0 M 1 ml L^{-1} and potassium phosphate dibasic 1.5 g L^{-1}) for 48 ha 28 °C. The concentration of each rhizobacteria was adjusted to 10^9 mL^{-1} cells by means of a spectrophotometer (Thermo Spectronic model 4001/1) at a wavelength of 660 nm and absorbance of 1.

Preparation of alginate microcapsules with *Pseudomonas putida*

Of the rhizobacterial cultures previously grown in B-King medium, 100 ml of each strain of *P. putida* was taken and mixed with 2.5 g of sodium alginate and agitated for 25 min at 350 rpm on a magnetic stirrer (IKA® modelo C-MAG). The microcapsules of alginate containing the strains individually and combined, were made by drop formation with a transparent polyethylene Pasteur pipette of 5 ml capacity, with which the alginate mixture was taken with bacterial culture and drops were formed that were deposited in a sterile 0.1 M of $CaCl_2$ solution. Agitated for 30 min at 100 rpm on a magnetic stirrer to promote the gelation of the microcapsules, they were removed from the $CaCl_2$ solution and washed three times with sterile 0.85% NaCl saline (p/v). A group of microcapsules called MIX_{mc} consisted of mixing the three rhizobacteria in equal volumes until obtaining 100 ml of culture. Finally, alginate microcapsules of approximately 4 mm in diameter were preserved in sterile 0.1 M of $CaCl_2$ solution at room temperature for 72 h until inoculated into the plants.

Production of red pepper seedlings

Seeds of red pepper (*Capsicum annuum* L.) var. California Wonder (Hortaflor), productive plant with habit of determined growth and semi-precocious cycle with thick and sweet square fruits. For the production of seedlings, a 200-well polystyrene germination tray (2.5 x 2.5 x 6 cm) disinfected with 3% sodium hypochlorite solution was filled with a mixture of substrates based on vermicompost, tepetzil and sand. (2:1:1 v/v), sterilized with a sanitizing solution and liquid disinfectant of Anibac 580® (i. a. quaternary ammonium [1st generation] at 8.6% and quaternary ammonium [double chain] at 3.7%) in doses of 5 ml L^{-1}. One seed per cavity was placed at a depth of 1 cm, the germinating tray was kept in a greenhouse for 35 days at an average temperature of 26 °C and a relative humidity of 60%, until obtaining seedlings with an average height of 17 cm at the time of transplanting.

Transplant and application of microcapsules and liquid culture of *P. putida* in red pepper

Prior to the transplant, the roots of the red pepper seedlings were washed, then at the time of the transplant, 50 microcapsules (mc) of sodium alginate (equivalent to 5 ml of bacterial liquid culture at the concentration of 10^9 cells mL^{-1}) and in another group 5 ml of the rhizobacterial liquid culture (cl) of each strain was applied to the root. For both forms of application, a group of seedlings was inoculated with the combination of the three rhizobacterial strains called MIX. A randomized block experimental design was used with nine treatments: FA-8_{mc}, FA-56_{mc}, FA-60_{mc}, MIX_{mc}, FA-8_{cl}, FA-56_{cl}, FA-60_{cl}, MIX_{cl} and control (plants without inoculation) with four repetitions and two plants per experimental unit.

The plants were kept in a greenhouse for 100 days in black polyethylene bags of 8 kg capacity, containing as substrate 6 kg of tepetzil, previously disinfected with liquid solution of Anibac 580® in a dose of 5 ml L^{-1}. During the experiment an average temperature of 26 °C and 60% relative

humidity was recorded. All the plants were fertilized from the transplant until the end of the experiment with nutrient solution (g L^{-1}) composed of: $Ca(NO_3)_2$ $4H_2O$ (1.43), $Mg(NO_3)_2$ (0.95), KNO_3 (0.38), KH_2PO_4 (0.35) and micronutrients Tradecorp®AZ [Fe, Zn, Mg, B, Cu y Mo] (0.03), adjusting the pH of the solution to 6 At the end of the experiment the height, stem diameter, root volume, root length, fresh and dry biomass and colony forming units in fresh roots (UFC), for fruit yield and total soluble solids content (°Brix) in ripe fruit juice (two fruits per plant) quantification was carried out in six harvests during the last three weeks of the crop cycle.

Rhizobacterial population in root

To determine the rhizobacterial populations by means of colony forming units (UFC), at the end of the experiment a 3 g sample of fresh root was collected from the inoculated plants with each rhizobacteria and control, the roots were placed in a Petri dish with sterile saline solution NaCl at 0.85% (p/v). Subsequently, according to the methodology proposed by Holguin and Bashan (1996) the roots were macerated by means of a sterile glass rod, 1 mL of the sample was collected and deposited in a test tube containing 9 mL of sterile saline solution. 0.85% (p/v) of which serial dilutions were made for plate count with B-King solid culture medium in triplicate and incubated for 72 h at 28 °C. The population of each rhizobacterial strain was expressed as UFC 10^8 g^{-1} root (Gamalero *et al*., 2002).

Statistical analysis

The data obtained were processed through the analysis of variance and the multiple comparison test of Duncan means ($p< 0.05$) with the statistical program SAS version 9.4 for Windows.

Results and discussion

The obtained results indicate that the red pepper plants inoculated with the microcapsules and liquid culture of the three rhizobacterial strains individually and combined showed significant differences ($p< 0.05$) in the morphological variables of the plant, yield and content of total soluble solids of fruit (Table 1). The strain FA-56_{mc} applied to the red pepper plants by means of microcapsules of alginate significantly increased ($p< 0.05$) the height, root volume, dry biomass of the plant and fruit yield in 31.50, 32.56, 71.06 and 45.10% respectively, in comparison with the control plants. The plants inoculated with the strain FA-60mc and the combination of the three strains (MIX_{mc}) applied in microcapsules obtained significant increases ($p< 0.05$) for root length and stem diameter of 32.56 and 29.89% respectively, compared with plants without inoculation.

In contrast, for the fresh plant biomass variable, the treatment that showed the best response with respect to the control was the strain FA-60_{cl} in liquid form, obtaining a significant increase of 30.2% ($p< 0.05$). Regarding the percentage content of total soluble solids (°Brix) in mature fruits of red pepper, there are significant differences ($P< 0.05$) between treatments, highlighting the strain FA-56 applied by microcapsules of alginate and liquid culture with increments of 12.5% in both cases in relation to the fruits of control plants.

The use of rhizobacterial strains of different genera such as *Pseudomonas*, *Bacillus*, *Azospirillum*, *Serratia*, among others, have been widely studied, particularly for their quality to promote vegetative growth and productivity in red pepper crops (Gupta *et al*., 2015), tomato (Agrawal and Agrawal, 2013), rice (García *et al*., 2010), soybeans (Husen *et al*., 2011), maize (Hungria *et al*., 2010) and potatoes (Arseneault *et al*., 2015).

Table 1. Effect of microcapsules and liquid inoculation of three strains of *P. putida* on the growth and productivity of red pepper plants var. California Wonder in greenhouse.

Treatments	Height (cm)	Stem diameter (mm)	Radical length (cm)	Radical volume (cm^3)	Fresh biomass (g)	Dry biomass (g)	Fruit yield (g)	Soluble solids °Brix (%)
Cepa FA-8_{mc}*	116.25 ab	13.16 ab	38.75 ab	102.38 ab	340 ab	100.75 ab	363.35 ab	8.6 bc
Cepa FA-56_{mc}	123.63 a	13.99 a	40.85 ab	107.13 a	334.13 ab	113.5 a	427.5 a	9 a
Cepa FA-60_{mc}	112.13 ab	12.64 ab	44.25 a	90.75 abc	314.5 ab	99.88 ab	318.88 b	8.5 cd
MIX_{mc}†	96.88 b	14.08 a	40 ab	87.5 bc	302.55 bc	90.4 ab	381.75 ab	8.5 cd
Cepa FA-8_{cl}‡	112.63 ab	13.58 ab	37 ab	88.5 bc	280.5 cd	74.5 bc	309.68 b	8.5 cd
Cepa FA-56_{cl}	104.38 ab	12.25 ab	41.88 ab	96 abc	288.75 cd	80.8 bc	336.63 ab	9 a
Cepa FA-60_{cl}	110.88 ab	13.42 ab	38.13 ab	89.75 bc	343.35 a	106 ab	321.35 b	8.6 bc
MIX_{cl}	99 ab	12.32 ab	35.88 ab	84.63 cd	259 d	64.5 c	326.8 ab	8.5 cd
Testigo	94 b	10.84 b	33.38 b	74.5 d	263.7 d	66.35 c	294.63 b	8 d

*= alginate microcapsules (mc); †= MIX= mixture of the three rhizobacterial strains of *P. putida*; ‡= liquid bacterial culture (cl). Average values within the same column with different letters show significant differences with the Duncan test ($p< 0.05$).

This stimulating activity is related to the ability of rhizobacteria to synthesize plant growth regulators such as auxins, gibberellins, cytokinins and vitamins, antagonistic metabolites such as siderophores and hydrocyanic acid, as well as for their ability to facilitate the assimilation of nutrients through the solubilization of phosphorus, biological fixation of atmospheric nitrogen and ion chelation (Aguado-Santacruz *et al*., 2012; Vacheron *et al*., 2013; Ahemad and Kibret, 2014; Bashan *et al*., 2014).

Regarding Sivakumar *et al*. (2014) when evaluating the effect of *Bacillus megaterium* by microcapsules of alginate supplemented with humic acids in rice plants, indicate having obtained differences in seed germination, root length and stem height in relation to the control plants, also mention that the growth of rice plants was stimulated by the metabolic activity of the *B. megaterium* strain; however, the addition of humic acids in the microcapsules provided chemical stability and an organic nutritional source of C and N, which maintained a higher number of live bacterial cells. For Ahirwar *et al*. (2015) the inoculation of the SS5 strain of *Pseudomonas fluorescens* in tomato plants showed significant increases in root length, plant height and fruit yield, indicating that the plants inoculated with the SS5 strain in pot and field surpassed the control plants in 19 and 57% yield, respectively.

On the other hand, Abo-Kora *et al*. (2016) inoculated tomato plants with microcapsules of alginate and bacterial suspension based on *Pseudomonas fluorescens, Azotobacter chroorcoccum, Bacillus polymyxa* and *Azospirillum brasilense*, finding favorable effects on the growth of plants with both forms of inoculation of the rhizobacterial strains, excelling the microcapsules of *P. fluorescens* and *A. brasilense*. In this regard Schoebitz *et al*. (2013) when inoculating wheat plants with microcapsules of sodium alginate and potato starch combined with sodium alginate, both combinations with strains of *Pseudomonas fluorescens* and *Serratia* sp., Found that these significantly promote plant height, dry biomass and foliar content of P, attributing that encapsulates improve the effect of rhizobacteria by acting as mini-reactors that give bacterial cells stabilization, protection, population increase and progressive release around the rhizosphere of the plants where they are applied.

In lettuce plants Rekha *et al*. (2007) applied microcapsules of sodium alginate and liquid suspension containing the rhizobacteria of the species *P. putida* CC-FR2-4 and *B. subtilis* CC-pg104, in both forms of inoculation the plants treated with the strains showed significant increases in length radical and stem height in relation to the control plants, conclude that the inoculation of the lettuce plants with *P. putida* and *B. subtilis* by means of alginate encapsulates vegetative growth is promoted in a similar way to the liquid suspension, so which the technique of encapsulation of rhizobacteria could be feasible for its use in the agricultural industry of biofertilizers.

Regarding the content of soluble total solids °Brix, some reports attribute their increase in fruits from biofertilized plants due to the capacity of the rhizobacteria to facilitate the assimilation of essential nutrients and stimulation by means of growth regulators, such metabolic activities propitiated directly by the action of the rhizobacteria induce the production of ethylene (Gamalero and Glick, 2015), which promotes the synthesis of sugar-reducing enzymes present in the cell wall of the fruit, generating simple sugars that increase the concentration of total soluble solids (°Brix) in the fruits during the maturation process (Ordookhani and Zare, 2011; Vázquez-Ovando *et al*., 2012).

The presence of rhizobacterial populations (UFC) quantified in roots of red pepper plants, inoculated with microcapsules of alginate and liquid culture with strains of *P. putida* showed significant differences ($p< 0.05$) between treatments (Figure 1). For microcapsules, the highest population of UFC (10^8 g^{-1} of fresh root tissue) was found in the three strains individually and in combination, highlighting the population obtained from strain FA-56 with 590 UFC (10^8 g^{-1}). This same strain also stood out in the condition of liquid bacterial culture when obtaining the highest population number with 492 UFC (10^8 g^{-1}). Research conducted on the use of rhizobacteria, indicate that the population rate of bacterial cells present in the rhizosphere of plants depends on the organic compounds produced by the radical exudates of these, among which are amino acids, organic acids, phenols, phytohormones (auxins, gibberellins and cytokinins), sugars, vitamins and enzymes (Berg, 2009; Adhikari *et al*., 2013; Bashan *et al*., 2014).

The quantity and quality of the radical exudates promotes in the rhizobacteria the competition for these metabolites, as well as for the place they occupy on the root of the plant, being the junctions between the epidermal cells and the area where the roots emerge with greater attraction, adhesion, activity and microbial population (Lugtenberg and Kamilova, 2009, Vacheron *et al*., 2013).

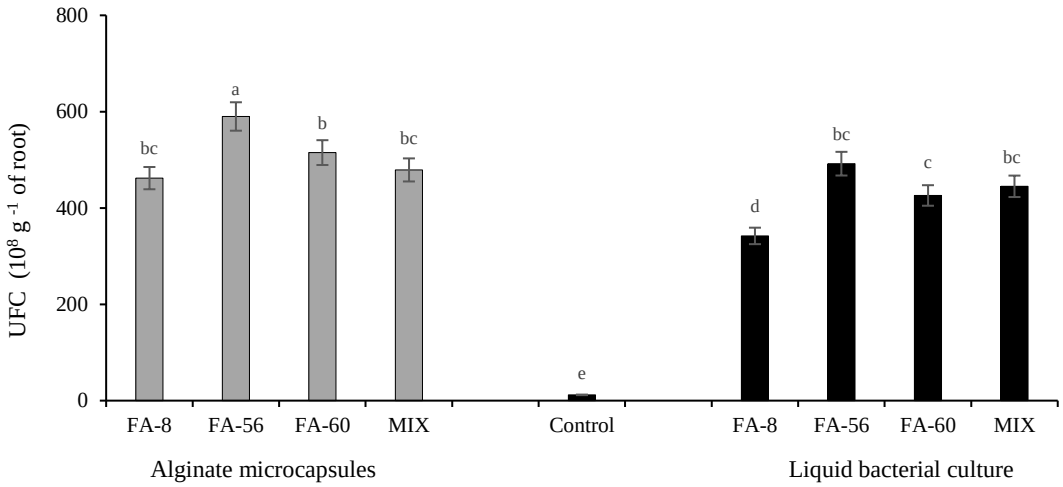

Figure 1. Colony forming units (UFC) quantified in root of red pepper plants var. California Wonder, inoculated with alginate microcapsules and liquid culture based on *P. putida* strains FA-8, FA-56, FA-60, MIX (mixture of the three strains) and control (plants without rhizobacteria only inorganic fertilization) at 100 days after the transplant in the greenhouse. Different letters on the bars show significant differences according to Duncan's multiple range test ($p< 0.05$).

Conclusions

The response of the red pepper plants inoculated with the FA-56 strain in the form of microcapsules showed the greatest increases in height, root volume, dry biomass, yield and content of soluble fruit solids. The same strain encapsulated and in liquid suspension had the largest rhizobacterial population in root (UFC).

The UFC populations determined in the microcapsule treatments indicate the possibility that the application of *P. putida* rhizobacteria by means of the microencapsulation technique facilitates the gradual release of the cells, improving their adhesion and colonization in the roots, inducing better effect as promoter agents of plant growth, which can be a viable alternative as biofertilizer for the cultivation of red pepper and its sustainable production.

More studies are needed to corroborate the potential of the strains of *P. putida* microencapsulated for their use as agents that stimulate the growth and productivity of red pepper plants.

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