Effect of *Pseudomonas Fluorescens* in the Germination and Growth of *Prosopis Laevigata* under Greenhouse Conditions

Abdul Khalil Gardezi[1*], Rolando Trejo-Pérez[2], Guillermo Carrillo-Castañeda[3], Héctor Flores-Magdaleno[4], Sergio Roberto Márquez Berber[5], Eduardo Valdés Velarde[6], Manuel Ortega Escobar[7], Mario Ulises Larqué-Saavedra[8], Gabriel Haro Aguilar[9].

[1,4,7,9]Postgraduate College, Agricultural Science, Department of Hydrosciences, Montecillo Campus, Km. 36.5 Carretera Fed. México-Texcoco, Montecillo, Texcoco, Estado de México, México, 56230.

[2]Institut de recherche en biologievégétale (IRBV) de l'Université de Montréal. 4101 Sherbrooke Est, Montréal, QC H1X 2B2, Canada

[3]Postgraduate College, Genetic Resources and Productivity Area of Molecular Genetics, Montecillo Campus, Km. 36.5 Carretera Fed. México-Texcoco, Texcoco, Estado de México, México, 56230.

[5]Crop Science Department, Chapingo Autonomous University, Km 38.5 Carretera Fed. México-Texcoco s/n, Col. Chapingo, Texcoco, Estado de México, Mexico, 56230.

[6]Agroforestry Center for Sustainable Development,Chapingo Autonomous University, Km 38.5 Carretera Fed. México-Texcoco s/n, Col. Chapingo, Texcoco, Estado de México,Mexico, 56230.

[8]Metropolitan AutonomousUniversity, Azcapotzalco. Mexico.

[*1]Corresponding author: Dr. Abdul Khalil Gardezi, Postgraduate College, Agricultural Sciences, Department of Hydrosciences, Montecillo Campus, Km. 36.5 Carretera Fed. México-Texcoco, Texcoco, Estado de México, México, C.P. 56230. Tel. (595) 9520200 ext. 1165, 1167 or 1171

Abstract— *Mesquite (Prosopis laevigata) is a tree of arid and semi-arid areas of northern and central Mexico. This species allows erosion control, atmospheric nitrogen fixation, and improves soil quality. Pseudomonas fluorescens is a rhizobacterium that favors plant growth-promoting rhizobacteria (PGPR). Also, promotes seed germination and development of Mesquite plants under adverse environmental conditions.*

The aim is to evaluate the role of bacterial strains A7 and Sv of P. fluorescens, using two types of soil (vertisol and phaeozem), and adding vermicompost (0, 25, 50, 75 and 100 tons/ha) in the germination and growth stages of mesquite (Prosopis laevigata).We tested the characteristics developed by the plants over 180 days. A randomized experimental design with four repetitions was used to test the seed germination rate and 16 more variables in the greenhouse, such as morphology, dry biomass accumulated, and morphological indices through the randomized factorial experimental design with three factors, 2x3x5x3.

Regarding the control treatment, the use of the bacterial strain A7 of P. fluorescens inhibited the germination of mesquite seeds, while the strain Sv favored seedlings development. We observed opposite effects; inhibition and growth in the germination stage, and development of the seedlings observed at 180 days when using the A7 and Sv strains of P. fluorescens.

Keywords*— *Arid and semi-arid areas, Bacterial strain, Biofertilizer, Mesquite, Plant growth-promoting Rhizobacteria (PGPR), Pseudomonas fluorescens, Vermicompost.

I. INTRODUCTION

The mesquite [*Prosopis laevigata* (Humb. Et Bonpl ex Wild.) M.C. Johnst], a multi-purpose tree (Rodríguez-Sauceda *et al.*, 2014), is used as a source of energy, a natural barrier, feed for livestock, getting gums and for medicinal (Prieto-Ruíz *et al.*, 2013). In addition, it has important ecological functions because it allows erosion control, atmospheric nitrogen fixation and improves soil quality (Stanton *et al.*, 2001; Buendía-González *et al.*, 2012; Palacios-Romero *et al.*, 2017). All these characteristics make it a species of interest to exploit and cultivate in arid and semi-arid areas, since according to Villanueva-Díaz *et al.*, (2004), cited by Palacios-Romero *et al.*, (2017), this woody species has a wide distribution in arid and semi-arid areas of northern and central Mexico.

However, cultivating species in arid and semi-arid environments requires generating new technologies (Prieto-Ruíz *et al.*, 2013). Mia *et al.*, (2012) stated that the development of any plant species depends on various factors such as the vigor of the seed for effective germination, and the rapid establishment of the plant (Bécquer *et al.*, 2013). In this sense, with the purpose of new production options, the use of plant growth-promoting microorganisms (PGPM) as biofertilizers (Afzal and Bano, 2008) they considered a solution and an alternative to promote plant growth and nutrition (Vessey, 2003; Jaiswal *et al.*, 2016). In fact, the use of such fertilization sources has attracted the attention of researchers because of their success in crop development and their low ecological footprint (Egamberdieva, 2008; Karakurt *et al.*, 2011; Radhapriya *et al.*, 2015) in relation to chemical fertilizers (Vessey, 2003; Dadrasan *et al.*, 2015).

Among the plant growth-promoting microorganisms, it gives great emphasis to the study of the group of fluorescent Pseudomonas bacteria, either to increase agricultural production or for its various related benefits by the association of bacteria on plant roots (Carrillo-Castañeda *et al.*, 2000; 2011). For leguminous species, many authors report that *P. fluorescens,* selected as plant growth-promoting Rhizobacteria, are a source of nutrients, where plant root colonization (Siddiqui *et al.*, 2001; Egamberdieva *et al.*, 2013) stimulates growth (Vessey, 2003) and direct or indirect production (Shaukat *et al.*, 2006, Afzal and Bano, 2008; Iqbal *et al.*, 2012).

They have shown the plant growth-promoting capacity of bacteria of the genus *Pseudomonas* through the "biofertilizing effect" and also for its ability to antagonize multiple pathogenic microorganisms (Pal *et al.*, 2001). In addition, they have considered that natural reforestation in eroded areas of the desert is very difficult because, in these degraded soils, the surface layer of the soil has lost the microorganisms that promote the development of plants. That is why, by artificially reforesting these areas, we recommend inoculating the seeds of the plants with microorganisms to return to the soil part of their fertility and potential for the development of plants. Drezner, (2006) mentioned that only good irrigation with water will not restore fertility and soil microbial communities.

That is why we considered in this work pertinent to determine the specificity and positive or negative effects of bacterial cells associated with mesquite seeds and roots as an ecological strategy to promote the growth of plants within an utilization perspective of natural and biological fertilizers. Thus, in this study, we tested the significant importance of an inoculation with cells of the bacterial strains A7 and Sv of *P. fluorescens* in two physiological stages, germination, and the development of mesquite. In addition, we suggested two hypotheses. The first is that the use of bacterial strains favors the germination process of mesquite. Meanwhile, the second one is that the use of these plant growth-promoting microorganisms favors the initial development stage of this tree species.

II. MATERIALS AND METHODS

2.1 Soil collection site

The soils used originate from two agricultural production sites, within the low micro-basin of the Rio Grande, Tulancingo, state of Hidalgo, northeast of Mexico City. The geographical coordinates and the surface of the collection sites (soil and soil samples) were 20° 11'39.96' 'N, 98° 26' 38.62" W and 0.86 ha for the vertisol soil, while for the Phaeozem soil was 20° 12'27.18' 'N, 98° 27' 21.50" W and 1.18 ha. We carried out the soil classification according to the edaphological vector data set, scale 1: 250 000 series II of the INEGI (Valdez-Pérez *et al.*, 2016).

We collected the soils in November 2016. Each sampling site comprised one hundred and twenty soil samples, with two hundred and forty samples, with forty sub-samples for section 0-5 cm deep, forty for section 5-10 cm and forty for that corresponding to 10-40 cm (Gardezi *et al.*, 2009). We describe the physicochemical characteristics in (Table 1), which shows that phosphorus and nitrogen levels were three times higher in the vertisol soil in relation to the phaeozem soil, than and twice as important as potassium.

2.2 Vermicompost used as organic matter

We prepared the vermicompost, used as organic matter, with 60 kg of bovine manure (Bos taurus), 25 kg of melon residues (*Cucumis melo*) and 15 kg of wheat residues (*Triticum* sp.), which interacted with Californian earthworm (*Eisenia fetida*) during the months of September to December 2016. Table 1 includes the physicochemical characteristics of vermicompost.

TABLE 1
PHYSICAL-CHEMICAL PROPERTIES OF VERMICOMPOST AND TWO AGRICULTURAL SOILS OF THE LOW MICRO-BASIN OF THE RIO GRANDE, TULANCINGO, MEXICO

Properties	Vermicompost	Soil samples					
		Vertisol			Phaeozem		
		0-5 cm	5-10 cm	10-40 cm	0-5 cm	5-10 cm	10-40 cm
Organic matter (%)	7.93	5.51	3.03	3.63	3.36	3.16	3.23
pH	7.08	6.99	7.16	7.27	6.39	6.28	6.35
Electrical conductivity dSm^{-1}	2.52	0.14	0.12	0.12	0.05	0.06	0.05
Total Nitrogen (N)%	0.34	0.27	0.06	0.13	0.11	0.06	0.07
Assimilable Phosphorus (P) mg kg^{-1}	145.68	39.77	28.85	26.36	11.95	8.79	8.55
Exchangeable potassium (K) mg kg^{-1}	8543	5659	5394	5203	2714	2741	3323
Lead (Pb) mg kg^{-1}	2.55	3.89	0.77	2.25	2.89	3.48	4.9
Chrome (Cr) mg kg^{-1}	0	0	0	0	0	0	0
Cadmium (Cd) mg kg^{-1}	0	0.26	0	0	0	0	0
Nickel (Ni) mg kg^{-1}	1.03	0.49	0.56	0.43	0.9	1.26	0.8
Cobalt (Co) mg kg^{-1}	0.12	0.14	0.11	0.12	0.14	0.08	0.09

2.3 Origin of mesquite germplasm and bacterial strains of *P. fluorescens*

The biological material used had two different origins. We collected the seeds of *P. laevigata* from the trees in September 2016, and the vertisol and phaeozem soils came from the micro-basin of the Río Grande, Tulancingo. We got the bacterial strains A7 and Sv of *P. fluorescens* from the collection of the Molecular Biology Laboratory, Institute of Genetics, Postgraduate College, Montecillo Campus.

2.4 Experimental design

In January 2017, we inoculated the *P. laevigata* seeds with cells of the Sv and A7 strains of *P. fluorescens*, subsequently transferred to the greenhouse for germination and growth. We used a randomized experimental design with four repetitions, used in the inoculation and germination of seeds, comprised three treatments, the first corresponded to the inoculation of *P. laevigata* roots with the bacterial strain A7 of *P. fluorescens*, and the second consisted of the inoculation of the roots of *P. laevigata* with the bacterial strain Sv of *P. fluorescens*; and the control treatment. The *P.fluorescens* culture was carried out in the King B base culture medium prepared in a liquid state (Carrillo-Castañeda *et al*., 2011).We incubated this cultures in a shaker at 150 opm at an average temperature of 30°C for 72 hours. The turbidity of the bacterial cultures was adjusted to turbidity (660 nm) of 1.8 for strain Sv and 1.2 for strain A7.

Prior to the inoculation, we treated the *P. laevigata* seeds with 120 mL of 70% alcohol for one minute, followed by washing with distilled water at an average temperature of 60°C (Quiñones-Gutiérrez *et al*., 2013) for five minutes and drying for four hours. In addition, twelve 15 ml bottles were prepared (four bottles per treatment) to perform the inoculation of the seeds and added 10 ml of bacterial cell suspension (A7 or Sv) for the two treatments (eight bottles), distilled water and 70 seeds. The twelve bottles (four with strain A7, four with strain Sv, and four without bacterial strain) were put to rest for two hours. Then we transferred the seeds of each bottle to pre-identified Petri dishes and provided them with a triple layer of absorbent paper.

2.5 Greenhouse experiment

We placed the twelve Petri dishes prepared in the laboratory in a greenhouse germination chamber of the Postgraduate College at an average temperature of 18.9°C and a humidity of 57.3%. We watered the seeds every 24 hours with 3 mL of distilled water. The daily germination percentage of *P. laevigata* seeds was determined for 20 days. We placed the seeds in germination beds, and after thirty days, the homogenous seedlings were selected. Then transplanted into polyethylene bags filled with nine kilograms of soil.

We implemented the greenhouse phase from January to July 2017, with the help of a randomized experimental design with a 2x3x5 factorial arrangement with three repetitions. The study factors were bacterial strains A7 and Sv of *P. fluorescens*, soils vertisol, phaeozem, and 0, 25, 50, 75, and 100 tons/Ha of vermicompost. We measured sixteen dependent variables, divided into three groups, in the experimental units or calculated after 180 days, either in group A «morphological variables (height (cm), diameter (mm), the number of leaves and root length (cm)) », group B« dry biomass (dry biomass weight of leaves (g),

leaf litter (mg), stems (g), roots (g), nodules (mg), aerial (g), underground (g) and total (g)) »and group C« indicators and indices (leaf area (cm^2), air weight ratio between radical weight, slenderness index, and Dickson index) ».

2.6 Statistical analysis

We performed the data analysis for the two experimental designs using R software (R Core Team, 2013). We subjected these to the homogeneity tests of variances by Bartlett's test and graphic methods (residual against predicted). In addition, we corroborated their normality by the Shapiro-Wilk test. Various transformations combined with the "power transform" function of Box-Cox (Table 2) were used to satisfy the assumed assumptions (Gurka *et al*., 2006) of the models used. As suggested by Rodríguez-Sauceda *et al*., (2019), the Tukey multiple comparisons ($p \le 0.05$) were used to match the effects of the control treatment, strain A7, and strain Sv of *P. fluorescens* of the experimental design used in the germination stage. For the greenhouse experiment, we determined significant differences ($p \le 0.05$) of the bacterial, soil, and vermicompost factors. We compared the means of the three levels of the bacterial strains factor, the factor of interest in this study.

TABLE 2
VARIABLES OF TRANSFORMATION: COMPLIANCE WITH THE ASSUMPTIONS OF THE MODELS OF ANALYSIS OF VARIANCE (ANOVA)

Determinations at 456 h	Variables of transformation
Random experimental design	
Variables	$y = (456h)^{9.8098}$
Random factorial experimental design	
A. Morphological variables	
Diameter (mm)	$y = (((Diameter\ (mm)) - 7)^2)^{0.241}$
Number of leaves	$y = ((\log(Number\ of\ Leaves) - 5.9)^2)^{1.1539}$
B. Dry biomass	
Dry weight of leaves (g)	$y = (((\mathrm{Log}(Dry\ weight\ of\ leaves\ (g) + 0.7)) - 1)^2)^{0.3156}$
Dry weight of leaves litter (Mg)	$y = (Dry\ weight\ of\ leaf\ litter\ (g))^{0.2551}$
Dry weight of stems (g)	$y = ((\mathrm{Log}(Dry\ weight\ of\ stems\ (g)) - 1)^2)^{0.3552}$
Dry weight of nodules (Mg)	$y = 1/(Dry\ weight\ of\ nodules\ (Mg) + 1)^{8.4901}$
Aerial dry weight (g)	$y = ((\mathrm{Log}(Aerial\ dry\ weight\ (g)) - 2)^2)^{0.1909}$
Total dry weight (g)	$y = ((\mathrm{Log}(Total\ dry\ weight\ (g)) - 2)^2)^{0.3686}$
C. Indicators and morphological indices	
Dickson Index	$y = (Dickson\ index)^{0.0292}$
Slenderness index	$y = ((\mathrm{Log}(\mathrm{Slenderness\ index}) - 3)^2)^{0.1337}$
Aerial/radical weight ratio	$y = 1/(\mathrm{Aerial}/radical\ weight\ ratio)^{0.3766}$
Foliar area (cm^2)	$y = ((\mathrm{Log}(Foliar\ area\ (cm^2)) - 6)^2)^{0.074}$

III. RESULTS

3.1 Germination of *P. laevigata* seeds

When analyzing the trend during the germination stage from initial to end, the results allowed us to discard our first hypothesis proposed at the beginning of this study for two reasons. First, the germination of *P. laevigata* seeds inoculated with cells of the bacterial strains A7 and Sv, was inhibited, 22.81 and 18.16% respectively, showing the results of the analysis of variance showed significant effects ($p \le 0.05$) between treatments (Table 3). The second, the absence of significant favoring or inhibition of germination at 120 hours, and for the period 168-360 hours (Table 3). Corresponding to our results of the inhibition of the germination process, the tendency of the observations agreed with those made by Carrillo-Castañeda *et al*., (2000; 2011), who concluded that there is a capacity for an inhibition of specific strains of *P. fluorescens* for certain plant species. However, among the limitations found in this study were the exclusive use of the A7 and Sv strains of *P. fluorescens*, which suggest future experimentation with other bacterial strains to corroborate the tendency of these plant growth-promoting microorganisms (PGPM) in the germination stage of mesquite (*P. laevigata*).

TABLE 3
GERMINATION OVER A PERIOD OF 456 HOURS OF *P. LAEVIGATA* SEEDS INOCULATED WITH CELLS SUSPENSIONS OF THE INDICATED BACTERIAL STRAINS

Determination of germination after the number of hours indicated	ANOVA Pr(>F)	Comparison of means: germination percentage		
		WI	A7	Sv
24	0.0270 *	5.71 a	3.22 ab	1.43 b
48	0.00755 **	31.07 a	11.79 b	15.72 b
72	0.0237 *	43.22 a	18.93 b	26.79 ab
96	0.0933.	50.00 a	29.29 b	40.72 ab
144	0.0700.	67.15 a	42.14 b	53.57 ab
384	0.0671.	92.50 a	83.22 b	88.57 ab
408	0.0822.	92.50 a	83.57 b	88.57 ab
432	0.0672.	92.86 a	83.57 b	88.57 ab
456	0.0394 *	93.22 a	83.57 b	88.57 ab
120, 168-360	>0.3186	NS	NS	NS

The means with an identical letter in the same row are statistically equal (Tukey). NS = Not significant. WI = without inoculation. Significance code: '': 0.001, '**': 0.01, '*': 0.05, '.': 0.1, '': 1.***

3.2 Seedling growth of *P. laevigata*

When dimensioning and quantifying separately the effect of the three factors used in the corresponding experimental design in the greenhouse growth stage, the soil and vermicompost factors showed significant differences (see p value in table 4) for 81.25% and 68.75% of the 16 variables tested (Table 4), respectively. When considered the bacterial factor, we only observed significant differences (p≤0.05) in 56.25% of the analyzed variables (Table 4). Although in third position, when compared with the soil and vermicompost factors, the use of strains A7 and Sv together open the way to explore the development of environmentally friendly production technologies focused on the use of plant growth-promoting bacteria(PGPB), which have been widely used to improve plant growth (Egamberdieva, 2008; Karakurt *et al*., 2011; Radhapriya *et al*., 2015).

TABLE 4
ANALYSIS OF VARIANCE OF MORPHOLOGICAL VARIABLES, DRY BIOMASS AND MORPHOLOGICAL INDICATORS AND INDICES AT 180 DAYS OF GROWTH IN THE GREENHOUSE OF *P. LAEVIGATA*

Variable	Soil	Bacteria	Vermicompost
A. Morphological variables			
Height (cm)	1.81e-05 ***	0.2395	0.0305 *
Diameter (mm)	0.000963 ***	0.024451 *	0.000487 ***
Number of leaves	0.32762	0.00473 **	0.02485 *
Root length (cm)	0.0932.	0.5814	0.0565.
B. Dry biomass			
Dry weight of leaves (g)	1.08e-08 ***	0.2903	0.0580.
Dry weight of leaves litter (Mg)	0.4109	0.0509.	0.1004
Dry weight of stems (g)	< 2e-16 ***	0.00679 **	0.00182 **
Dry weight of roots (g)	3.48e-10 ***	0.0148 *	1.18e-05 ***
Dry weight of nodules (Mg)	1.14e-07 ***	0.0214 *	0.4127
Aerial dry weight (g)	9.58e-05 ***	0.04279 *	2.71e-06 ***
Underground dry weight (g)	1.55e-09 ***	0.0162 *	1.40e-05 ***
Total dry weight (g)	<2e-16 ***	0.1848	0.5536
C. Indicators and morphological indices			
Dickson Index	5.57e-05 ***		4.57e-06 ***
Slenderness index	0.141	0.344	0.793
Aerial/radical weight ratio	0.00131 **	0.47089	0.19293
Foliar area (cm^2)	3.18e-09 ***	0.2957	1.48e-05 ***

Significance code: '': 0.001, '**': 0.01, '*': 0.05, '.': 0.1, '': 1.***

Among the 56.25% of variables classified as significant, we observed two patterns of prime effect that allowed us to respond to our second hypothesis. The first, favoring, since in relation to the control treatment, the inoculation of the mesquite seeds with cells of the **Sv** strain of *P. fluorescens* in mesquite roots (*P. laevigata*) improved significantly(see p value in table 5) 43.75% of the variables analyzed in this study. Specifically, for group A) morphological variables: an increase of 17.89% and 19.48% in diameter (mm) and the number of leaves; Group B) dry biomass: an increase between 23.81% and 44.27% of the dry weight of leaves litter (mg), stems (g), roots (g), and underground (g). Finally, group C) indicators and morphological indices: the same trend, an increase of 28.02% in the Dickson index (Table 5). While, for the second pattern, inhibition, in relation to strain Sv, we found that strain A7 significantly decreased (see p-value in table 5) 12.5% of the analyzed variables, 48.10% and a 16.73% for the dry weight of nodules (mg) and aerial (g), respectively (Table 5). Only the first pattern of this research work related to the trend of inoculation work with plant growth-promoting bacteria (PGPB) in legumes reported in the literature, for example, for the growth of the air section of Bashan *et al*., (2012), cited by Radhapriya *et al*.,(2015), height (Iqbal *et al*., 2012), and anhydrous weight (Dileep Kumar *et al*., 2001).

The results obtained allowed us to delimit the degree of action corresponding to the use of bacterial strains as a natural source of nutrients (Afzal and Bano, 2008; Iqbal *et al*., 2012) to increase the growth of mesquite trees. However, although the Sv strain was the pioneer treatment when the effects of the bacterial factor were significant in relation to the control treatment (43.75% of the variables), we must take into account that this factor is in the last position if we consider the effects of the soil and vermicompost factors.

TABLE 5
COMPARISON OF BACTERIAL FACTOR MEANS OF MORPHOLOGICAL VARIABLES, DRY BIOMASS AND MORPHOLOGICAL INDICATORS AND INDICES AT 180 DAYS OF GROWTH IN THE GREENHOUSE OF *P. LAEVIGATA*

Variable	Bacteria		
	Witness	**A7**	**Sv**
A. Morphological variables			
Height (cm)	83.44 a	82.46 a	93.29 a
Diameter (mm)	5.22 b	5.74 ab	6.35 a
Number of leaves	36 b	50 a	45 a
Root length (cm)	58.20 a	60.14 a	55.83 a
B. Dry biomass			
Dry weight of leaves (g)	2.25 a	2.47 a	2.85 a
Dry weight of leaves litter (Mg)	101.62 b	131.74 ab	182.34 a
Dry weight of stems (g)	4.89 b	5.27 b	6.41 a
Dry weight of roots (g)	3.61 b	4.10 ab	4.86 a
Dry weight of nodules (Mg)	85.74 ab	45.46 b	87.59 a
Aerial dry weight (g)	7.24 ab	7.87 b	9.45 a
Underground dry weight (g)	3.70 b	4.14 ab	4.95 a
Total dry weight (g)	10.94 a	12.01 a	14.40 a
C. Indicators and morphological indices			
Dickson Index	0.67 b	0.79 ab	0.93 a
Slenderness index	16.13 a	14.44 a	15.87 a
Aerial/radical weight ratio	2.14 a	1.94 a	2.00 a
Foliar area (cm^2)	273.13 a	293.60 a	334.41 a

The means with an identical letter in the same row are statistically equal (Tukey).
Significance code (p value): '': 0.001, '**': 0.01, '*': 0.05, '.': 0.1, '': 1.***

IV. DISCUSSION

4.1 Germination of *P. laevigata* seeds

In this study, we observed a significant decrease ($p \leq 0.05$) of 9.65% of the germination percentage of mesquite seeds inoculated with cells of the bacterial strain A7 in relation to the seeds without inoculation after 456 hours (Table 3). However, our results opposed previous research by authors such as Bashan *et al*.,(2012), Radhapriya *et al*., (2015), Elekhtyar (2015), and Kumar (2016), who pointed out that plant growth-promoting Rhizobacteria (PGPR), including *P. fluorescens*,

increase seed germination. However, Villegas-Espinosa *et al*., (2014) mentioned that in different investigations carried out with plants and beneficial microorganisms, they observed inhibitory or positive effects on germination.

4.2 Seedling growth of *P. laevigata*

Another aspect to consider is that the results obtained only apply to plant production under greenhouse conditions described in the method of this document. Given the need to find answers for different environmental conditions, as stated by Bécquer *et al*., (2013) and Villegas-Espinosa *et al*., (2014)**.** It requires future research at the field level to assess the effects of the interaction of plant growth-promoting microorganisms (PGPM) and mesquite trees, both exposed, for example, to the environmental conditions of the site of origin.

V. CONCLUSION

This study allowed us to reach two main conclusions regarding the control treatment. The first one: the use of bacterial strains called plant growth-promoting rhizobacteria showed opposite effects, inhibition-favoring, in the germination and growth stage tested at 180 days; however, the benefit of the cells associated with the roots of the plants could be presented in the phenological stages of the crop. The second conclusion: the use of bacterial strains proved to be an alternative as a biofertilizer to stimulate the growth of 43.75% of the tested variables, which shows that the plant-bacterial interaction of *P. laevigata* and *P.fluorescens* can be used as a biological method to contribute to the balance of soil fertility.

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