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"El saber de mis hijos hará mi grandeza"

GROWTH AND OIL YIELD PARAMETERS OF THE *Capsicum annuum* VAR *aviculare* ASSOCIATED TO THE BENEFICIAL BACTERIUM *Bacillus amyloliquefaciens* AND *Azospirillum halopraeferens* UNDER FIELD CONDITIONS

PARÁMETROS DE CRECIMIENTO Y RENDIMIENTO DE *Capsicum annuum* VAR *aviculare* ASOCIADA A LAS BACTERIAS BENÉFICAS *Bacillus amyloliquefaciens* Y *Azospirillum halopraeferens* EN CONDICIONES DE CAMPO

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ABSTRACT

Capsicum annuum var. *aviculare* is one of the most extremely hot chilies, measuring between 50,000 and 100,000 Scoville Units. In the present work we studied the growth and development of chiltepin (Mazocahui), under field conditions, inoculated with previously selected and cultivated native strains of *Bacillus amyloliquefaciens* and *Azospirillum halopraeferens*. The seed was inoculated by the vacuum technique, and later at 34, 120, 180 and 210 days after germination. A drip irrigation system with a salinity (TDS) = 0.8 ppt was used. The results show that germination is significantly favored by the promoter effect caused by the beneficial bacteria. These inoculants increased some parameters of growth and development. We found significant differences regarding the control for the total weight and length of root and stem of the plants, as well as the total content of proteins, and in some parts of the plant analyzed as stem and leaf. Both bacteria increased fruit yield in the Mazocahui genotype. Our findings suggest that the application of strains of *Bacillus amyloliquefaciens* and *Azospirillum halopraeferens*, influence the increase in yield of *Capsicum annuum* var. *aviculare*, under field conditions. In addition, the data show the usefulness of the inoculation of chiltepin seed for agricultural producers in semi-arid areas where salinity is a problem.

Key words: *Azospirillum halopraeferens, Bacillus amyloliquefaciens*, biofertilizers

RESUMEN

Capsicum annuum var. *aviculare* es de los chiles extremadamente mas picosos, midiendo entre 50.000 y 100.000 Unidades Scoville. En el presente trabajo se estudió el crecimiento y desarrollo del chiltepin (Mazocahui), en condiciones de campo, inoculándose con cepas nativas previamente seleccionadas y cultivadas de *Bacillus amyloliquefaciens* y *Azospirillum halopraeferens*. La semilla fue inoculada por la técnica del vacío, y posteriormente a los 34, 120, 180 y 210 días después de la germinación. Se utilizó un sistema de riego por goteo con una salinidad (TDS) = 0.8 ppt. Los resultados arrojan que la germinación se ve favorecida significativamente por el efecto promotor causado por las bacterias benéficas. Estos inoculantes incrementaron algunos parámetros de crecimiento y desarrollo. Se encontraron diferencias significativas respecto al control para el peso total y la longitud de raíz y tallo de las plantas, así como el contenido total de proteínas, y en algunas partes de la planta analizada como tallo y hoja. Ambas bacterias aumentaron el rendimiento de fruto en el genotipo Mazocahui. Nuestros hallazgos sugieren que la aplicación de cepas de *Bacillus amyloliquefaciens* y *Azospirillum halopraeferens*, influyen en el aumento del rendimiento de *Capsicum annuum* var. *aviculare*, bajo condiciones de campo. Además, los datos muestran la utilidad de la inoculación de la semilla de chiltepin para los productores agrícolas en zonas semiáridas donde la salinidad es un problema.

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Palabras clave: *Azospirillum halopraeferens, Bacillus amyloliquefaciens, biofertilizers*

INTRODUCTION

Sonora as one of the most arid states of Mexico (annual precipitation averages of 60 mm), lacks of surface water resources as lakes or rivers. Therefore, agriculture activities are dependent of underground water extraction. Unfortunately, unsound cultural practices as an unbalanced extraction/ recharge equilibrium and inadequate use of fertilizer had promoted salinization of agriculture soil, which presents problem for the production of traditional crops. *Capsicum annuum* var. *aviculare* is a pseudohalophyte which grows in natural form on dry arid zones of Sonora, México. This *aviculare* variety of *C. annuum* called as Chiltepin (Chiltecpin from the Nahuatl Mexican word meaning "flea") is the most extremely hot chili, measuring between 50,000 and 100,000 Scoville Units (Tarazón *et al*., 2010). The tiny chili peppers of Chiltepin are red to orange-red, usually slightly ellipsoidal, and about 1 cm in diameter. Some chili enthusiasts argue that the chiltepin can potentially be hotter than the habanero or red savina, supported with the numbers reported from Craig Dremann's Pepper Hotness Test scores. Apart from giving smell and flavor to the Mexican food, the chili has curative and preventive properties of diseases; it is the powerful antimicrobial. According Tarahumaras and Papagos Indians the chiltepin has certain properties such as appetizer, is a tonic that manages offset pains, is laxative, relieves colic discomforts, and is a diuretic, antiseptic and anti-irritant. It is reported that those persons who base their diet on the consumption of chili do not easily fall ill due to bacterial infections. Chiltepin is a perennial shrub that usually grows to a height of around 1 m, but sometimes reaches 3 m (9.8 ft) and in areas without hard frost in winter, plants can live 5 - 40 years. Tarahumara and Papago Indians area and farmers of Sonora desert are the main population to obtain the fruit of plants that growth in natural conditions and lived from this activity. For the last four years the native Indians and farmers are finding the best way to incorporate C. a. var. *aviculare* into traditional agriculture since it is an everlasting plant, to help support the agricultural economy of those areas affected by salinity. One of the main alternative approaches for the production is the development of crops irrigated with high contents of salt or even the use of diluted solutions of seawater, to select and assay salt-tolerant plants, already inhibiting marsh areas, and focusing on those that might make desirable crops. In the Mexican Sonora state, the chiltepin has a wide distribution along the central part of Sonora State. This plant was identified from among many plant species tested for possible domestication for the potential as a new economic resource and an agro industrial commodity.

Several studies have been conducted to study the nutritional conditions to foster the growth of Chiltepin (Votava *et al*., 2002). However, it has been reported that its nitrogen fertilizer needs are extremely high and at long run that potentially would promote some adverse pollution effects.

One way to avoid such harm is the inoculation of crop plants with N_2-fixing bacteria or plant beneficial bacteria as fertilizer source to introduce and promote their application to improve the growth of plants in normal soils affected by severe saline conditions (Hamdi 1999). One of the most studied bacteria to promote growth is *Azospirillum* spp. This genus has proven to be effective for several crops.

In this study, we present data on the effects of applying two N_2-fixing bacteria to the rhizosphere of one *Capsicum annuum var aviculare* genotype (Mazocahui). We *applied Azospirillum halopraeferens*, reported as salt tolerant, and a purified endemic N_2-fixing bacterium, which it has properties as a N_2-fixing bacteria, identified as *Bacillus amyloliquefaciens* isolated from Craters of volcanoes in Sonora desert.

The aim of this study was to measure how these bacteria affect the growth and development of Mazocahui genotype of *Capsicum annuum var aviculare*. We focused on height, biomass, and yield production as well as in biochemical parameters.

MATERIALS AND METHODS

Location and plant material

The study was developed in the Agriculture Department at Universidad de Sonora, Mexico, at coordinates 29º 00'47" N and 110º 08' 00" W´. We studied one genotype of *Capsicum annuum var aviculare* (Mazocahui). Chinaleña Company of Hermosillo, Sonora, México provided this genotype. We evaluated the inoculation of two N_2-fixing beneficial bacteria (*Bacillus amyloliquefaciens* and *Azospirillum halopraeferens*) in Mazocahui genotype, under field conditions.

Previously to sowing, the wild seed was sifted to separate the mature seeds, cleaned from fruits dry and select the bigger size seeds, uniform color and without apparent mechanical damages. The studied genotype was treated with a 3% ($^v/_v$) bleaching agent (CLORALEX) for 30 s. Seeds were washed three times with sterile distilled water and dried with sterile drying paper. After the last wash, we tested viability on genotype, as suggested by Pérez (1995).

Bacteria preparations

Both bacteria were grown in N-free medium containing 0.12 M NaCl denominated OAB media (Reinhold *et al*., 1987). Bacteria concentration was adjusted to 1 x 10^9 colony-forming units (CFU/mL) using a spectrophotometer (master spectrum FISHER SCIENTIFIC 415) when the culture was in a logarithm phase (14 to 16 h). We added 0.5 g of seeds (approximately 590 seeds ± 7) to each bacterial solution. Seeds were inoculated according to Carrillo *et al.* (1998) and then plated in growth 1 m^2chambers, containing 7 cm of fine sand and covered with a fine cap (3 mm ± 1) of peat-moss (Sunshine, Sun I Cry Horticulture Canada, Ltd.) at 19 ± 3 °C. Once seeds germinated, seedlings were selected and planted on the field (50 cm distance between plants, and 75 cm between rows, from each treatment). Special care was taken to avoid mechanical damage to the root system. For analytical purposes, seedlings were located under an experimental

array based in a completely randomized design and five replications of 60 seedlings per replicate. The bead inoculation program was applied during the vegetative development of Chiltepin. This program started at the seedling phase, continued during the development of branches, then at the pre-flowering and flowering stages. This means 34, 120, 180 and 210 days after germination, respectively. Inoculation was accomplished by placing 1 g of bacteria-containing beads, within the soil sub-surface, as near to the root system of each plant as possible.

Plants were irrigated with potable water using a drop irrigation system (pH = 7; salinity (TDS) = 0.8 ppt; E.C. = 1.194 $ds \cdot cm^{-1}$; NO_2 = 0.10 and NO_3 = 87.2 $mg \cdot L^{-1}$). The irrigation frequency in this stage was every ten days, applying an amount of 1.5 ± 0.5 $L \cdot m^{-2}$ under a 60 min lapse.

The average temperature (°C) monthly values were 18 ± 4° C for the initial three months of cultivation and 35 ± 4° C for the next three months, and 44 ± 5° C for the summer season (July and August). At the same time, the mean values of the relative humidity (R.H.) were 35% for the initial four months, and 50 to 55% for the next months (CONAGUA, 2016).

Microbiological analysis of micro-parcels

In order to determine the status of the soil microbiology, we applied a widely used technique for the detection of nematodes according to Thorne (1961), and fungi, according to Manovsky (1982). For this purpose, we collected soil samples from the experimental parcels according to SARH-DGSV (2015).

Analysis of variables

We measured the germination rate and percentage, and recorded once the seedlings emerged from the substrate (8 days after sowed). The number of germinated seeds was recorded by readings (evaluations) every third day (Germination Rate), and finally the percentage of germination (%) was determined after the 30th day of trial. The germination rate was calculated according to Maguire (1962) by means of the equation:

M = n1/t1, n2/t2, …n30/t30;

where n1, n2, … n30 are the number of germinated seeds at the time t1, t2, … t30 (in days). The data of germination percentage were analyzed taking into account a transformation to the arc-sin (Sokal and Rohfl, 1988). The germination rate, which it is the sum of counted germinated seeds per day, was previously transformed for its analysis.

In order to obtain the growth curve, 35 plants of each treatment were randomly choose in order to measure plant height, on monthly basis. At the physiological flowering stage, we collected five plants per treatment, sectioned in three parts: root, stem and aboveground portion. For stem analysis, we considered the first three branches from the plant base, jointly with its lateral branches. The aboveground portion was considered from the 4th lateral branch and upward where the nitrates content in plant sap ($N\text{-}NO_3$ $mg \cdot mL^{-1}$ of sap) was obtained by means of the procedure from Coombs *et al.* (1988), and analyzed according to Wood (1967). Finally, weights of fresh and dry root were determined only at flowering stage. The plants and root system length were measured using a hand scale micrometer (General, 143, General Tools, Manufacturing Co., Inc. New York, USA). The dry weight was measured once each organ was dried at 110° C for 36 hours. We assayed proteins, carbohydrates, and ashes content for every plant organ. Proteins were assayed by the micro-Kjeldahl method, ashes by difference of weight, burning the sample for 24 h at 500º C (Barnes and Blackstock, 1973).

At 210 days before the sowed, 10 plants were randomly collected per treatment to quantify the variable "fruits production per plant" ($g \cdot plant^{-1}$). For the variable "fruits yield" in $g \cdot plant^{-1}$, we multiplied in each treatment the average production of seed per plant by the total number of plants that were sown. The dry matter was evaluated per parcel (dry matter in $g \cdot plant^{-1}$). The biochemical composition of the Chiltepin fruit was determined after harvest and drying, for the nutritional quality with the basis of the techniques recommended by the A.O.A.C. (1993).

Statistic analysis

The previously outlined variables were analyzed applying the procedure of Analysis of Variance (ANOVA), and a test of F to determine the statistical difference (Snedecor, 1956). The dampness, proteins, and ashes data were analyzed after transforming values to arc-sin values (Sokal and Rohfl, 1988). The least significant difference was estimated by Duncan's Multiple Range test at *P*=0.05. The statistical tests were performed through the SAS computer program (SAS, 2012).

RESULTS AND DISCUSSION

Results on microbial content of microparcels

Our analysis showed a low population of nematodes of the Dorylaimida Order (7 ± 3 specimens: 100 g^{-1} of soil). We also found fungal microorganisms, including *Rhizoctonia* spp, *Alternaria* spp, *Fusarium* spp, and *Aspergillus* spp, with no evident effect on growth and developed of the genotype.

3.2 Germination

Evaluation of the final percentages of germination and germination rate showed that Mazocahui genotype of *Capsicum annuum* var. *aviculare* took between 12-25 days to reach their maximum germination. The highest impact (P< 0.5) on final percentages of germination were on those inoculated with *Azospirillum halopraeferens* and *Bacillus amyloliquefaciens* (93 and 95%, respectively) while the control treatment showed lowest values (83%) (Table 1). It seems that *Capsicum annuum* var. *aviculare* keeps this strategy to cope with the changing environment of saline areas. The presence of *Azospirillum halopraeferens* and *Bacillus amyloliquefaciens* affected positively the germination rate as well as the final germination percentage on Mazocahui genotype.

Table 1. Influence of *Bacillus amyloliquefaciens* and *Azospirillum halopraeferens* on the germination (%), the physiological flowering stage, height (cm), Fresh weight (g·plant), Dry weight (g·plant) and Length root (cm) of Mazocahui genotype of *Capsicum annuum* var. aviculare, under field conditions.
Tabla 1. Influencia de *Bacillus amyloliquefaciens* y *Azospirillum halopraeferens* sobre la germinación (%), fase fisiológica de floración, altura (cm), peso fresco (g·plant), peso seco (g·plant) y la longitud raíz de (cm) del genotipo Mazocahui de *Capsicum annuum* var. *aviculare*, bajo condiciones de campo.

Inoculant (Bacterium)	Germination (%) 30th day of trial	Height (cm) At the physiological flowering stage	Fresh weight (g.plant) At the physiological flowering stage	Dry weight (g.plant) At the physiological flowering stage	Length root (cm) At the physiological flowering stage
CONTROL	83 ± 4 b	85 ± 14 b	1320 ± 124 b	45 ± 12 b	15 ± 12 b
Azospirillum halopraeferens	93 ± 4 a	115 ± 24 a	2250 ± 214 a	75 ± 14 a	25 ± 12 a
Bacillus amyloliquefaciens	95 ± 3 a	118 ± 23 a	2342 ± 253 a	90 ± 12 a	20 ± 10 a

The capacity to promote higher rates of seed germination in the studied genotype, is explained on the basis of the bacteria capacity to influence the synthesis of hormonal compounds, such as acetic acid or giberellin GA_3, as has been published elsewhere (Zexun and Wei, 2000).

The observed biotic seems important in order to increase for the success of these pseudohalophyte populations like *Capsicum annuum* var. *aviculare*, which have only one opportunity in their annual life history for its reproduction and population dispersion, tasks that are highly dependent on the germination responses of seeds. It has been observed that under natural conditions, the germination process of Chiltepin occurs during a period when the soil or water salinity levels are reduced (Rozema, 1975). However, the presence of high temperatures, play an important role to degrade the membrane present in solanaceas like *Capsicum annuum*. These results are evidence that the presence of beneficial microorganisms could play a main role in different vegetative stage, such as the germination process of diverse halophytes populations (Goodfriend *et al.*, 2000; Bagwell *et al.*, 2001; Garbeva *et al*., 2011).

After replanting, seedlings showed a typical sigmoidal growth curve during its growth cycle. In the late phase of the experimental growth, we observed the increment on height affected by *Bacillus amyloliquefaciens* on Mazocahui genotype (Table 1). The height of the studied genotype was stimulated around 30% ($p< 0.05$), when the inoculant was *Bacillus amyloliquefaciens*. In addition, plants inoculated with *A. halopraeferens* were ($P<0.05$) affected compared with those plants none inoculated. The behavior of the non-inoculated plants were very similar to those reported elsewhere (Rozema, 1975; Jefferies, 1981; Mc Graw and Ungar, 1981; Stumpf *et al.*, 1986; Momonoky and Kamimura, 1994; Blom *et al*., 2011).

Plant flowering stage

According to the variable "fresh weight", during the physiological flowering stage, the obtained results showed a highly significant difference between treatments, with $P< 0.5$ (Table 1), where Mazocahui + *Bacillus amyloliquefaciens* showed the maximum values, followed by those plants inoculated with *A. halopraeferens.* Similarly, Mazocahui inoculated with *Bacillus amyloliquefaciens* was the best treatment for the variable "dry weight", followed by the treatment of Mazocahui + *A. halopraeferens*. The treatments without any kind of inoculation were not stimulated or inhibited (Table 1).

Treatments differed significantly in their inherent ability to sustain a root growth, but after the application of beneficial bacterial in our study, we observed significant differences at $P< 0.5$ (Table 1). The treatment based on Mazocahui inoculated with *Bacillus amyloliquefaciens* showed the highest values for "root growth", followed by the association of *A. halopraeferens.* It seems probable that plant growth substances produced by *Bacillus amyloliquefaciens* improve plant growth by their direct effects on metabolic processes. However, since they induce proliferation of lateral roots and root hairs and thus increase nutrient absorbing surfaces, this may lead to greater rates of nutrient absorption. This in turn would be expected to increased plant growth, which is in agreement with Tarazon *et al.* (2010).

Either inoculation of Chiltepin of Mazocahui genotype plants with *A. halopraeferens* or with *Bacillus amyloliquefaciens*, stimulated total protein content at root, stem and "aboveground portion" though at different levels of concentration. At root level, both bacteria stimulated total protein content to around 26% (Table 2); at stem of "aboveground portion", the stimulation capacity was in a 38% and 17% respectively, compared with the control (Table 2). The increment in protein influenced either by *Bacillus amyloliquefaciens* or *A. halopraeferens*, seemed to be reflected with an increment in total plant biomass, as proven to be found influenced in other micro-plant systems (El-Shatnawi *et al.*, 2001; Rueda *et al.*, 2010; Agaras *et al*., 2016).

The above results suggest us that growth of *Capsicum annuum* var. *aviculare*, during the vegetative development, can be promoted by beneficial bacteria to finally help the plant promoting in some physiological mechanisms, based on hormonal activities and mechanisms as N_2 fixation, as a production of undefined signal molecules, an enhanced mineral uptake and an improved root growth.

Table 2. Influence of Bacillus amyloliquefaciens and Azospirillum halopraeferens on proteins (%) in three partes (root, steam and above-ground portion) and Content of NO3 (mg.mL-1) in sap at the flowering stage of Mazocahui genotype of Capsicum annuum var. aviculare, under field conditions. Values are mean of a triplicate. Letters indicate significance if they are different (P< 0.5).
Tabla 2. Influencia de Bacillus amyloliquefaciens y Azospirillum halopraeferens sobre las proteínas (%) en tres partes (raíz, tallo y porción superior) y contenido de NO3 (mg.mL-1) en la savia en la etapa de floración del genotipo Mazocahui de Capsicum annuum var. aviculare, bajo condiciones de campo. Los valores son la media de un triplicado. Las letras indican significancia si son diferentes (P <0.5).

Inoculant [Bacterium]	Root	Stem		Above-ground portion
	Protein	Protein	NO_3.mL (mg. mL^{-1})	Protein
Control	2.09± 0.6 b	2.98± 0.2 b	1.3± 0.5 a	4.99± 0.1 c
Azospirillum halopraeferens	2.79± 0.9 a	4.57± 0.5 a	1.1± 0.2 a	5.89± 0.1 b
Bacillus amyloliquefaciens	2.85± 1.0 a	4.76± 0.1 a	1.1± 0.2 a	6.01± 0.0 a

In relation to the NO_3^- content in sap at the flowering stage, the results showed no significant difference, where the treatments with bacterium showed the highest values (Table 2), while the lowest content of NO_3 were expressed by the inoculated treatments. These effects could be due to the capacity to metabolize into other products, in order to be assimilated and sustain biomass production, where the NO_3 can be detected in another form such as total nitrogen (Table 2). Similar results were obtained in agriculture precision studies with *Zea mays* plants (Blackmer and Mallarino, 1996; Berendsen *et al*., 2012). These authors applied different levels of N_2 fertilizer, where the results showed low concentrations of NO_3 in steam; they concluded that the assimilation of the applied N_2 was limited by the growth in plant mature stage at the final physiological growth of plants.

Plant mature physiological stage

Lack of available nitrogen in soil is a limitation in plant productivity. Application of nitrogen could be expensive depending on the source. The application of bacterial inoculants such as *A. halopraeferens* and *Bacillus amyloliquefaciens* showed high values in the productivity of *Capsicum annuum* var. *aviculare*. Table 3 shows that both, *A. halopraeferens* and *Bacillus amyloliquefaciens*, increased yield of Mazocahui genotype and bromatological analysis, where the high protein values were in those plants inoculated. These results agree with data published elsewhere (Bashan *et al*., 2000; Ortiz-Castro *et al*., 2013) though at seems to contrast with others observed in different plant systems.

CONCLUSIONS

The variables analyzed along the different phenologycal stages in *Capsicum annuum* var. *aviculare* Mazocahui genotype showed to be sensitive to the bacterial inoculation. The rate of germination percentage was influenced positively, but the final percentage was no affected. The parameters evaluated were found to be appropriate for determining differences between *Bacillus amyloliquefaciens* and *A. halopraeferens* in Mazocahui genotype. Under our experimental conditions, the best bacterium was *Bacillus amyloliquefaciens* for Mazocahui genotype.

Table 3. Mean values of seed production (g.plant-1) and Bromatological analysis (Ceniza, Extracto etéreo, Fibra cruda, Proteína cruda, ELN) of Mazocahui genotype of Capsicum annuum var. aviculare, as influenced by the beneficial bacteria Bacillus amyloliquefaciens and Azospirillum halopraeferens, under field conditions. Values are mean of a triplicate. Letters indicate significance if they are different (P< 0.5).
Tabla 3. Valores medios de la producción de semillas (g.plant-1) y análisis bromatológicos (Ceniza, Extracto etéreo, Fibra cruda, Proteína cruda, ELN) del genotipo Mazocahui de Capsicum annuum var. aviculare, influenciado por las bacterias beneficas Bacillus amyloliquefaciens y Azospirillum halopraeferens, en condiciones de campo. Los valores son la media de un triplicado. Las letras indican significancia si son diferentes (P <0.5).

Inoculant (Bacterium)	Seed						
	Yield/plant g.plant-1	Ceniza	Extracto etéreo	Fibra cruda	Proteína cruda	ELN	Total
CONTROL	190.8± 23.4 a	4.67	14.89	31.3	12.9	36.3	100
Azospirillum halopraeferens	256.4± 20.8 a	4.55	15.65	33.2	13.9	32.7	100
Bacillus amyloliquefaciens	270.5± 29.0 a	4.78	15.23	32.9	13.2	33.9	100

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