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Effect of Rhizobacteria Indole producing on the Development of *Capsicum annuum* var. jalapeño M

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Abstract

The capacity which some rhizobacteria have for producing compounds of the indole group makes them important for consideration like plant growth promoting rhizobacteria (PGPR). These compounds havean important rolein the development of vegetation. The aim of this study was to evaluate the effect of rhizobacteria producing indole in the development of Capsicum annuum var. jalapeño M. The bacterial strains were isolated from Capsicum sp.and their capacity to produce indole was determined in vitro. Variations in height, diameter of the stem, fruiting and plant appearance,were determined. The bacteria were identified as enterobacter ludwigii, Serratia quinivorans, Lysinibacillus sphaericus, Aeromonas media and pseudomonas poae. The individual application of these bacteria showed no significant difference in any of the variables evaluated. The plants treated with a mixture of these six bacteria did increase the number of fruits produced by C. annuum var. jalapeño M. demonstrating the existence of a synergic effect between the strains ($F_{-7, 47}$ = 11.144, p = 0.035).

Keywords: PGPB, YIB, plant, microorganism interaction, biofertilizers, microbial synergy.

Introduction

Chili (*Capsium* sp.) is considered to be one of the most important horticultural crops in the world. According to data obtained from FAO the estimated production for the year 2011 was 29,601,175 t. yielding 16.11 t/ha. In northern Europe, yields of 260.64 t/ha have been achieved showing values far superior to those obtained by countries such as China (21.97 t/ha) and Mexico (14.76 t/ha) which are renowned for their large annual production. This may be attributed to the use of more adequate technology in countries such as Holland and Belgium which contributes towards a better application of irrigation and fertilization, whilst in China and Mexico cropping systems still employ open field, which makes this application more difficult.

The use of chemical fertilizers has permitted the obtentionof high yields and high quality in the production of various agricultural crops[1]. However, their excessive use tends to degrade the environment and produce noxious effects[2,3,4]. In spite of this fact, it is estimated that their use is increasing day by day in accordance with the increase in alimentary demand. Based on the foregoing, several studies have been developed with the aim of substituting these chemicals with the use of beneficial microbes as a biological alternative for increasing agricultural production in a more sustainable way[5-7].

Rhizobacteria that promote plant growth (PGPR) are considered as microbes which are capable of increase plant growth[8,9].They have the potential of making nutrients available to the plants by fixing atmospheric nitrogen (N_2), of phosphate solubilization, and producing siderophores and synthesizing growth regulators, such as auxinas[10-13].Amongst the latter, compounds which produce one group of indole are outstanding and responsible for stimulating the formation of lateral roots and adventitious of the plants, which favours their capacity to absorb nutrients and minerals and the exudation of compounds that stimulate the proliferation of bacteria on the roots[14].

Species of bacteria which produce indole have been studied as PGPR in the case of some commercial crops. Amaresan *et al.*[15]found that the inoculation of *Bacillus* sp. and *Serratia* sp. increased the length of roots and shoots in crops such as *Lycopersiconesculentum* and *Capsicum annuum*, as well as increasing the number of secondary roots, whilst Adesemoye *et al.*[16] observed the fact that inoculation with *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4 on*L.*

Esculentum plants reduced the need for chemical fertilizers by 20%.

In this way, the use of PGPR to increase the production of crops in great demand, such as *Capsicum,* is vital in an attempt to work towards sustainable agriculture. The aim of our study was to isolate bacteria which produce indolebelonging to the rhizosphere of *Capsicum* sp. and to evaluate their capacity in promoting development in *C.annuum* var. jalapeño M.

Material and Methods

The isolation of bacterialstrains: *Capsicum* sp. plants showing vigorous growth were collected in Tolome, Veracruz, Mexico, situated at 19°16′00″N and 96°23′00″W at 25 MASL. The bacterial strains were isolated from suspensions of roots previously washed in sterile distilled water which were macerated in a porcelain mortar with 3mL of sterile distilled water. In order to achieve this,20 µL of this suspension was inoculated on 4 culture mediums - LG medium for *Azotobacter* and *Azomonas*[17], B de King (KB)medium, nutrient agar(NA) and a modified medium for *Bacillus* spp.[18]. The culturesin the KB, NA and *Bacillus* medium were incubated at 28 ± 2°C for 48h and 120 h in the case of the LG medium. Under a stereoscopic microscope (Karl Zeiss, Stemi 1000), it was observed that various types of colonies developed. These were later transferred to a new medium to obtain pure cultures.

Detection of bacteria strains indole producing: The production of indole for each bacteria strain was determined by studying any development taking place in tubes containing a culture medium made up of peptone free of indole (10g), sodium chloride (5g) and distilled water (1L). After incubating the cultures at 28°C ± 2 for 24 h, 3 drops of the reagent Kovac[19] were applied. The tests were performed in duplicate and a culture of *Escherichia coli* was employed as a positive control and a sterile peptone medium as a negative control.

In addition, the capacity of the bacteria to produce siderophores was determined by presenting fluorescent pigments under UV light after cultivating them in a medium of KB (28°C ± 2 for 24h). The capacity for fixing N_2 was also determined resulting from the development of colonies in the LG medium[17] and lastly, the capacity for solubilizing phosphates according to the method developed by the National Botanical Research Institute[20].

Inoculation of the bacteria in C. annuum plants var. jalapeño M: An evaluation was carried out using *C.annuum* var. jalapeñoM from the distributor Crown Seed de Mexico, S.A. de C.V. The experiment was set up in a nursery in the municipality of Las Vigas de Ramírez, Veracruz, with the following coordinates 19°38'00"N, 97°06'00"W and at an elevation of 2,420 MASL.

The seeds were placed in trays (20 by 10 cavities) using local soil as the substrate for germination. Twenty days after, the plantlets were transplanted to black plastic bags filled with soil (30 by 40 cm).

Cellsuspensions were prepared at a concentration of≈1.2 X 10^8 UFC from each bacterial strain capable of producing indole. A further suspension was prepared which consisted of a mixture of all the bacterial isolates together at concentration which was proportionally the same.

The inoculation of the suspension was carried out on the plants; 15 days after transplanting took place. Six treatments were evaluated which corresponded to the six individual strains as well as one treatment with a mixture of all six strains and a control where only sterilized distilled water was used. This was repeated 20 times for each treatment. The plants were distributed at random in four grooves in the nursery. The inoculation was performed using 3 mL of bacterial suspension in the surrounding soil from the stem of the plant.

Evaluation of bacterial strain as PGPR for C. annuum var. jalapeño M: The variables height, diameter and plant appearance, were measured after 15, 30, 50 and 99 days following the application of the treatments. The numbers of fruits were counted after 99 days. The height of each plant was measured from the base of the stem up to the apical leaves of the central axis. The diameter was measured at the base of the stem. The appearance of the plants was determined as: i. dead plant; ii. plant with a poor aspect (more than 50% of leaves yellow, flaccid and fragile distributed all over the plant); iii. plant with a normal appearance (less than 20% of basalleaves yellowing, short distance between knots and shriveled leaves); and iv. good appearance (vigorous plant).

Identification of bacterial species: The bacterial isolates were identified using the analysis the 16S rRNA gene. The genomic ADN extraction was carried out according to the method employed by Cheng and Jiang[21].

The amplification of 16S rRNA genewas carried out using the following reagents: 100ng of genomic ADN, Buffer PCR 1X, $MgCl_2$ 2.5 mmol, dNTP-Mix 0.25 mmol, 8f primer15 pmol (5'CACGGATCCAGACTTTGATYMTGGCTCAG3'), 1512r primer 15 pmol (5 GTGAAGCTTACGGYT AGC TTG TTACGACT 3') and Platinum® Taq DNA polymerase 1.5 U. The reaction was carried out in 25 µL of the total volume in a thermocycler (personal Master cycler Eppendorf) following a thermal program of: initial denaturalization at 94°C for 2 min; followed by 35 cycles with denaturalization at 94°C for 10 s, annealing at 59°C for 20 s and an extension at 72°C for 2 min; and finally, an extension phase at 72 °C for 4 min.

The amplified products were analyzed in an agarose gel (Pronadisa CONDA) 1.6% (p/v) in a 0.5% TBE buffer in Power Pac Basic (BioRad) equipment for electrophoresis. The

amplified products were purified with Wizard® SV Gel and PCR Clean Up System (Promega) and were quantified using a spectrophotometer Nano Drop (ND 1000). The process of sequencing was carried out at the Instituteof Biotechnology at the Universidad Nacional Autónoma de México.

Statistical analysis: The data covering the death of plants and number of fruits formed was analyzed using a variance analysis Kruskal-Wallis. Height, diameter and appearance were analyzed using ANOVA with repeating measurements. Significant variables were analyzed in pairs tests Mann-Whitney. Statistical analysis was carried out using the SPSS statistic program (v. 19).

Results and Discussion

A total of 90 bacterial strains were isolated on the roots of *C. annuum* L. of these, only six isolates were capable of producing indole. No significant differences were found for variations in height ($F_{-21,\ 213} = 0.71$, p= 0.82), diameter ($F_{-21,\ 213} = 0.93$, p = 0.55) and appearance ($F_{-21,\ 213} = 0.74$, p = 0.79), in the plants inoculated with individual bacterial strains, however, the mixture of all six rose significantly ($F_{-7,\ 47} = 11.144$, p = 0.035) in the number of fruits produced, see figure-1. It is possible that this result is due to a synergic effect because of the interaction of the various bacterial species. These results coincide with those reported by Datta *et al.*[22] who found that the inoculation of *Bacillus* sp. and *Streptomyces* sp. increased the yield (number of fruits and their weight) of *C. annuum* L. when compared with plants which were not inoculated or inoculated with only one bacterial specie.

In a study carried out by Lim and Kim[23], it was observed that the joint application of auxins, such as indoleacetic acid, indolebutiric acid, (IBA) and indole propionic acid (IP), isolated from *Bacillus subtilis* AH18 and *Bacillus licheniformis* K11, increased the length of the stem and roots of red pepper and tomatoes, when compared with plants which were not treated with auxins. In fact, the results proved more effective than individual applications of these hormones and commercial presentations such as IAA and IBA. In this sense, a similar effect could have taken place in our study if we consider that the diverse species of bacteria employed were capable of synthesizing indole. In order to confirm this fact, it is considered necessary to carry out further studies which permit us to identify the auxins produced by the bacterial isolates employed.

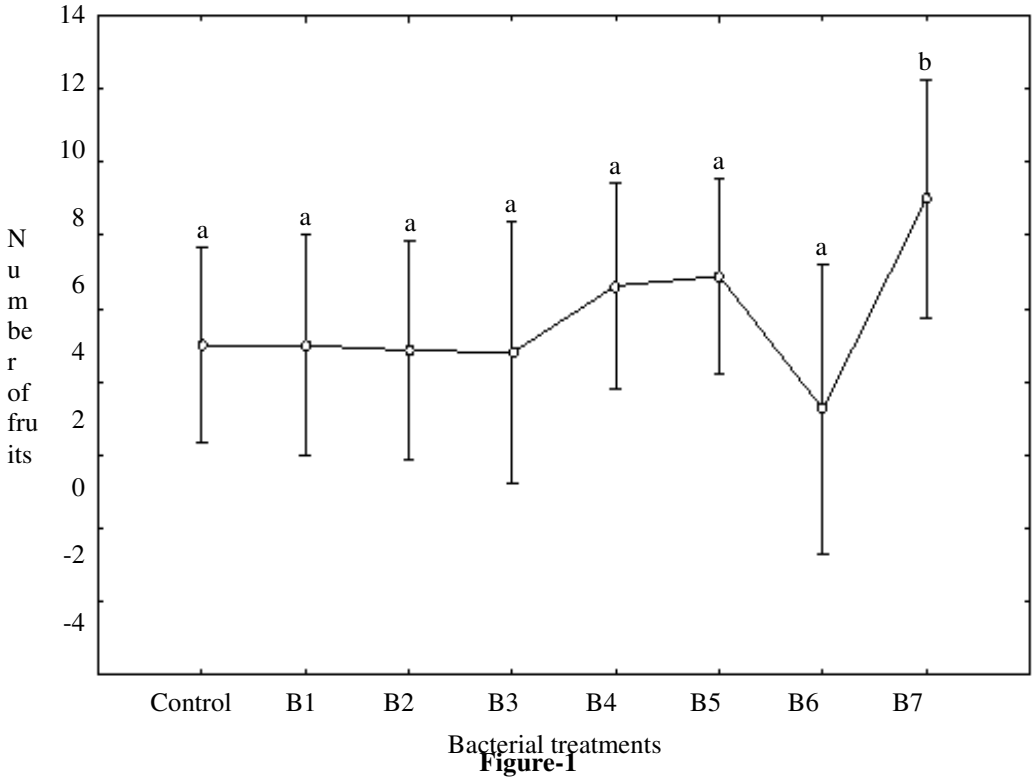

Figure-1

Thefruiting of *Capsicum annuum* var. jalapeño M with the inoculation of various bacterial treatments capable of producing indole: B1; *Enterobacter ludwigii,* B2; *Serratia quinivorans,* B3; *Enterobacter ludwigii*, B4; *Lysinibacillus sphaericus*, B5; *Aeromonas media,* B6; *Pseudomonas poae,* B7; a mixture of all the bacterial isolates. The B7 treatment shows an increase in the number of fruits produced ($F_{7,47} = 11.144$, p = 0.035) when compared with treatments B1, B2, B3, B4, B5, and B6. Different letters indicate significant differences

Apart from the production of indole, the production of siderophores, the solubilization of phosphate and N_2 fixing constitute some of the attributes which should be taken into account when determining the potential use of these bacterial strains as PGPR[24], thus, we evaluate them for each of the six bacterial isolates, see table-1. Based on the foregoing, B4 could prove to be a promising strain. Nevertheless, its application alone did not produce significant differences in the variables analyzed here when compared with the control, which supports the fact that a synergic effect must have taken place amongst all these or some of the bacterial isolates to produce an increase in the number of fruits.

Table-1
Production of siderophores, N_2 fixing and solubilization of phosphate by bacteria producing indole. Colony development directly proportional to the capacity for N_2 fixing (+ low colony development, ++ medium colony development, +++ abundant colony development)

Bacterial isolate	Production of siderophores	N_2 Fixing	Solubilization of phosphate (mm)
B1	NO	++	0.5
B2	NO	+	1
B3	NO	+++	0.5
B4	YES	++	3.5
B5	NO	+	0
B6	NO	+	0

Pierson and Weller[25] mention that one of the motives that could cause synergic effects is due to the fact that a more diverse bacterial community is possibly more stable and is capable of completely colonizing roots and survive any biological, chemical or physical changes that might occur.

Molecular results enabled us to identify bacterial species such as *Enterobacter ludwigii* (B1), *Serratia quinivorans* (B2), *Enterobacterludwigii* (B3), *Lysinibacillus sphaericus* (B4), *Aeromonas media* (B5) and *Pseudomonas poae* (B6).

Some reports exist relating to the capacity of promoting plant growth by some species of bacteria included in the study; such is the case of *E. ludwigii* which increased the number of new shoots and the density of the roots of *Lolium perenne,* and this was attributed to its capacity to solubilize tricalcium phosphate ($Ca_3(PO_4)_2$) and synthesize IAA[26]. In addition, the *E. ludwigii strain* of this study showed its capacity for fixing N_2. With regard to *L. sphaericus,* Yasmin *et al.*[27] reported that strains of this species were capable of increasing the dry weight of storage roots as well as shoots in the case of sweet potato crops, whilst Wahyudi *et al.*[28] mention its capacity for solubilizing phosphate and producing IAA and siderophores which also coincides with our report, see table-1.

In the case of *Serratia* genus, the capacity to synthesize IAA is reported[29] as well as the capacity of some strains to increase the number and weight of fruits in *L.esculentum* Mill cv. Rutgers[30] and their use as a biocontrol agent for phytopathogen fungi[31], while that for*S. Quinivorans* there are no reports onthis aspect.

For *P. poae,* there are no reports on its use as a PGPR, but there are reports that it is capable of producing siderophores[32]as well as solubilizing tricalcium phosphate[33], which confers some attributes for including it in an evaluation of PGPR. No reports have been found for the use *of A. media* as a PGPR.

Conclusion

The joint application of suspensions of *S. quinivorans, E. ludwigii, L. sphaericus, A.media and P.poae* produced an increase in the number of fruits in the case of *C.annuum* crops var. jalapeño M, whilst the length and diameter of the stem and the appearance of the plants did not result in a determining factor for increasing the production of fruits.

It is considered necessary to evaluate possible mixtures which could be established between the bacterial isolates reported in this study, with the aim of defining which bacterial species are required to stimulate fruiting or whether is necessary a joint application to produce this effect. The foregoing will permit us to identify which group of bacterial species is capable of acting as a PGPR and is able to provide a biological alternative for increasing yields obtained in the cultivation of *C annuum*.

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