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Effect of Inoculation with Three Phytohormone Producers Phytobacteria with ACC Deaminase Activity on Root Length of *Lens esculenta* Seedlings

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ABSTRACT

Plant-associated bacteria that inhabit the rhizosphere may influence the plant growth by their contribution to the endogenous pool of phytohormones and by the activity of ACC deaminase to decrease the ethylene concentration. The aim of this study was to analyse the root length growth by the promoting effect of indole acetic acid producers phytobacteria with ACC deaminase activity, on inoculated seeds of *Lens esculenta* as synergistic effect on root elongation. In this study, although the roots of *L. esculenta* seedlings do not show a significant promotion, these phytobacteria could be recommended to treat plants analyzing their added inoculum to increase plant biomass and retard the effect of ethylene on cultures supplied with Tryptophan and ACC.

Keywords: Plant Growth-Promoting Bacteria; *Lens esculenta*; Root Elongation Test; Indole Acetic Acid; ACC Deaminase Activity

1. Introduction

The 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACC deaminase) hydrolyses the 1-aminocyclopropane-1-carboxylate (ACC) into ammonia and α-ketobutyrate instead of its conversion into ethylene; the uptake and cleavage of ACC by this enzyme decrease its amount and as consequence the ethylene concentration in plants [1-5]. Authors reported that the ACC deaminase trait has been extensively studied in numerous soil microorganisms, but it is most common among plant growth promoting rhizobacteria to protect plants from both biotic and abiotic stresses and favor the increase of plant biomass through the regulation of ethylene synthesis in inoculated plants [5-14]. Bacteria that inhabit the rhizosphere also may influence the plant growth by their contribution to the endogenous pool of phytohormones, such as auxins in plants; the production of the auxin Indole Acetic Acid (IAA) is reported among plant-associated bacteria [15]. The aim of this study was to analyze the root length growth by the promoting effect of indole acetic acid producers phytobacteria with ACC deaminase activity on inoculated seeds of *Lens esculenta* as synergistic effect on root elongation.

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2. Materials and Methods

2.1. Evaluation of the IAA Production of the Selected Phytobacteria

The phytobacteria employed: *Lemna* 2 strain, U-M1-4 strain and U-M1-5 strain, were isolated from the aquatic plant *Lemna gibba*, collected from the Lake Xochimilco, México. The selected phytobacteria were analyzed by their Indole Acetic Acid (IAA) production [16,17] using the Salkowski reagent according to the method of Bric *et al.* [18] and Melo *et al.* [19], taking 4.9 mL of sterile Luria-Bertani (LB) liquid media, added to culture tubes (10 × 15 cm); the culture tubes were inoculated with 0.1 mL of each phytobacteria with an adjusted inoculum by optical density of 5×10^7 cells/mL in sterile distilled water and incubated at 28˚C for 120 h. After the incubation,

the cultures were centrifuged at 3,500 rpm, at 25˚C for 45 minutes to discard the bacteria pellets, the supernatant was recovered and 2 mL of each supernatants were mixed with 2 mL of Salkowski's coloring reagent, the development of a pink color indicates IAA production and was quantified reading its absorbance at 535 nm. The concentration of IAA was estimated by a standard curve and the assays were performed by triplicate.

Evaluation of the ACC deaminase activity of the selected phytobacteria The ACC deaminase activity of the isolates was assayed according to the method of Penrose and Glick [20] and Khandelwal and Sindhu [21], with ACC and $(NH_4)_2SO_4$ as sole nitrogen source. The assay was done using agar plates with DF minimal medium [22] supplemented with ACC (1mM) or $(NH_4)_2SO_4$ (2 g/L), equally divided into 24 sectors and spot inoculated with a sterile toothpick each colony of the selected phytobacteria. The plates were incubated at 28˚C for 48 to 72 h and the presence of ACC deaminase activity, was recorded by the measurement of the diameter of the colonies and compared between the two nitrogen conditions. The assays were performed by duplicate.

2.2. Effect on Root Growth of *Lens esculenta* Seedlings Inoculated with the Selected IAA Producers with ACC Deaminase Activity Phytobacteria

Bacterial inoculum were obtained by culturing the phytobacteria strains on plates with LB agar medium for 48 h at 28˚C and re-suspending in sterile distilled water to adjust by optical density an inoculum with cell density of 5×10^7 cells/mL; 0.1 mL of the bacterial suspensions were spread on Petri dishes containing mineral medium with phytagel, added with Tryptophan (2 mg/L) and/or ACC (1 mM). The plates were stand for 30 minutes for the inoculum absorption. Twenty five commercially seeds of *Lens esculenta* were surface-sterilized with 10% sodium hypochlorite and then thoroughly rinsed with sterile distilled water and placed in the Petri dishes, with the respective conditions. Petri dishes with mineral medium containing: 0.20 M $NH_4H_2PO_4$, 0.50 M NH_4NO_3, 1.15 M $Ca(NO_3)_2$, 0.26 M $CaCl_2$, 0.2 M $MgCl_2 \cdot 6H_2O$, 0.20 M $Mg(NO_3)_2 \cdot 6H_2O$, 0.40 M $MgSO_4 \cdot 7H_2O$, 0.20 M KH_2PO_4, 1.2 M KNO_3, 0.5 M K_2SO_4, 0.04 M $FeCl_3 \cdot 6H_2O$, 1.2×10^{-2} M H_3BO_3, 1.2×10^{-4} M $CuCl_2 \cdot H_2O$, 2.3×10^{-3} M $ZnCl_2$, 4.4×10^{-4} M $MnCl_2 \cdot 4H_2O$, 6×10^{-6} M $Na_2MoO_4 \cdot H_2O$, EDTA and $FeSO_4 \cdot 7H_2O$, pH = ±6.0, and 3 g of phytagel; uninoculated treatments were considered as blanks. All the experiments were performed by duplicate and maintained at 30 °C in a growth chamber in dark for 4 days. The plant root elongation promoting (PREP) activity assay was employed to analyze the promoting and synergistic effect of rhizobacteria strains on *Lens esculenta* seeds, according to the modified root elongation assay of Belimov *et al.* [23], the root length of the seedlings were measure and the Growth Index (GI), expressed as the ratio of the root lengths of plants grown in the presence and absence of the phytobacteria, was obtained [14]; GI = RLpb/RLc, where RLpb is the root length of plants grown in the presence of the specific phytobacteria inoculum and RLc is the root length of plants grown in absence of inoculum (control).

2.3. Statistical Analysis

All data were analysed by One-way ANOVA analysis of variance and the mean differences were compared applying a Tukey-Kramer post-test, using the statistics program Graph Pad Instat Ver. 3.10.

3. Results

3.1. IAA Production and ACC Deaminase Activity by the Tested Strains

Lemna 2 strain and U-M1-4 strain presented a higher ACC deaminase activity ($p < 0.001$) than U-M1-5 strain, according to their colonial diameter **(Figure 1)**. The employ in the metabolic assay of ammonium sulfate as nitrogen source, was only taking it as control, compared to the ACC assay, but in this study, the two phytobacteria mentioned had a minor colony diameter, compared to the ACC activity. The three phytobacteria presented an increase in the IAA production as the tryptophan supply in cultures increased, the basal production of this auxin without the aminoacid presented this order: U-M1-5 strain (32.67 μg/mL) > U-M1-4 strain (30.7 μg/mL) > *Lemna* 2 strain (19.32 μg/mL), all the bacterial isolates were classified according to Khalid *et al.* [24] as higher phytohormone producers **(Figure 2)**.

3.2. Root Elongation Test of *L. esculenta* and the Effect of Phytobacteria Inoculation

The response of *L. esculenta* roots showed that this species was susceptible to the presence of Trp, ACC and Trp + ACC, with a significant reduction on the root development compared to the roots grown on mineral medium: U-M1-4 strain 55% > U-M1-5 strain 36.17% > *Lemna* 2 strain 27.95% ($p < 0.001$). In general, inoculation with the three phytobacteria strains decrease the root length compared to the control roots (U-M1-4 strain > U-M1-5 strain > *Lemna* 2 strain). Particularly, the effect of the seeds inoculated with the strains and Trp showed that the order of response was as follows: U-M1-5 strain > U-M1-4 strain > *Lemna* 2 strain), the inoculated seeds and ACC: *Lemna* 2 strain > UM1-5 strain > UM1-4 and

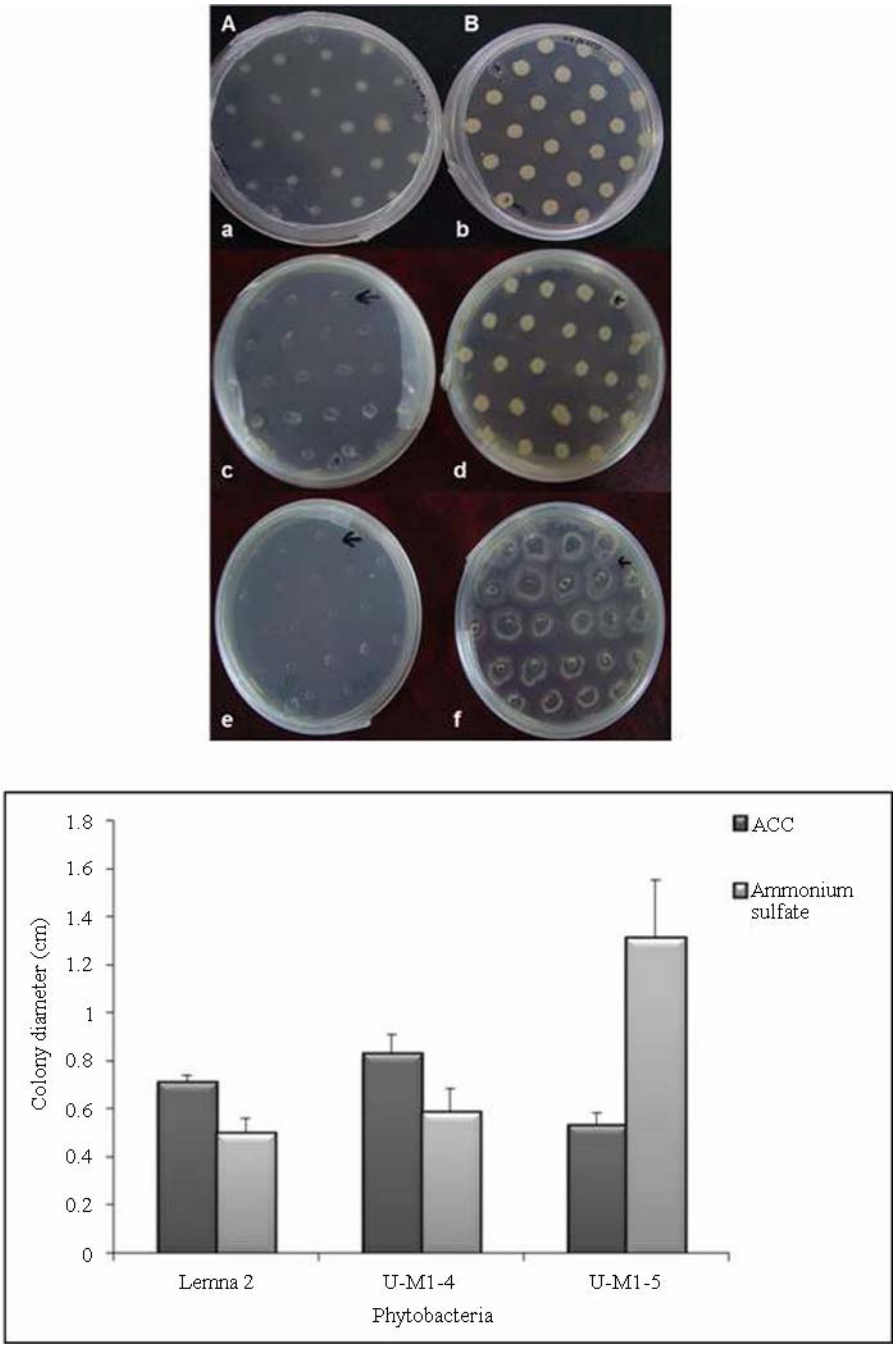

Figure 1. ACC deaminase activity of the phytobacteria tested: (A) DF minimal medium with ACC and (B) DF minimal medium with Ammonium Sulfate, where: "a and b" *Lemna* 2 strain, "c and d" U-M1-5 strain, "e and f" U-M1-6 strain. Mean values ± S.D. from 48 replicates.

the inoculated seeds and Trp + ACC: UM1-5 strain > *Lemna* 2 strain > U-M1-4 strain (**Figures 3** and **4**).

4. Discussion

Glick *et al.* [25] showed that the promotion of root growth is one of the principal markers by which the beneficial effect of plant growth-promoting bacteria is measured. It is known from application of exogenous IAA [26] or application of diluted culture extracts or low density inoculum of bacteria that produce high levels of IAA [27,28] that low concentrations of IAA can stimulate primary root elongation; it is important to note that the effect of bacterial IAA on plant growth depends on the size of the inoculum of a single strain, but not always,

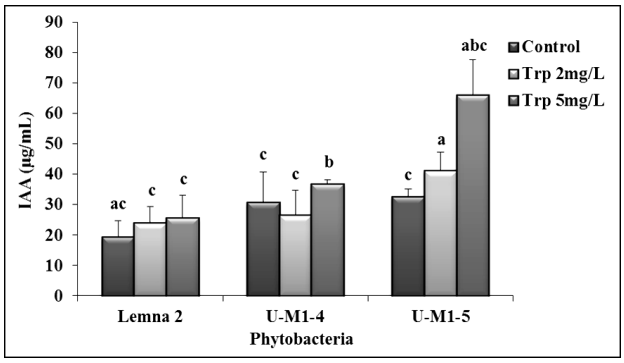

Figure 2. IAA *in vitro* production of the phytobacteria tested. Mean values ± S.D. from three replicates.

the inoculum density employed means that more IAA is available to the plant, and reports of bacterial mutants that overproduce IAA show a root growth-inhibiting effect [29-31]. Burd *et al.* [14] and Belimov *et al.* [32] reported that there is a number of plant growth promoting bacteria that contain the enzyme ACC deaminase and stimulate the root growth of different plant species; ACC is exuded from roots or seeds and cleaved by ACC deaminase to NH3 and a-ketobutyrate, the bacteria utilize the NH_3 as a source of nitrogen and thereby decrease ACC within the plant [9,33] with the concomitant reduction of plant ethylene [4,14,34,35]. Patten and Glick [36] demonstrated that the IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity. The authors mention that the IAA and ACC deaminase work together to stimulate the root elongation; this consideration is regarding to the exogenous IAA that increase the transcription and activity of ACC synthase; this enzyme catalyzes the production of ACC in plants and therefore ACC stimulates the ACC deaminase activity in bacteria [6,37], while the IAA produced by the bacterial inoculum, stimulates the root elongation or the formation of lateral and adventitious roots [29-31,38]. In this study, the correlation between the IAA production and the ACC deaminase activity by the phytobacteria tested, showed a relationship between the U-M1-4 and U-M1-5 strains that were joined together as one group by this attribute, separated to the *Lemna* 2 strain; probably the density of the inoculum produced a high IAA concentration that generated a inhibiting effect on root growth, joined to the ACC metabolism activity presented by the phytobacteria. This attribution is related with the visible short and thick appearance of the roots showed in the treatments with ACC and Trp + ACC. The effect of the inoculation with these three phytobacteria strains on *Lens esculenta* seeds, doesn't show an evident increase in the root length of seedlings; but particularly was the response of two of the isolates: *Lemna* 2 strain and

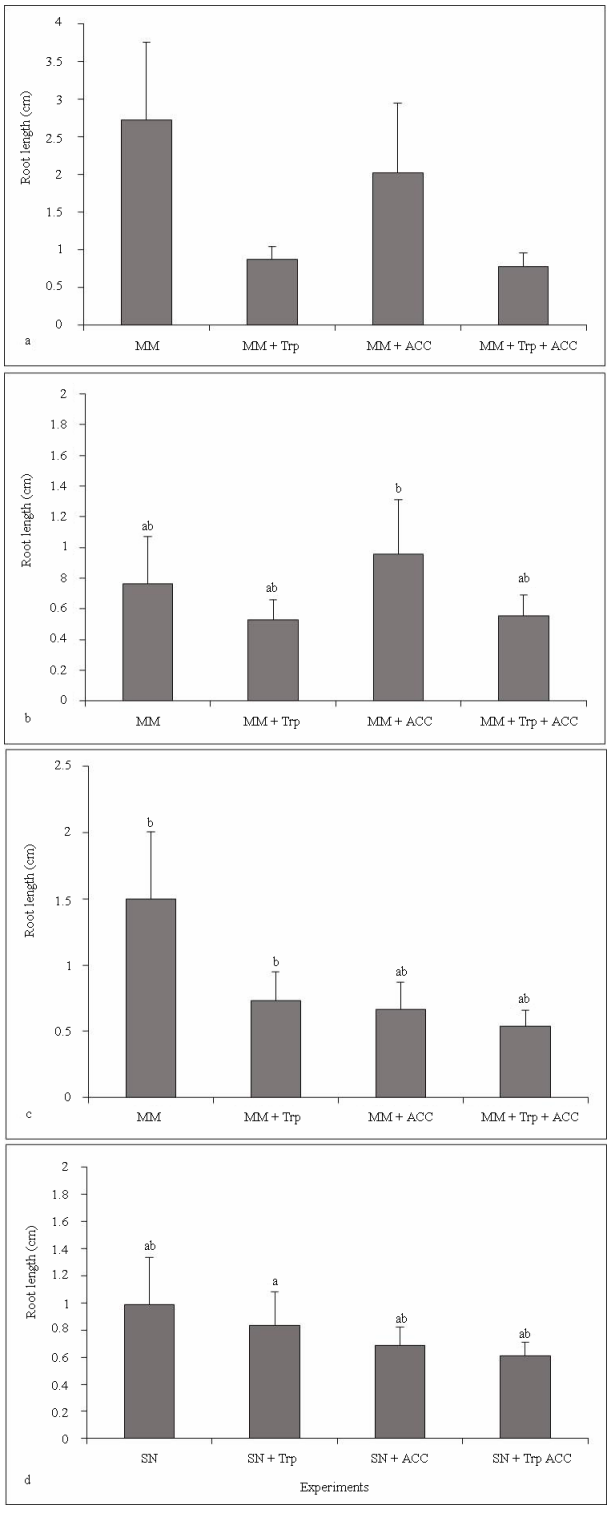

Figure 3. Root length measurement of *Lens esculenta* seedlings: (a) Control experiments, (b) Trp, ACC and Trp + ACC inoculated with *Lemna* 2 strain, (c) Trp, ACC and Trp + ACC inoculated with U-M1-4 strain and (d) Trp, ACC and Trp + ACC inoculated with U-M1-5 strain. Mean values ± S.D. from 50 replicates, the different bold letters show the significant differences between experiments ($p < 0.001$).

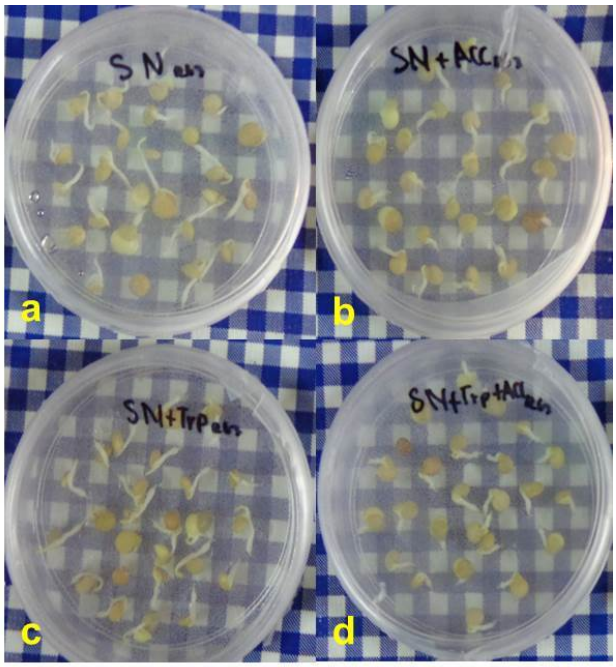

Figure 4. Root length experiments of *Lens esculenta* seeds inoculated with U-M1-5 strain: (a) Mineral Medium, (b) Mineral Medium + ACC, (c) Mineral Medium + Trp and (d) Mineral Medium + Trp + ACC.

U-M1-5 strain with a relationship according to their measured attributes: *Lemna* 2 strain with a high ACC activity (with a 47.32% of root growth compared to the control roots) and U-M1-5 strain with the highest *in vitro* IAA production (with a 94.98% of root growth compared to the control roots) especially with the presence of Trp (2 mg/L). Even the root length of the seedlings was lesser than control seedlings for both strains; the seeds' treatment with Trp + ACC showed that the response of the root length was higher in the experiments with TRP with U-M1-5 strain (with a 78.26% of root growth compared to the control roots) than *Lemna* 2 strain (with a 71.09% of root growth compared to the control roots).

These results were according to the results obtained by Zafar-Ul-Hye *et al.* [39]; these authors screened rhizobacteria containing ACC deaminase to promote lentil growth under axenic conditions and by Shaharoona *et al.* [5], who analyzed different strains of rhizobacteria with variations in their ACC deaminase activity, capability of the rhizobacterial isolates of IAA production in the presence and absence of tryptophane and isolates also varied in their ability to colonize etiolated pea roots; both according to the reports by Shaharoona *et al*. [40] and Zafar-Ul-Hye *et al.* [39] where the bacteria with more ACC deaminase activity had more ability to decrease the intensity of the called "ACC-induced classical triple response", the ACC deaminase activity of bacteria was responsible for the decrease of endogenous and exogenous ACC supply in inoculated plant with the inhibition on the root length of *L. esculenta* seedlings (*Lemna* 2 strain to the control roots). Finally, although the roots of *L. esculenta* seedlings do not show a significant promotion with the presence of the strains, Trp and ACC in medium; and even though these responses were adverse, the results obtained in this work suggested a synergistic effect between the two bacterial attributes probed and the phytobacteria tested and could recommended that it is important to consider the inoculum density to the increase of plant biomass and retard the effect of ethylene on cultures supplied with Tryptophan and ACC.

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