In vitro root induction and growth of *Lens esculenta* and *Physalis ixocarpa* shoot explants by plant growth promoting rhizobacteria

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Abstract— *Direct effects of plant growth promoting rhizobacteria are associated with the production of phytohormones and clearly the root growth promotion is one of the major markers by which the beneficial effect of plant growth-promoting bacteria is measured. Recent studies reported that treatments of stem cuttings with beneficial microorganisms such as Agrobacterium, Alcaligenes, Azospirillum, Azotobacter, Bacillus, Pseudomonas and Streptomyces species, induce on rhizogenesis, growth promotion of in vitro cultured plants by the natural auxin production of these bacteria and their Inoculation in tissue culture eliminate many of the difficulties associated with the rooting of stem cuttings and protect the micropropagated plants against biotic and abiotic stress. The aim of this work was to analyze the effects of the auxin rhizobacteria producer Pseudomonas sp. strain C2 on rooting and shoot elongation of Lens esculenta and Physalis ixocarpa stem cuttings. In this work, two particularly responses were obtained: root production and shoot elongation in Physalis ixocarpa and only shoot elongation in Lens esculenta stem cuttings. In both plants their mass clonal propagation response was clearly related to their genetic nature, although there was evident the stimulation of growth by the presence of the inoculated Pseudomonas sp. strain C2.*

Keywords—plant growth-promoting rhizobacteria, Physalis ixocarpa, Lens esculenta, stem cuttings, in vitro plants.

I. INTRODUCTION

As some authors noted [1-4], PGPR (Plant Growth Promoting Rhizobacteria) have gained worldwide importance and acceptance. Mechanisms involved in plant growth promotion by PGPR's produce direct and indirect effects; one of those direct effects is the production of phytohormones. Ramos-Solano et al. [5] mentioned that the modification of a plant's physiology by plant growth regulator production is a very important mechanism, not only because it alters the principal mechanism of plant growth regulation but also because it is based on the evolutionary development of common metabolic pathways in plants and bacteria. Glick et al. [6] showed that the promotion of root growth is one of the major markers by which the beneficial effect of plant growth-promoting bacteria is measured; it is related to rapid establishment of roots that is advantageous for young seedlings to obtain water and nutrients from their environment [7]. Recent studies confirm that the treatments of seeds or cuttings inoculated with these kind of beneficial microorganisms such as *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas* and *Streptomyces*, reported the effect on rhizogenesis, growth promotion and reduction of hyperhydricity of *in vitro* cultured plants [8-14]; that induce root formation because of natural auxin production by these bacteria [4, 15, 16]. Although the mechanisms are not completely understood, root induction by PGPR's is the accepted result of auxin production [17]. Microbial production of the auxin indole-3-acetic acid (IAA) has been extensively reported [18,19]. Most studies using microorganisms that produce IAA have reported a link between IAA production and root development and morphology [20,21]. Many different bacterial species can produce IAA through various mechanisms. *Aeromonas* spp., *Azospirillum brasilense* and *Comamonas acidovorans* are among the many IAA species that promote plant growth in rice [22], wheat [23] and lettuce [24]. Kapulnik et al. [25] and Lifshitz et al. [26], reported that considering the numerous interactions between the different hormonal signaling pathways in plants, it is difficult to assess which of these pathways is the primary target of PGPR's, but it is known that these microorganisms modify phytohormonal pathways by the different morphological changes observed, like the lateral root elongation and root hair development. One of the more characteristic effects of PGPR's is the increased elongation and initiation rate of lateral roots, giving a more branched root system. These authors finally mention that the employ of PGPR's for nursery material multiplication may be important for obtaining organic nursery material. Nowak and Shulaev [27], Vestberg et al. [28] and Larraburu et al. [29] reported that the inoculation with PGPR's in tissue culture eliminate many of the difficulties associated with the rooting of stem cuttings and protect the micropropagated plants against biotic and abiotic stress.

The aim of this work was to analyze the effects of the auxin rhizobacteria producer *Pseudomonas* sp. strain C2 on *in vitro* rooting and shoot elongation of *Lens esculenta* and *Physalis ixocarpa* stem cuttings.

II. MATERIAL AND METHOD

2.1 Characteristics of the IAA producer rhizobacteria employed in this study

The rhizobacteria employed: *Pseudomonas* sp. strain C2, was isolated from the rhizosphere of *Sporobolus indicus* grown in a metal contaminated soils located in Villa de la Paz in the state of San Luis Potosí, México by Melo et al. [30], it was characterized by the same authors as higher IAA producer with 25 µg/mL in culture medium supplemented with 2 mg/L of L-tryptophan (IAA precursor) instead of its lower production without Trp (18.7 µg/mL). Bacterial inocula were obtained by culturing the rhizobacteria strain on plates with Luria-Bertani (LB) agar medium for 48 h at 28ºC and re-suspending in sterile distilled water to adjust by optical density an inoculum with cell density of 5x107 cells/mL; 5mL of the bacteria suspension was employed to inoculate the stem cuttings of both plant species, as follows.

2.2 Establishment of in vitro plants for the explants collection

Commercially obtained certified seeds of *Lens esculenta* Moench and *Physalis ixocarpa* Brot were used to obtain the shoot explants which were used for the present study. Seeds were surface-sterilized with 10% sodium hypochlorite solution, rinsed with deionized sterile water and twenty seeds of each species, were placed separately in baby food flasks with Magenta SIGMA caps (by quintuplicate) with 25 mL of mineral medium containing: 0.20 M $NH_4H_2PO_4$, 1.15 M $Ca(NO_3)2$, 0.26 M $CaCl_2$, 0.40 M $MgSO_4 \cdot 7H_2O$, 1.2 M KNO_3, 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 6% bacteriological agar. Flasks were kept at +36°C in a growth chamber with a 12:12 photoperiod for fifteen days.

2.3 Adventitious root induction from stem explants

2 cm stem cuttings from each plant species (*L. esculenta* (Len) and *P. ixocarpa* (Phy)) were excised using sterile blades from the 15-day old in vitro aseptic seedlings of both plant species. These were transferred in sterile conditions, to culture flasks that contained rooting medium without phytohormones, considering it a 50% salts concentration of MS (Murashige and Skoog) [31] basal medium supplemented with 2% sucrose and 3g/L Phytagel. Four stem explants considered as cuttings were deposited per flask and replicates were performed by quadruplicate. Treatments were considered as follows: Treatment I: control stem cuttings grown in only 50% MS medium, without KH_2PO_4, Treatment II: stem cuttings grown in 50% MS medium supplemented with KH_2PO_4 (85mg/L), Treatment III: stem cuttings grown in 50% MS medium inoculated with 0.1mL of the *Pseudomonas* sp. strain C2 suspension and Treatment IV: stem cuttings grown in 50% MS medium inoculated with 0.1mL of the *Pseudomonas* sp. strain C2 suspension and supplemented with KH_2PO_4 (85mg/L). Experimental units were kept at +36°C in a growth chamber with a 12:12 photoperiod for twelve days until rooting stems were obtained.

2.4 Analysis of shoot and root growth of stem cuttings

The cultured stem cuttings were collected from the media; total plant length and number and length of adventitious roots were recorded. Foliar fresh weight was measured after rinsing with sterile water and blotting away surface water and chlorophyll content was extracted from leaves of each plant species, ground with 5mL of acetone, incubated at 4°C for two hours, centrifuge and the absorbance of supernatant collected was recorded at 663 and 645nm. Total chlorophyll content was calculated using the following formulae: Total Chlorophyll (mg/g fresh weight) = $(8.02 \times A_{663}) - (20.2 \times A_{645})$. Determination of plant growth ratio was calculated considering the R/S length ratio (Root/Shoot) and BN/PL ratio (Branch Number/Plant Length).

2.5 Statistical analysis

All the results were analysed by ANOVA test, and Tukey-Kramer Method using the statistics program Graph Pad Instat Ver. 2.03. A numerical comparative analysis considering all the treatments was done; a distance matrix was built using the conventional standard distance coefficient, a phenogram was build using the unweighted pair group method of arithmetic averages (UPGMA) method and correlation coefficient of Pearson was obtained using the NTSyS-PC version 2.11T (Numerical Taxonomy and Multivariate Analysis System) software.

III. RESULTS AND DISCUSSION

3.1 General response of stem cuttings

There was evident a particularly response of each plant species; where only *P. ixocarpa* plants showed a rooting effect in all the tested conditions for stem cuttings. *L. esculenta* cuttings do not shown a rooting effect and only there was an increase in

stem branch number and shoot elongation. This was the first sign about particularly response regarding to root induction of both plant species. Even there was only one complete response of plant species, regarding to the in vitro rooting effect and shoot growth in *P. ixocarpa* stem cuttings; there was a clear evidence of shoot growth promotion of both species. Comparing both data, *L. esculenta* plants showed the highest promotion of plant growth, not only induced in control stem cuttings; but also it was evident in stem cuttings supplemented with KH_2PO_4 salt and inoculated with the PGPR (Fig. 1). Regarding to total chlorophyll content, only treatments with the inoculated rhizobacteria showed a slightly increase for both plant species, without statistical significance between treatments ($p<0.001$); control *P. ixocarpa* and *L. esculenta* plantlets: 56.6+44.4 and 36.47+4; inoculated *P. ixocarpa* and *L. esculenta* plantlets: 69.48+8.5 and 52.39+5.2, respectively.

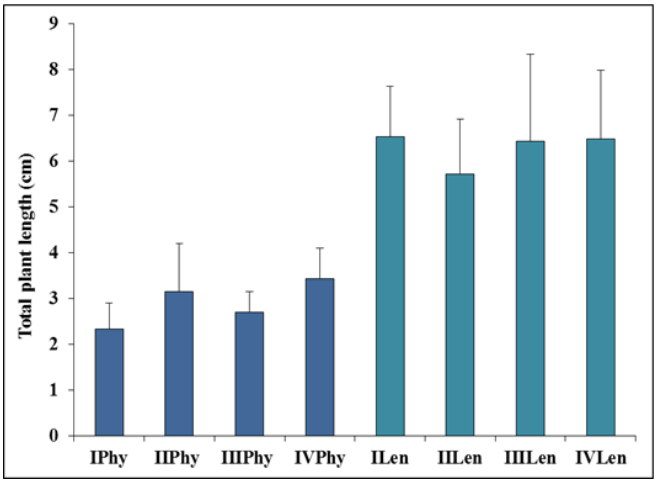

FIG.1. TOTAL PLANT LENGTH MEASUREMENT OF *PHYALIS IXOCARPA* AND *LENS ESCULENTA* IN VITRO PLANTLETS.

Mean values + S.D. from four replicates of four plantlets per replicate. No significant differences were found between plant species treatments (p < 0.001).

3.2 Rooting and root growth of *P. ixocarpa* stem cuttings

Data obtained regarding to R/S length ratio (Fig. 2), showed that response of plants in each treatments was: Treatment III (1.39, 58%), Treatment I (0.56, 36%), Treatment IV (0.33, 24%) and finally Treatment II (0.31, 23%). Results about the average of roots number and total root length obtained for each treatment in *P. ixocarpa* stem cuttings are showed in Fig. 3. In this plant species there was evident the effect of the presence of rhizobacteria and the addition of phosphate salt; where the number of total roots obtained was greater than control plants and in treatments with the inoculum and KH_2PO_4 only. Average of total root length in all treatments showed that roots do not increased in length between the tested conditions; even these results were almost the same, little diminished values were obtained in treatments with the added phosphate salt and inoculum present. Thus, PGPR's presence in stem cuttings had only significant higher number of roots, but a diminished root growth, compared with the other treatments.

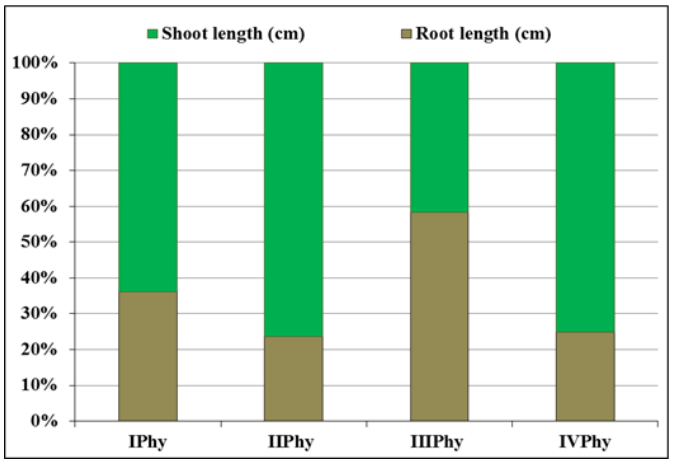

FIG. 2. SHOOT/ROOT PERCENTAGE OF *PHYALIS IXOCARPA* IN *VITRO* PLANTLETS.

Mean values from four replicates of four plantlets per replicate.

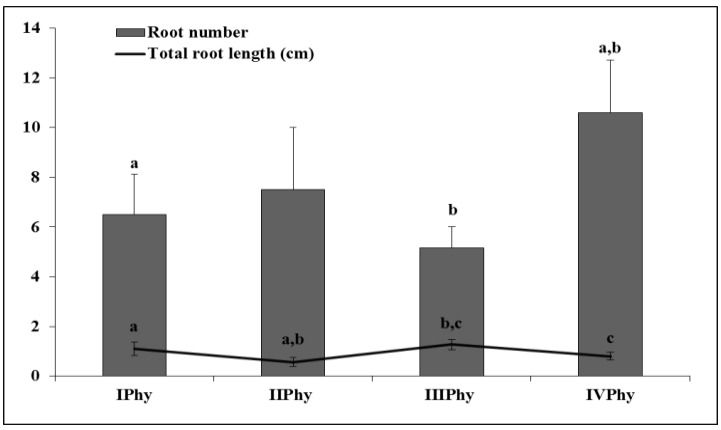

FIG. 3. TOTAL ROOT LENGTH AND ROOT NUMBER MEASUREMENT OF *PHYALIS IXOCARPA IN VITRO* PLANTLETS

Mean values + S.D. from four replicates of four plantlets per replicate. Different bold letters showed the significant differences between treatments (p < 0.001).

3.3 Shoot growth response of *L. esculenta* stem cuttings

Particularly response was obtained in *L. esculenta* stem cuttings, where the number of stem branches increased only in stem cuttings treated with 50% MS medium without phosphate salt (control plants) and in inoculated medium with the IAA producer rhizobacteria. The other treatments do not shown an increase in stem branch number. Data obtained regarding to BN/PL ratio (Fig. 4), showed that response of plants in each treatments was: Treatment I (0.83, 47%), Treatment III (0.67, 40%), Treatment II (0.55, 37%) and finally Treatment IV (0.42, 30%). Comparison against the latest treatment showed a significant statistical difference ($p<0.001$) between it and Treatments I and II.

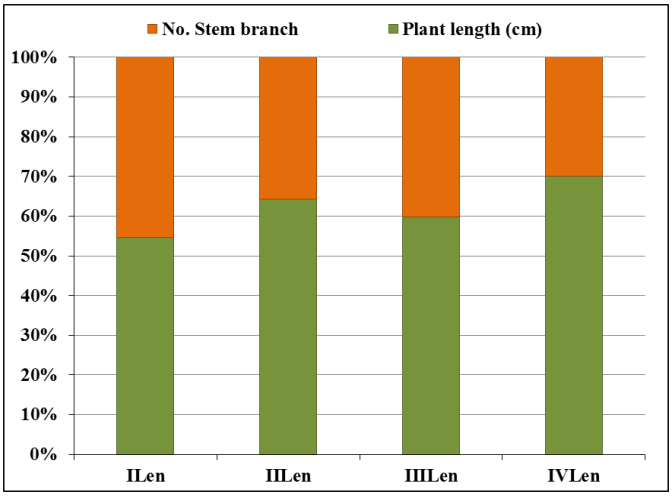

FIG. 4. NUMBER OF STEM BRANCHES/PLANT LENGTH PERCENTAGE OF LENS ESCULENTA IN VITRO PLANTLETS.

Mean values from four replicates of four plantlets per replicate.

3.4 Rooting response of both plant species with PGPR co-cultive

It is known that PGPR's inoculation substantially increased root growth and development of tissue-cultured plantlets, where the initial response was observed during root hair formation. Baset et al. [7], mention that initiation of more root hairs might be due to bacterial interactions with the root surface of the host plant; these results were found in different cereal crops and tomato seedlings where PGPR's inoculation enhanced the appearance of root hairs reported by Okon [32] and Hadas and Okon [33], also inoculation of wheat with these kind of microorganisms reported by Levanony and Bashan [34] enhanced cell division in the root tips while Hartmann et al. [35] mention that in maize there was an increase in diameter and length of lateral roots. Molla et al. [36] probed that *Azospirillum* strain Sp7 has the potential to synthesize plant hormone which can replace IAA to stimulate root growth in vegetable soybean. Regarding to these kind of plant compounds, Döbbelarere et al.

[19] suggested that liberation of plant growth promoting substances such as auxins, gibberellins and cytokinins by the bacteria could be responsible of plant growth effects. In this work, complete plant's response regarding to rooting and shoot elongation was obtained in *P. ixocarpa*, compared to *L. esculenta* stem cuttings, without rooting promotion. Rooting response in *P. ixocarpa* stem cuttings not only could be related with the medium culture characteristics and inoculation with the rhizobacteria; Erturk et al. [37] mention that other explanation could be that the cuttings can produce auxin themselves after PGPR's inoculation and also Nelson [38] noted that this microorganisms are able to exert a beneficial effect on plant growth increasing root's growth and weight. Mafia et al. [39] and Zhang et al. [40] conclude that there is evident that the inoculation of cuttings of different plant species will give a particularly response depending of their genotype, this was reflected also in this study for both plant species tested. Shoot elongation instead of rooting and root elongation was reported by some authors in plants inoculated with PGPR's. Khan et al. [40] reported in their results that the application of endophytic bacterial strain MPB 2.1 significantly enhanced the development of tomato seedling, where shoot length, shoot weight, root length, and chlorophyll contents increased. Length of shoots and roots were almost the same as no-inoculated plants. These authors particularly reported that strain MBP 2.1 produced a significant effect on shoot weight. In this study, even *L. esculenta* stem cuttings do not notably produced roots under the tested conditions, it showed a slightly differentiation evidence in cutting zones, with the appearance of callus, it could presume that plants needed more time to induce rooting response; also this plant species shown particularly effect regarding to shoot elongation and increase in stem branching in the presence of the *Pseudomonas* sp. strain C2. The comparison of all plant growth parameters measured (total plant length, foliar fresh weight and total chlorophyll content) for both plant species, shown in Fig. 5 a particularly response that associate them in two established groups: a diverse Group I conformed by treatments II and IV from both species and another small group (Group II) that associate treatments III from the two plant species. More similar response were obtained between stem cuttings treated with phosphate salts added to medium culture and the stem cuttings inoculated with the rhizobacteria and supplemented with K_2HPO_4.

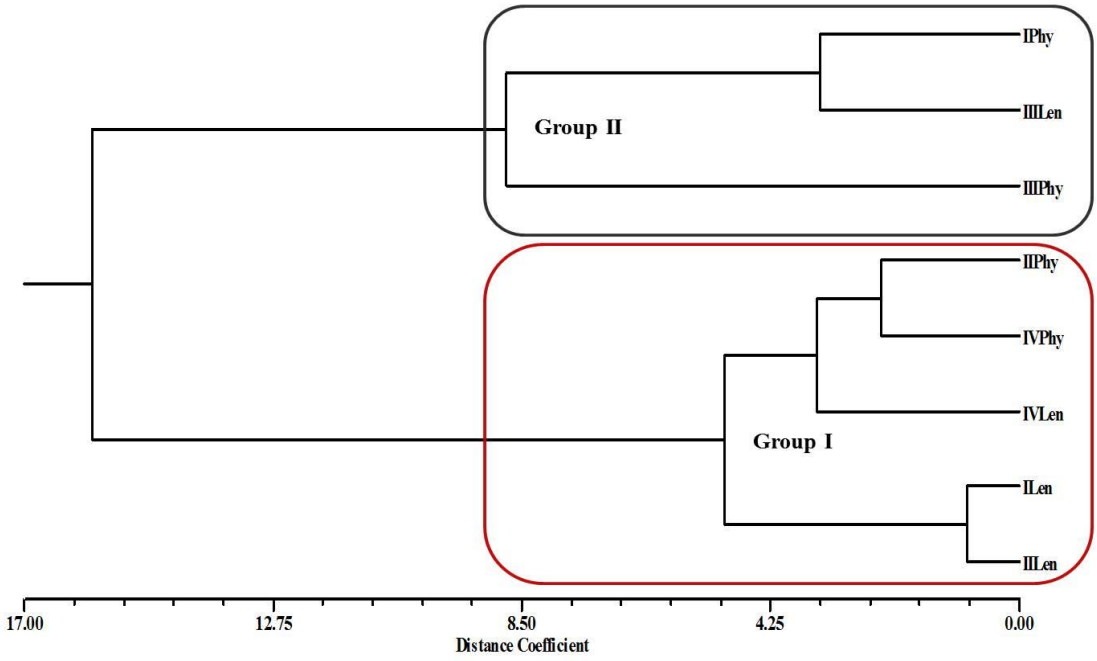

FIG. 5. PHENOGRAM OF THE RESPONSE OF *PHYALIS IXOCARPA* AND *LENS ESCULENTA* STEM CUTTINGS RELATED WITH THEIR PLANT GROWTH TRAITS (r = 0.83).

IV. CONCLUSION

Finally as a conclusion of this work, there was a plant growth promoting effect of *Pseudomonas* sp. strain C2, an auxin producer. Where two particularly responses were obtained: root production and shoot elongation in *Physalis ixocarpa* and only shoot elongation in *Lens esculenta* stem cuttings. In both plants their mass clonal propagation response was clearly related to their genetic nature, although there was evident the stimulation of growth by the presence of the inoculated *Pseudomonas* sp. strain C2.

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