

## Effect of Phytohormones and Rhizobacteria on *Epithelantha micromeris* Callus Growth

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### ABSTRACT

The employ of *in vitro* systems allows the dissection of complex interaction between plants and microorganisms, by the analysis of stress factors involved in the plant's response. Sterile cell cultures are reproducible during long periods and help to the measurement of physiological activities. Plant growth promoting rhizobacteria (PGPR) are a group of microorganisms that are involved in plants' growth promotion. This study evaluated the effect of particular phytohormones and a plant growth promoting rhizobacteria on the growth and viability of *Epithelantha micromeris* callus culture. *E. micromeris* callus growth without the inoculant, diminished in Murashige-Skoog (MS) + naphthalene acetic acid (NAA) medium (34.78%) and MS + indole acetic acid (IAA) (26.08%) experiments. In inoculated callus (B) there was a little promoting effect in: MS+NAA+B (17.39%), MS-VITAMINS+B (30.43%); MS+B (47.82%) experiments, and particularly case was MS+IAA+B experiment, where callus gain 30.43% biomass above the control values obtained. In this work, the presence of some particular auxins and their concentration on non-inoculated and inoculated *Epithelantha micromeris* callus with a plant growth promoting rhizobacteria, showed a diminished response and even there wasn't a notably cell dead and the effect of the suggested bioauxins were not evident, the gain of callus fresh biomass was present.

#### Keywords

Indole acetic acid,  
Naphthalene acetic  
acid, Callus  
culture, PGPR.

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### Introduction

The use of *in vitro* systems allows the dissection of the complex interaction between plant, soil, and microorganisms, by the analysis of stress factors that affects metabolism and specific enzymes involved in the plant's response. Golan-Goldhirsh *et al.*, (2004) mention that the advantages of plant tissue cultures can be summarized in two principal aspects: their reproducible growth during long periods and sterile cultures allows the measurement of physiological activities

that can be controlled by environmental conditions. Plant growth promoting rhizobacteria (PGPR) are a group of microorganisms which colonize rhizosphere producing molecules and are able to increase plant growth and/or to protect plants against pathogens (Glick, 1995; Russo *et al.*, 2008); they are also involved in many important ecosystem processes, such as nutrient cycling, seedling establishment and soil quality (Barea and Azcón-Aguilar, 2002) and possess a

valuable potential in agriculture and forestry (Kloepper *et al.*, 1989; Chanway *et al.*, 1991); but not all PGPR's are equally effective in promoting plant growth (Kloepper *et al.*, 1988).

The ability to produce plants phytohormones (particularly auxins) is widespread among soil, epiphytic and tissue colonizing bacteria (Costacurta and Vanderleyden, 1995; Patten and Glick, 1996; Barazani and Friedman, 1999), The genera: *Azospirillum*, *Azotobacter*, *Enterobacter*, *Xanthomonas*, *Serratia*, *Pseudomonas*, among others; have shown that enhance plant growth by promoting the outbreak of secondary roots and acting as protectors' against phytopathogenic microorganisms via plant phytohormones release (Tien *et al.*, 1979; Fett *et al.*, 1987; Zimmer and Bothe, 1988; Minamisawa and Fukai, 1991; Amströen *et al.*, 1993; Glick, 1995; Patten and Glick, 1996).

Evans Blue is a non-permeating dye with low toxicity in plant cells. It is a commonly used chemical to selectively stain dead cells (Gaff and Okong'o-Ogola, 1971; Turner and Novacky, 1974). This technique has been used by Song *et al.*, (1999) to determine the relative number of dead cells in a plant cell culture population.

*Epithelantha micromeris* (Engelm.) F. A. C. Weber ex Britt. & Rose is an appreciated cactus by national a foreign collectors; as these balloon-like cacti are very rare, they are highly appreciated by ornamental plant producers and nursery workers as it is a demanded product by the national and international markets (Leszczyńska, 1990; Villavicencio-Gutiérrez *et al.*, 2012). This study evaluated the effect of the presence of particular phytohormones and a plant growth promoting rhizobacteria on the growth and viability of *Epithelantha micromeris* callus culture.

## Materials and Methods

### Callus culture inoculant

#### The rhizobacteria employed

*Pseudomonas* sp. strain C2, was isolated by Melo *et al.*, (2011), from the rhizosphere of *Sporobolus indicus* L. R. Br., grown in a metal contaminated soils located in Villa de la Paz in the state of San Luis Potosí, México and categorized by its *in vitro* production of indole acetic acid (IAA) according to Khalid *et al.*, (2004) as higher producer with 18.7 to 25 µg/mL.

Bacterial inoculum were obtained by culturing rhizobacteria strain on plates with Luria-Bertani (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  $5 \times 10^7$  cells/mL.

#### *Epithelantha micromeris* callus culture maintenance

*E. micromeris* callus tissue was grown and maintained on Murashige and Skoog (MS)<sup>1/4</sup> salts medium (Murashige and Skoog, 1962) supplemented with 1mg/L NAA (naphthalene acetic acid) and 10mg/L of 6-Benzylaminopurine (BAP) at 28°C and subcultured every 3 weeks.

#### *E. micromeris* callus bioassay

*E. micromeris* callus of 1cm<sup>2</sup> were fractioned and four callus pieces were deposited in Petri dishes with sterile filter paper of medium pore, in each experimental conditions considered: control treatment with 0.5mL of MS <sup>1/4</sup> salts medium (MS); 0.5mL of MS + NAA (only naphthalene acetic acid, 2mg/L); 0.5 mL of MS + IAA (only indole acetic acid, 2mg/L); 0.5mL of MS-VIT (without vitamins and phytohormones) and inoculated

treatments with 0.25mL of the prepared rhizobacteria inoculum (B) and 0.25mL of each treatment as follows: MS+B; MS+NAA+B; MS+IAA+B and MS-VIT+B.

Petri dishes were sealed with Parafilm to prevent water loss and incubated in dark at room temperature (30°C) for fifteen days. All the experiments were performed by quadruplicate and after incubation; each callus pieces were visually analyzed for the appearance of injury due to medium effect or inoculation, following the criteria of Souissi and Kremer (1998), regarding to tissue discoloration, growth reduction or tissue disintegration.

Callus pieces were recovered and fresh weight obtained. Finally, their cell viability, after treatments, was determined according to the method described by Souissi and Kremer (1994; 1998), modified by Toledo-Salas (2012) as follows: callus pieces were deposited in 10mL assay tubes with 1mL of 0.5% aqueous Evans Blue solution, incubated at room temperature for 10 minutes; solution was decanted and the excess of stain was removed with deionized water, until the final rinse was uncolored. Callus pieces were homogenized with a Potter-Elvehjem and suspended in 1mL of distilled water. The cell suspensions were centrifuge at 3,000 rpm at room temperature for 3 minutes; cell pellet was discarded and supernatant recover, and the final stain bound to dead cells was quantified spectrophotometrically by measuring the absorbance at 630 nm. Finally, the Evans Blue concentration was determined employing a standard curve of this reagent.

All data were analyzed by one-way analysis of variance and the mean differences were compared applying a Tukey-Kramer Method using the statistics program Graph Pad InStat Ver. 2.03. The relationship between the callus fresh biomass and Evans Blue concentration

of control and inoculated experiments was also analyzed by regression analysis using the Paleontological Statistics Software PAST Ver. 2.17b.

## **Results and Discussion**

### **Growth of *E. micromeris* callus culture**

Souissi and Kremer (1998) mention that these kind of bioassays are useful to distinguish between the effect of inoculant and the absence of it; showing if the presence of rhizobacteria produces an inhibitory or promontory effect on callus growth.

In this study, the highest visual damage observed in *E. micromeris* callus was in the presence of IAA, showing a diminished growth and browning color. Although the rating systems proposed by Souissi and Kremer (1994; 1998) based on visual observations of callus pieces are important; the most recommended and consistent measurement is the quantity of the fresh weight cell or callus biomass. Figure 1a shows *E. micromeris* callus growth in each experimental conditions; compared to the control where callus culture grown in MS medium; there was a notable effect between non inoculated callus and inoculated with the plant growth promoting rhizobacteria. Callus fresh weights were expressed taking as the 100% of non-inoculated control callus pieces; *E. micromeris* callus growth without the inoculant, diminished in MS + NAA (34.78%) and MS + IAA (26.08%) experiments. In inoculated callus there was a little promoting effect in: MS+NAA+B (17.39%), MS-VIT+B (30.43%); MS+B (47.82%) experiments, and particularly case was MS+IAA+B experiment, where the callus gain a 30.43% biomass above the control values obtained. This response was agree with the results reported by Souissi and Kremer (1998) whose mention that symptoms caused

by the presence of rhizobacteria as bioinoculants not always promote the cell growth; and according to Goodman *et al.*, (1986) they can elicit injury depending to the mode of action and biochemistry of plant-rhizobacteria interaction, particularly in the callus culture.

Smolenskaya *et al.*, (2007) reported that the presence of growth regulators and their proportions are important factors for the maintaining of cell lines in cultures. Regarding to this commentary, El-Mahrouk and Belal (2007) mention that the effect of growth regulator on tissue cultures can vary according to the chemical nature of the compound, plant species, type of culture and even the developmental state of the explant (Lakshmanan *et al.*, 2002).

In this study, the phytohormones assayed and their concentrations in *E. micromeris* callus culture; particularly, the IAA concentration inhibited the callus growth; even some authors reported that exogenous auxins are the most important growth regulators for the induction of embryogenic callus in the majority of the angiosperms (Roy and Banerjee, 2003).

Regarding to the NAA, Smolenskaya *et al.*, (2007) and Meftahizade *et al.*, (2010); reported that this phytohormone is a common auxin that ensures the growth and viability of cell cultures. In this study, the *E. micromeris* callus notably diminished their growth in 65% less than control callus and even more in MS+NAA inoculated callus by 83%.

This response could be related to the fact that NAA in cell cultures retards cell division but promotes cell elongation. Smolenskaya *et al.*, (2007) mention that the reason for its action is because it activates different pathway controls in cell division and cell elongation. In this study, *E. micromeris* callus biomass was higher when media contained the adequate

supply or concentration of growth regulators; but not vitamins. Bacteria inoculation in MS-VIT medium showed a slightly promoting effect.

### ***E. micromeris* callus viability**

Cell viability determined by the quantity of Evans Blue allows the measurement of non-viable cells proportion; showing in this study that there was a direct association between callus biomass and cell death; particularly in Ms+IAA experiment. Figure 1b showed that the increase in Evans Blue percentage reflects the cell death in the callus culture tested.

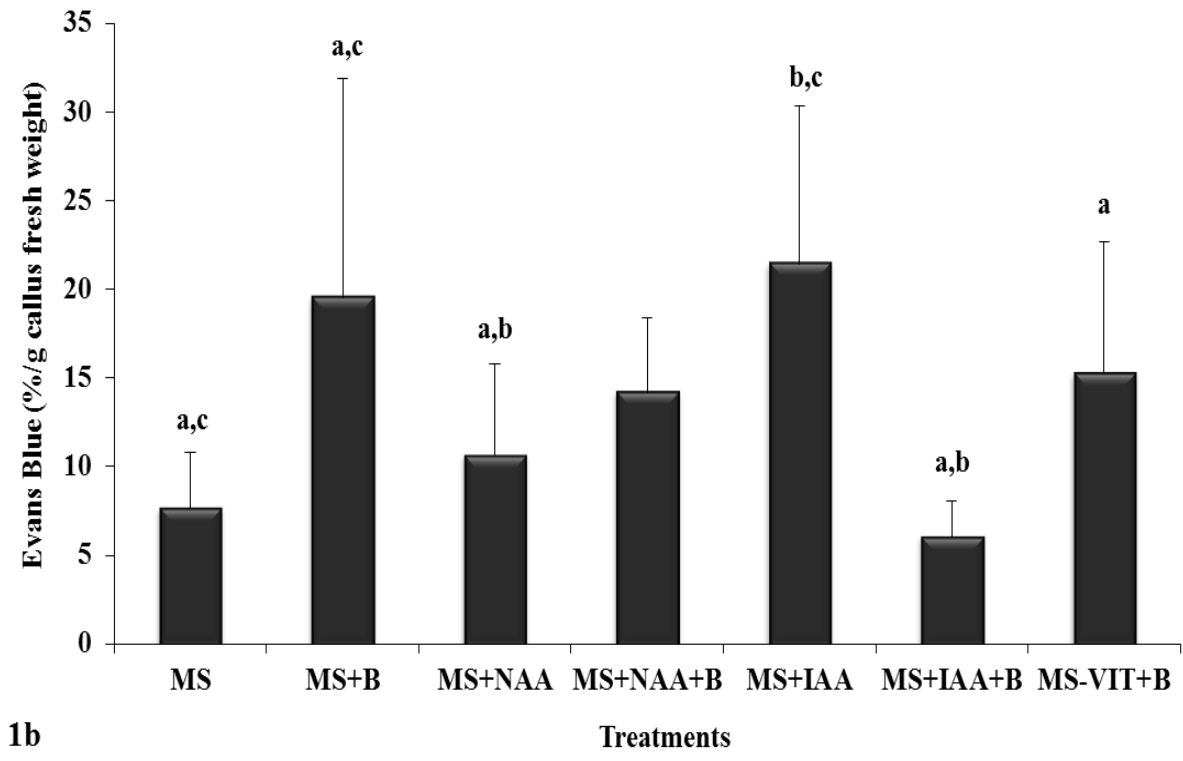
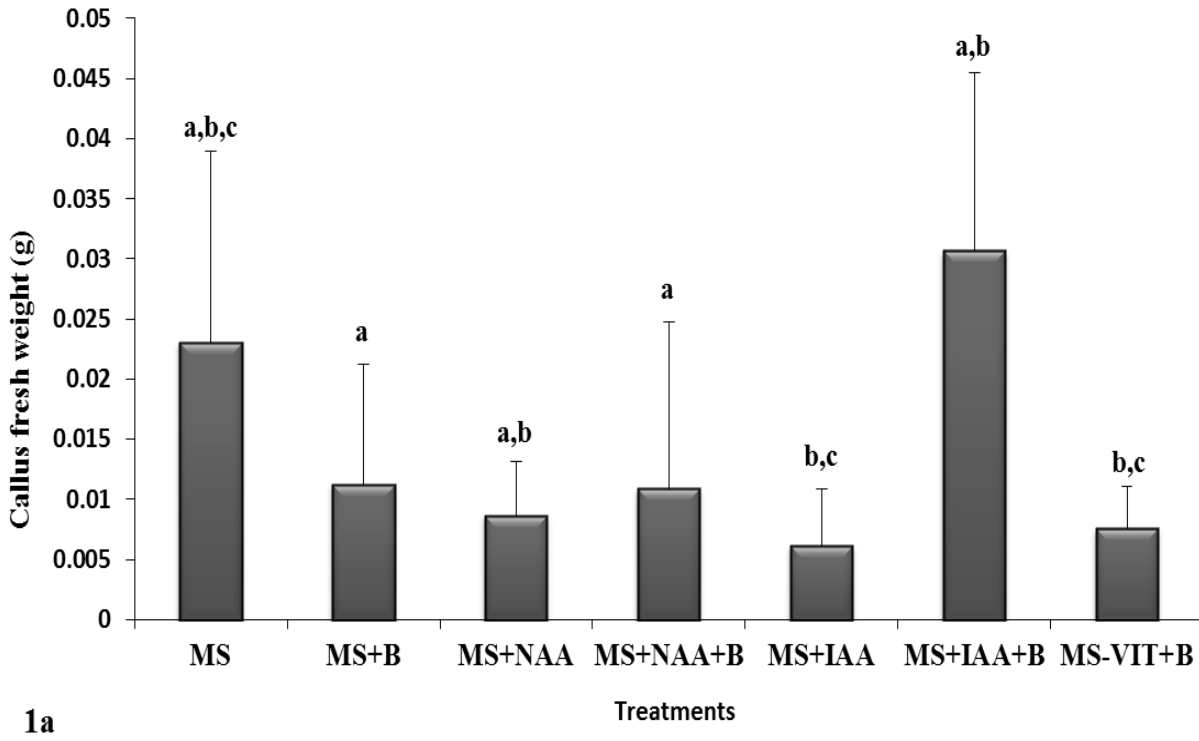
### **Direct effect on biomass and cell viability of *E. micromeris* callus**

Quantitative analysis of the responses of *E. micromeris* callus grown in different media conditions and inoculated with plant growth promoting rhizobacteria, is finally presented in callus biomass and quantitative cell viability measurements of Evans Blue solution at 630nm of callus extracts detecting their sensitivity according to Souissi and Kremer (1998).

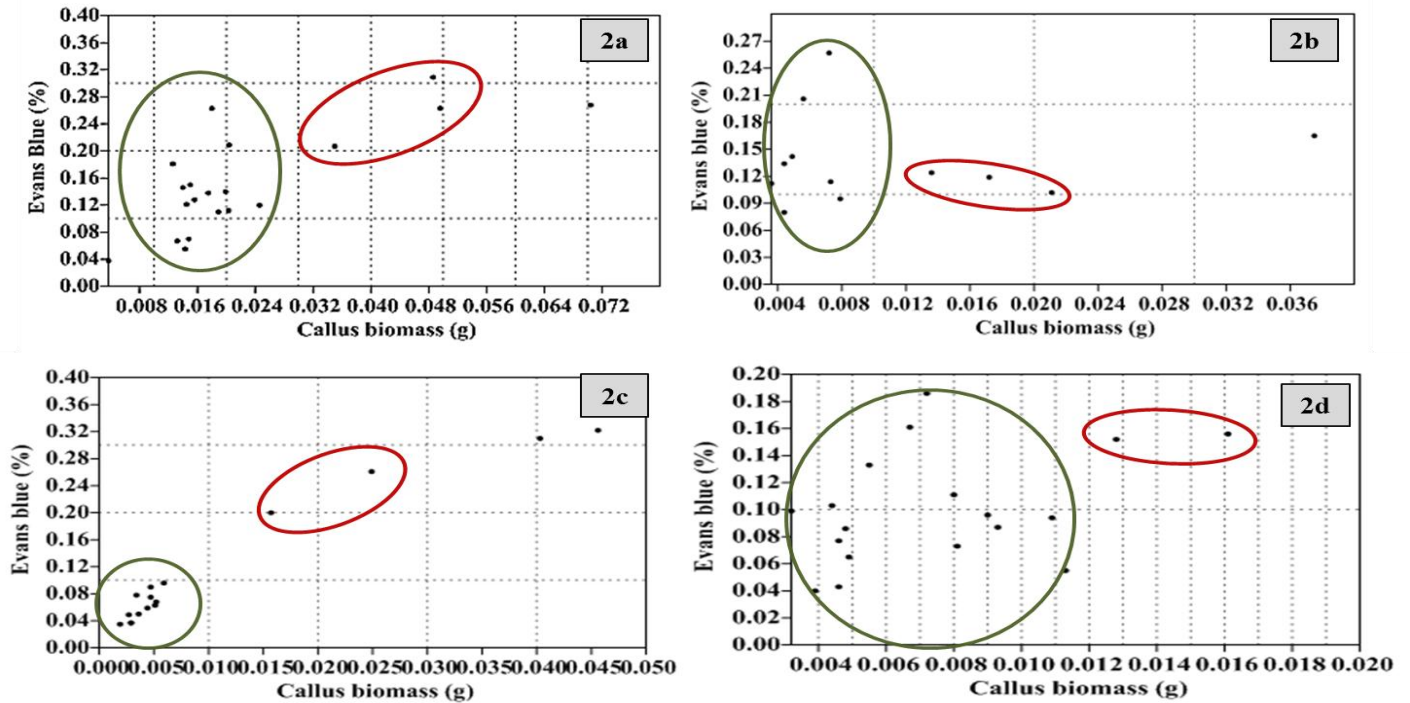
Regarding to the effect of the presence of *Pseudomonas* sp. strain C2 and *E. micromeris* callus growth; Figure 2 shows the regression analysis of all the bioassays inoculated with this rhizobacteria; all figures indicated a positive linear relationship between the Evans Blue concentration of callus extracts and callus fresh weight: MS + NAA + B ( $r = 0.96$ ); MS ( $r = 0.75$ ); MS-VIT+B ( $r = 0.38$ ) and MS+B ( $r = 0.031$ ).

These results are agree with Souissi and Kremer (1998) regarding to the confirmation of callus growth and quantitative cell viability measurements, showing callus sensitivity to the presence of the plant growth promoting rhizobacteria.

**Fig.1** *Epithelantha micromeris* callus growth. **1a)** callus fresh biomass and **1b)** dead cells quantified by Evans Blue reagent. (n=16, the different lower-case letters shows the significant differences founded [p < 0.001])



**Fig.2** Linear regression curves showing the relationship between *Epithelantha micromeris* callus biomass (g) and Evans Blue (%): **a)** MS ( $p < 0.05$ ,  $r = 0.75$ ), **b)** MS+B ( $p < 0.05$ ,  $r = 0.031$ ); **c)** MS+NAA+B ( $p < 0.05$ ,  $r = 0.96$ ) and **d)** MS-VIT+B ( $p < 0.05$ ,  $r = 0.38$ ). Groups of individual callus are shown in the plots



The comparison between callus fresh biomass obtained in control experiments (10 to 20mg) with the inoculated experiments, do not showed a notably increase of it. Even all the experiments presented almost the same values of Evans Blue concentration (0.04 to 0.20%); suggesting a low proportion of dead cells. Individual *E. micromeris* callus showed an increase of fresh biomass at the fifteen days of growth, with the following order: MS-VIT+B (4 to 10mg); MS+B (4 to 8mg) and finally, MS+NAA+B (1 to 5 mg). According to Chanway and Nelson (1991), whose mention that the composition of growth medium significantly affect callus biomass; the results reported in this study were agree with these authors regarding to the response of *Glycine max* L. (Merr.) var. Acme, where callus biomass diminished when grown on medium with NAA at low concentrations. They also reported the diminished effect on *G. max* callus inoculated with *Pseudomonas*

*putida* strain G2-8, with consistent biomass reduction in medium without vitamins; giving some possible causes of this effect associated to nutrients competition between plant cells and bacteria or the production of growth inhibitors by this bacteria. In this study, the presence of *Pseudomonas* sp. strain C2 did not promote the callus growth in this system and this could be associated to some rhizobacteria mechanisms that are not related to vitamins and/or auxins production, as these authors noted.

In this work, the presence of some particular auxins and their concentration on non-inoculated and inoculated *Epithelantha micromeris* callus with a plant growth promoting rhizobacteria, showed a diminished response and even there wasn't a notably cell dead and the effect of the suggested bioauxins were not evident, the gain of callus fresh biomass was present.

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