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Enzymatic response of *Lemna gibba* plants inoculated with endophyte bacteria

Luis Eduardo Gutiérrez-Andres, Jorge Adrián Paz-Delgado and Angélica Rodríguez-Dorantes *

Plant Physiology Laboratory, Botany Department, School of National Biological Sciences, National Polytechnic Institute, México City 11340, México.

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Abstract

Duckweeds a kind of aquatic plants classified as macrophytes, have been considered as nutrient pumps. Studies of endophyte associates focused to their functional analysis contributing to the knowledge of plant microbe interactions. This work analyzed the effect on selected phenoloxidases as a response of *Lemna gibba* plants inoculated with endophyte bacteria associated to their activity of growth promotion. Five isolated endophyte bacteria belonging to Bacillus genera and named: *Bacillus* **sp.** strain Fb1, *Bacillus* **sp.** strain Fb2, *Bacillus* **sp.** strain Fb3, *Bacillus* **sp.** strain Fb4 and *Bacillus* **sp.** strain Fb5 were employed as inoculants. According to the fresh weight of *Lemna gibba* fronds, the results obtained showing a suitable promoting activity by the endophyte bacteria tested; even the growth of fronds based on protein content was less, it showed growth promotion between 30 to 60% of the effect compared to control plants. In this work, *Lemna gibba* plants inoculated with five endophyte bacteria, showed that these ***Bacillus*** strains not only acting as biostimulants they also induced the activity of both enzymes tested (guaiacol peroxidases and hemeperoxidases), recommending this plant species to evaluate the toxicity effect of contaminants and xenobiotics.

Keywords: Plant growth-promoting endophyte bacteria; Phenoloxidases; *Lemna gibba;* Xenobiotics

1 Introduction

Bacteria are related with aquatic plants and affect them by the promotion or inhibition of their growth and are considered as adequate biotechnological strategy [1-4]. Some studies of *Lemna* and its associated bacteria were reported and included microscopic observations and isolation of bacteria from plant surfaces [5], showing that they promote plant growth by their bacterial production of plant hormones [6,7]. Duckweeds including *Lemna gibba* are small (1-15 cm) free-floating monocotyledon aquatic plants that belonging to the family Lemnaceae and can be used as an indicator species to assess ecotoxicity of waters polluted by contaminants [8-11]. They have a small size and rapid growth rate by clonal proliferation of their simple structure and morphology and high degree of homogeneity [10, 12-15]. Some authors reported that these plants are more sensitive to chemicals and can be employed as useful biomarkers [16-18], showing changes in biochemical and physiological processes at the cellular and tissue levels [19]. It is known that oxidative stress can be induced by oxygen deprivation with a direct photoreduction of O_2 to O_2^- by reduced electron transport associated with the photo-respiratory cycle [20]. The mechanisms existing in plant cells can also be stimulated to regulate the overproduction of reactive oxygen species (ROS) [21, 22], including the activation of antioxidant enzymes like peroxidases [23] and some antioxidative responses of *Lemna* under high concentrations of heavy metals [24] and organic compounds [25] showing their physiological tolerance [26]. The aim of this work was to evaluate the enzymatic response of *Lemna gibba* plants inoculated with endophyte bacteria related to their plant growth promoting effect in this plant species.

*Corresponding author: Angélica Rodríguez-Dorantes
Plant Physiology Laboratory, Botany Department, School of National Biological Sciences, National Polytechnic Institute, México City 11340, México.

2 Material and methods

2.1 Culture of *Lemna gibba* plants

Lemna gibba plants were hand collected from a selected area in the Xochimilco Lake characterized by water channels system related to its land use and environmental conditions according to Lopez-López et al. [27] and Ortega-Acosta et al. [28], with an agricultural zone adjacent to the water channels knowing as "Chinampa zone". Plants were surface sterilized with 10% sodium hypochlorite treatment (1minute), rinsed with distilled sterile water (3 times) and cultivated in Baby Gerber Flasks with Magenta B-caps (Sigma-Aldrich), containing 50mL of mineral medium diluted 1:4 containing: 0.20M $NH_4H_2PO_4$, 0.50M NH_4NO_3, 1.15M $Ca(NO_3)_2$, 0.26M $CaCl_2$, 0.20M $MgCl_2 \cdot 6H_2O$, 0.20M $Mg(NO_3)_2 \cdot 6H_2O$, 0.40M $MgSO_4 \cdot 7H_2O$, 0.20M KH_2PO_4, 1.20M KNO_3, 0.50M K_2SO_4, 0.040M $FeCl_3 \cdot 6H_2O$, 1.2 x 10^{-2}M H_3BO_3, 1.2 x 10^{-4}M $CuCl_2 \cdot H_2O$, 2.3 x 10^{-3}M $ZnCl_2$, 4.4 x 10^{-4}M $MnCl_2 \cdot 4H_2O$, 6 x 10^{-6}M $Na_2MoO_4 \cdot H_2O$, EDTA and $FeSO_4 \cdot 7H_2O$, adjusted pH= $\pm$ 6.0, in a growth chamber (28°C, photoperiod of 8h day/8h night cycle with a Philips Linear Fluorescent 32-Watt, 5000°K PLUS T8 Natural light bulb).

2.2 Inoculation of *Lemna gibba* plants with selected endophyte bacteria

The selected endophyte bacteria: *Bacillus* sp. strain Fb1, *Bacillus* sp. strain Fb2, *Bacillus* sp. strain Fb3, *Bacillus* sp. strain Fb4 and *Bacillus* sp. strain Fb5; were employed as inoculants and these were characterized by their Indole Acetic Acid (IAA) production according to Ortega-Acosta et al. [28], as higher producers. Bacterial inoculums were obtained by culturing the isolated endophyte strains on plates with Nutritive Agar medium for 48 h at 28°C. *Lemna gibba* plants from culture were selected and 5 plants of 1mm diameter per flask were transfer to small Petri dishes (35x15mm) containing 10mL of 1:4 diluted mineral medium. These were directly inoculated with a calibrate loop (1/1000 cells) of each selected endophyte bacteria in each flask. Experiments: Fb1, Fb2, Fb3, Fb4 and Fb5 and control plants considered as not inoculated; were performed by triplicate and maintained under controlled conditions in a growth chamber for 10 days. At the end of the bioassays, plants were harvested, excess water was absorbed in sterile paper towel, and *Lemna gibba* plants images of each experiment were obtained using Kodak Easyshare C713Zoom Digital Camera. Fresh weight of fronds was obtained and all were employed for the evaluation of protein content and enzymatic activity. The Effect of Plant Growth (EPG) by endophyte bacteria on *Lemna gibba* plants was determined at the end of the bioassays according to Ishizawa et al. [4] based on the fresh weight and protein content of fronds as follows: EPG-FW (%) = 100 x (FWb – FWc) / FWc, where: "FWb" was the fresh weight of fronds inoculated with endophyte bacteria and "FWc" was the fresh weight of control fronds; and EPG-Prot (%) = 100 x (Protb – Protc) / Protc, where: "Protb" was the protein content of fronds inoculated with endophyte bacteria and "Protc" was the protein content of control fronds.

2.3 Protein quantification and enzymatic analysis of *Lemna gibba* plants

Fronds from each experimental condition were homogenized in ice with a Potter-Elvehjem with 5 mL of 50 mM sodium phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH 6.0) containing 0.1 mL β-mercaptoethanol. The homogenates were centrifuged at 5000 rpm for 30 minutes at 4°C and supernatants were employed for protein quantification and enzymatic activities. Protein content was determined according to Bradford method [29] using 100 µL of supernatant and bovine serum albumin for standard curve preparation. Guaiacol peroxidases (GPX) activity was quantified according to the method of Guerrero and Rodríguez [30]; 100 µL of supernatant were deposited in tubes that contained 1mL of phosphate buffer 100mM pH = 6.0 + 32µL of guaiacol 0.2M + 32µL of H_2O_2 0.03M, this reaction mixture was incubated at room temperature for 15 minutes and the absorbance was read at 436nm. The activity of GPX consider the extinction molar coefficient of guaiacol (ε_{436}= 6,400 M/cm) and expressed as nM of oxidize guaiacol/min/ fresh weight g fronds. Hemeperoxidases (HPX) activity was quantified according to the method of Guerrero and Rodríguez [30] 2005); 100 µL of supernatant were deposited in tubes that contained 1mL of sodium acetate buffer 0.20M pH = 5.3 + 100µL of tetramethylbenzidine (TMBZ) 0.2M + 50µL of H_2O_2 0.17%, this reaction mixture was incubated at room temperature for 15 minutes and the absorbance was read at 652nm. The activity of HPX consider the extinction molar coefficient of TMBZ (ε_{652}= 39,000M/cm) and expressed as nM of oxidize TMBZ/min/ fresh weight g fronds.

2.4 Statistical analysis

All data obtained 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. A numerical comparative analysis considering the experimental conditions except the control was done; a distance matrix was built using the euclidian distance coefficient, a phenogram was build using the unweighted pair group method of arithmetic averages (UPGMA) method and correlation coefficient of Pearson, also a matrix plot was obtained, all using the PAST Software (Paleontological Statistics Software Package) Ver. 4.09.

3 Results and discussion

3.1 Effect of endophyte bacteria on *Lemna gibba* growth

The effect of endophyte bacteria inoculation of *Lemna gibba* plants showed at first sign an increase in fronds growth compared to the control plants (Figure 1a). According to Ishizawa et al. [4] and Wang et al. [23], changes in fronds can estimate the gain biomass at small experimental conditions and are useful as appropriated growth parameters. In this work, the EPG calculated by the fronds fresh weight and protein content analyzed was an adequate indicator of the effect of endophyte bacteria inoculum added to the culture of *Lemna gibba* plants (Figure 1b) and is important to consider here that according to the size of the inoculum of a single strain the response may vary [31-33].

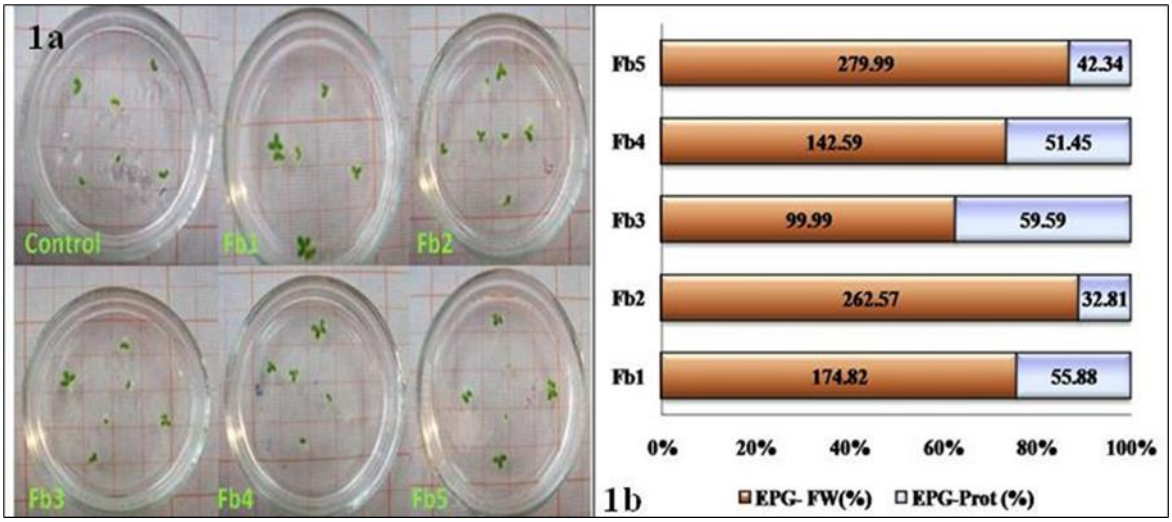

Figure 1 Endophyte bacteria and *Lemna gibba* plants response: 1a) selected image of *Lemna gibba* plants bioassay; 1b) promotion effect on *Lemna gibba* plants growth based on fresh weight (EPG-FW) and protein content (EPG-Prot) (values are mean values + SD from fifteen replicates; without significant difference between experiments: $p<0.001$)

According to the fresh weight of *Lemna gibba* fronds, the results of the EPG-FW obtained from the five endophyte bacteria were: *Bacillus* sp. strain Fb5 (279%) >*Bacillus* sp. strain Fb2 (262%) >*Bacillus* sp. strain Fb1 (174%) >*Bacillus* sp. strain Fb4 (142%) >*Bacillus* sp. strain Fb3 (99%), without statistical difference ($p\geq0.05$) showing a suitable promoting activity by the endophyte bacteria tested that could be associated with their IAA production and as Ishizawa et al. [3] and Glick et al. [35]noted; promotion of growth is one of the principal attributes of useful plant growth-promoting bacteria. The growth of *Lemna gibba* plants associated to protein content expressed by EPG-Protein percentage showed a growth response that even it was between 30 to 60% of the effect compared to control plants; *Bacillus* sp. strains Fb1, Fb3 and Fb4 induced almost the same percentage: 55, 59 and 51%, respectively. Followed by *Bacillus* sp. strain Fb5 (41%) and *Bacillus* sp. strain Fb2 (32%) (without statistical difference ($p\geq0.05$)).

3.2 Enzymatic response of *Lemna gibba* by the effect of endophyte bacteria

Figure 2 shows that GPX activity was notably higher than HPX activity in control plants, according to color degradation of the matrix plot for each enzymatic response. The presence of endophyte bacteria in cultures produce a remarkable effect on GPX (Figure 2a); *Bacillus* sp. strain Fb4 induced the highest activity followed by *Bacillus* sp. strains Fb3, Fb1, Fb5 and Fb2 with statistical difference between control and endophyte conditions tested ($p<0.001$). For HPX (Figure 2b) the control plants presented the less activity compared to the activity obtained by the inoculated plants: *Bacillus* sp. strain Fb3 followed by *Bacillus* sp. strains Fb4, Fb1, Fb5 and Fb2 (without statistical difference ($p\geq0.05$)). It is known that GPX activity may decrease if stress conditions overloaded the cellular defense system of plants and also may trigger enzyme inactivity [26, 36].

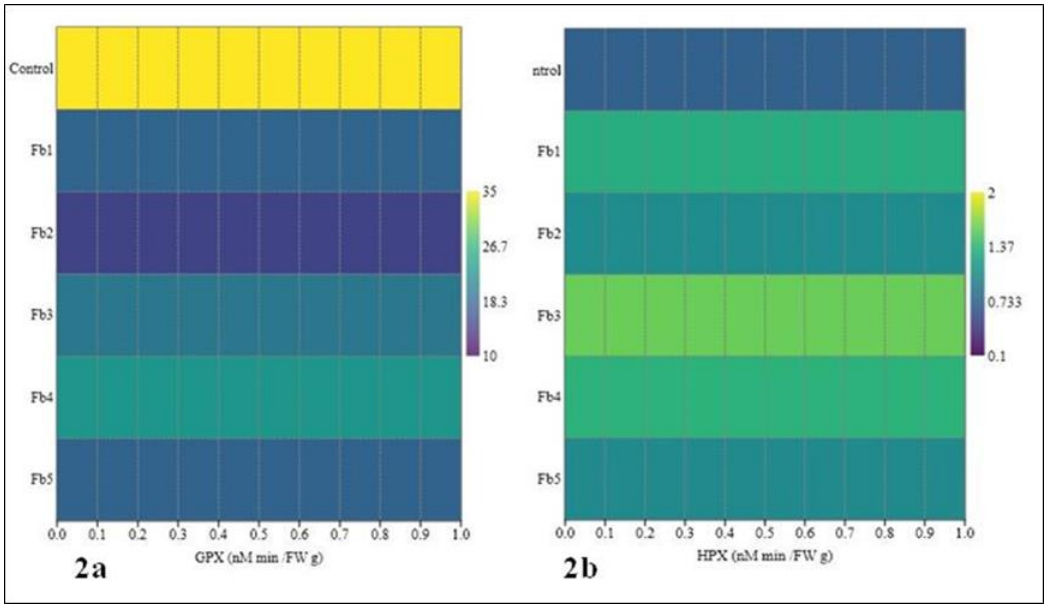

Figure 2 *Lemna gibba* matrix plot of enzymes activity: 2a) GPX and 2b) HPX (values are mean values from fifteen replicates)

3.3 Global response of *Lemna gibba* by the effect of endophyte bacteria

According to the numerical comparative analysis, in Figure 3a there was the association of groups according to the nature of the measured parameters; where two groups was forming at first: group I made only by EPG-FW and group II that includes all the rest of the parameters tested (Group IIa: GPX, Prot, FW and HPX) and EPG-Protein is separated of them (Group IIb), according to the colored scale of the matrix plot with a closer response. Figure 3b showed the relationship between the response of *Lemna gibba* plants inoculated with the selected bacteria; even *Bacillus* sp. strain Fb2 and *Bacillus* sp. strain Fb5 induced the highest EPG-FW value; their effect on enzymatic response was slight in all the experimental conditions tested. *Bacillus* sp. strain Fb1, *Bacillus* sp. strain Fb3 and *Bacillus* sp. strain Fb4 almost showed the same response in all the analyzed parameters.

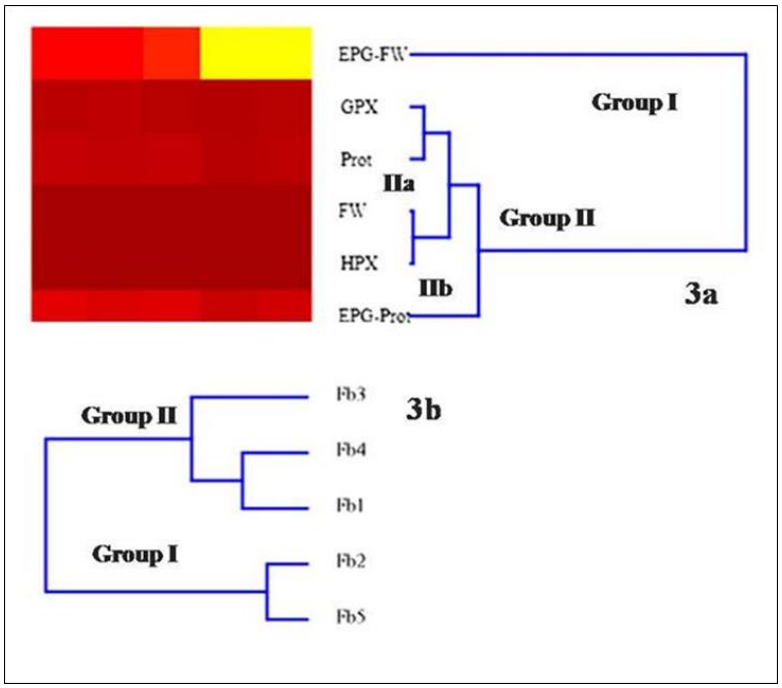

Figure 3 Phenogram comparing the experimental conditions tested (r = 0.87): 3a) parameters phenogram with matrix plot and 3b) endophytes phenogram

4 Conclusion

In this work, *Lemna gibba* plants inoculated with five endophyte bacteria, showed that these *Bacillus* strains not only acting as biostimulants for plant growth; they also induce the enzymatic activity of an important phenoloxidases markers that could apply to evaluate the toxicity effect of contaminants and xenobiotics in this kind of plants, particularly the hemeperoxidases response.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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