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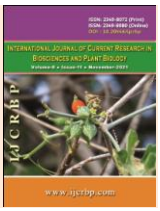

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# Endophyte bacteria growth promotion effect on *Lemna gibba* plants

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**Abstract**

Duckweeds a kind of aquatic plants classified as macrophytes, have been considered as nutrient pumps that can help to reduce the eutrophication effects. Studies of endophyte associates focused to their functional analysis contributing the knowledge of plant microbe interactions. This work compared the phytohormones *in vitro* production efficiency of endophyte bacteria isolated from *Lemna gibba* plants and their plant growth promoting effect in this plant species. Five 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 isolated. The IAA quantified as basal and inherent production without the addition of the amino acid tryptophan showed that *Bacillus* sp. strain Fb1, *Bacillus* sp. strain Fb3 and *Bacillus* sp. strain Fb5, were considered as higher producers (24.21, 25.44 and 30.8 µg/mL, respectively). Even the effect of plant growth (EPG) on *Lemna gibba* plants was between 40 to 70 %; a suitable promoting activity by the endophyte bacteria tested was obtained, and could be associated with their IAA production. This work contributes to the knowledge of the phytobacteria diversity in aquatic plants, particularly in Lemnaceae species; here the five endophyte bacteria could be candidates to be recommended as biostimulants.

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## Introduction

Duckweeds including *Lemna gibba* are small (1-15 cm) free-floating monocotyledon aquatic plants that belonging to the family Lemnaceae considered as macrophytes (Appenroth et al., 2017; Yoneda et al., 2021) are nutrient pumps (Leng, 1999; Hasan and Chakrabarti, 2009) that also help to eutrophication effects providing oxygen from their photosynthetic activity (Megateli et al., 2009; Verma and Suthar, 2015). It is known that they grow fast by clonal proliferation of their simple plant body structure involving fronds, floating juvenile tissue lacking stem and single or multiple root(s) (Appenroth et al., 2016; Chang et al., 2016; Okada et al., 2017; Yamakawa et al., 2018). Collection and analyses of plant-associated microorganisms, including Plant Growth Promoting Bacteria (PGPB) in this plant species is few an undeveloped (Yamaga et al., 2010; Suzuki et al., 2014; Tang et al., 2015; Appenroth et al. 2016; Berg et al. 2016; Yamakawa et al., 2018). It has been recognized that bacteria co-existing with aquatic plants

significantly affect the biomass production through growth promoting and growth-inhibiting effects on the host aquatic plants and are adequate as biotechnological strategy (Underwood and Baker, 1991; Lugtenberg and Kamilova, 2009; El-Deeb et al., 2012; Ogata et al., 2013; Ishizawa et al., 2017; Ishizawa et al., 2019a,b). There were reported some studies of *Lemna* and its associated bacteria that included microscopic observations and enumeration of bacteria on plant surfaces as well as several culture-dependent studies (Landolt, 1986), also it is known that bacteria promote plant growth by their bacterial production of plant hormones such as indole-3-acetic acid (IAA) (Rajkumar and Freitas, 2008; Sharma et al., 2013), cytokinins, and gibberellins (Idris et al., 2007). The aim of this work was to compare the phytohormones *in vitro* production efficiency of endophyte bacteria isolated from *Lemna gibba* plants and analyze their plant growth promoting effect in this plant species.

## Materials and methods

### Isolation and selection of endophyte bacteria from *Lemna gibba* plants

Three samples of *Lemna gibba* L. plants (100 g) with a phytoplankton net (60 cm x 25 μ) were 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. (2006) and Ortega-Acosta et al. (2017), with an agricultural zone adjacent to the water channels knowing as "Chinampa zone". These samples were deposited in Ziploc bags and transported in cold (4 °C) to the laboratory. The endophyte bacteria were isolated according to Ortega-Acosta et al. (2017) taken 10g of *L. gibba* plant biomass. Plants were surface sterilized with 10 % sodium hypochlorite for few seconds, rinsed several times with sterile distilled water and finally deposited in sterile mortar and pestle to homogenize them with 10 mL of sterile distilled water. The plants homogenize was transfer to sterile bottles containing 90 mL of sterile distilled water and the plant suspension from each sample were analyzed by appropriate dilutions ($10^{-1}$ and $10^{-2}$), taking 0.2 mL from each sample and placed on plates containing Nutrient Agar (NA) medium. The plates were incubated at 28 °C for 24 h and the endophyte bacteria were selected, maintained and preserved on NA medium plates, for their conventional bacterial analyzes (colonial morphology, microscopic morphology, Gram-staining). The endophyte bacteria isolates were identified by the determination of gene 16S rRNA sequences. Colony PCR was performed from live cell cultured on agar NA medium plates. Cells were harvested after 24 h and processed for DNA isolation using the Allers and Linchen procedure (2000). Using the purified genomic DNA, the molecular target gene 16S rRNA was amplified using universal primer set fD1 and rD1 designed by Weisburg et al. (1991). Aliquots of PCR reaction products were electrophoresed in 1 % agarose gel and then stained with ethidium bromide. These PCR products were purified and sequenced by the Unidad de Biotecnología y Prototipos of FES-Iztacala (UNAM). The sequences were then compared to similar sequences in the databases using BLAST analysis (Basic Logical Alignment Search Tool, BLAST at NCBI).

### Measurement of IAA production by the endophyte bacteria of *Lemna gibba*

The selected endophyte bacteria isolated from *L. gibba* plants were analyzed by their Indole Acetic Acid (IAA) production with the addition of the amino acid Tryptophan according to Ortega-Acosta et al. (2017), employing Salkowski reagent (Bric et al., 1991). The assays were done taking 4.9 mL of sterile nutritive broth media, added to culture tubes (10 x 15 cm) and supplemented with L-Trp at final concentrations of 2 and 5 mg/L. The culture tubes were inoculated with 0.2 mL of each endophyte bacteria inoculum: 5 x $10^7$ cells/mL in sterile distilled water and incubated at 28 ºC for 120 hrs. After the incubation, the cultures were centrifuged at 3,500 rpm, at 25 ºC for 45 minutes to discard the bacteria pellets and to recover the supernatant where the auxins were excreted; 2 mL of each supernatants were mixed with 2 mL of Salkowski's coloring reagent and the development of a pink color indicates IAA production and was quantified reading its absorbance at 535 nm and the concentration was estimated by a standard IAA curve. The assays with and without L-Trp were performed by triplicate.

### Culture of *Lemna gibba* plants

*Lemna gibba* plants also were hand collected from the study zone of Xochimilco, prior to plant growth promotion bioassays. About 0.5 g of plants were previously aseptically cultured for 15 days; these 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 50 mL of mineral medium diluted 1:4 contained: 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 16h/8h day/night cycle with a Philips Linear Fluorescent 32-Watt, 5000 °K PLUS T8 Natural light bulb).

### Effect on *Lemna gibba* plants growth with the selected endophyte bacteria

Bacterial inoculums were obtained by culturing the isolated endophyte strains on plates with NA medium for 48 h at 28 ºC. *Lemna gibba* plants from culture were selected and 10 plants of 1mm diameter per flask were transfer to baby Gerber Flasks and Magenta B-Caps containing 50 mL 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 quintuplicate 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 C713 Zoom Digital Camera. Number of total fronds was counted, total area of plants was measured ($mm^2$) and fresh weight of them was obtained. Finally, the growth promotion by endophyte bacteria on *Lemna gibba* plants was determined at the end of the bioassays according to Ishizawa *et al.* (2019b) analysis by the Effect of Plant Growth (EPG) as follows: EPG = 100 x (FNb – FNc) / FNc. Where: "FNb" is the frond number of endophyte bacteria treated plants and "FNc" is the frond number of endophyte bacteria free control plants.

### 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. Principal Component Analysis (PCA) was done with matrix data set of all the experimental conditions of bioassays at 10 days; employing the Pearson correlation with PAST (Paleontological Statistics Software Package) Ver. 2.17b.

## Results and discussion

### Endophyte bacteria from *Lemna gibba* plants: identification and their IAA production

The five isolated endophyte bacteria from *L. gibba* plants identified based on its 16S rDNA sequence homology analysis 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. The isolated endophyte bacteria were screened for their ability to produce plant growth regulator the IAA, recording without and with different concentrations of tryptophan (0, 2 and 5 mg/L) as inducer and precursor of IAA (Fig. 1a). The IAA quantified as basal and inherent production without the addition of the amino acid showed that in the endophytes bacteria: *Bacillus* sp. strain Fb1, *Bacillus* sp. strain Fb3 and *Bacillus* sp. strain Fb5, were considered as higher producers (24.21, 25.44 and 30.8 µg/mL, respectively). The increase of IAA production by the isolates as the concentration of Trp increased, was present only in *Bacillus* sp. strain Fb2. *Bacillus* sp. strain Fb1 and *Bacillus* sp. strain Fb3, showed the same tendency to increase their IAA production at 2 mg/L Trp concentration; with a decrease at 5 mg/L Trp concentration. Also *Bacillus* sp. strain Fb4 and *Bacillus* sp. strain Fb5 increased their IAA production with the highest Trp concentration. Even there was a less IAA production by *Bacillus* sp. strain Fb2, all of the endophyte bacteria according to Khalid et al. (2004) classification were considered as higher producers between 23 and 30.8 µg/mL. The total IAA production taking all the experimental conditions showed the next order of the isolated endophyte bacteria production: Fb5 (82.77 µg/mL) > Fb1 (77.14 µg/mL) > Fb3 (76.89 µg/mL) > Fb2 (69.03 µg/mL) > Fb4 (66.7 µg/mL).

### Biossays of *Lemna gibba* and the effect of endophyte bacteria inoculation

The effect of endophyte bacteria inoculation of *Lemna gibba* plants showed at first sign an increase in fronds growth compared to the control plants. Fig. 2 shows evident differences between control plants (1.8$\pm$2.1 $mm^2$) and inoculated plants regarding to the area reported from each frond at initial time (0 days) and final time (10 days), with the next order: *Bacillus* sp.

strain Fb5 (6.09±5.19 $mm^2$), *Bacillus* sp. strain Fb2 (5.73±5.2 $mm^2$), *Bacillus* sp. strain Fb1 (5.72± 6.6 $mm^2$), *Bacillus* sp. strain Fb4 (5.56±6.4 $mm^2$) and *Bacillus* sp. strain Fb3 (5.15±4.3 $mm^2$) without statistical difference ($p \leq 0.05$). The effect of plant growth (EPG) was determined and clear calculation of it showed the effect of endophyte bacteria inoculum added to the culture of *Lemna gibba* plants (Fig. 1b). It is important to note here that according to the size of the inoculum of a single strain the response may varied and the rationale for this note is that an adequate inoculum density probably shows more IAA concentration available to the plant as some authors reported (Loper and Schroth, 1986; Harari et al., 1988; Selvadurai et al., 1991; Evans et al., 1994; Sawar and Kremmer, 1995; Xie et al., 1996). The results of the EPG obtained from the five endophyte bacteria were: *Bacillus* sp. strain Fb2 (69.08 %) > *Bacillus* sp. strain Fb4 and *Bacillus* sp. strain Fb5 (54.54 %) > *Bacillus* sp. strain Fb1 (49.08 %) > *Bacillus* sp. strain Fb3 (39.99 %).

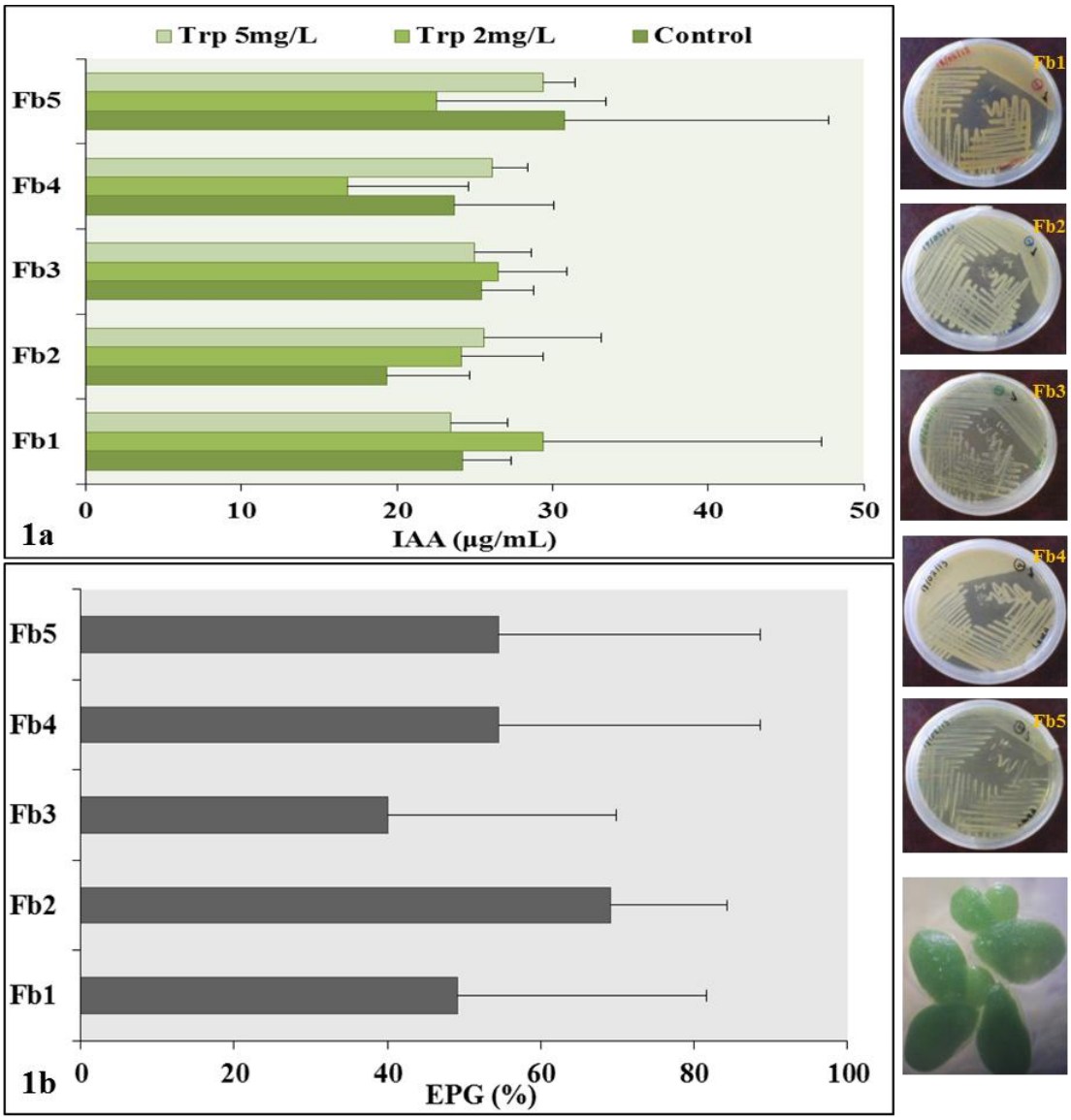


**Fig 1:** Endophyte bacteria and *Lemna gibba* plants response: **1a)** IAA production of the endophyte bacteria isolated (values are mean values + SD from three replicates; without significant difference between experiments: $p \leq 0.001$); **1b)** promotion effect on *Lemna gibba* plants growth (EPG) (values are mean values + SD from five replicates; without significant difference between experiments: $p \leq 0.001$).

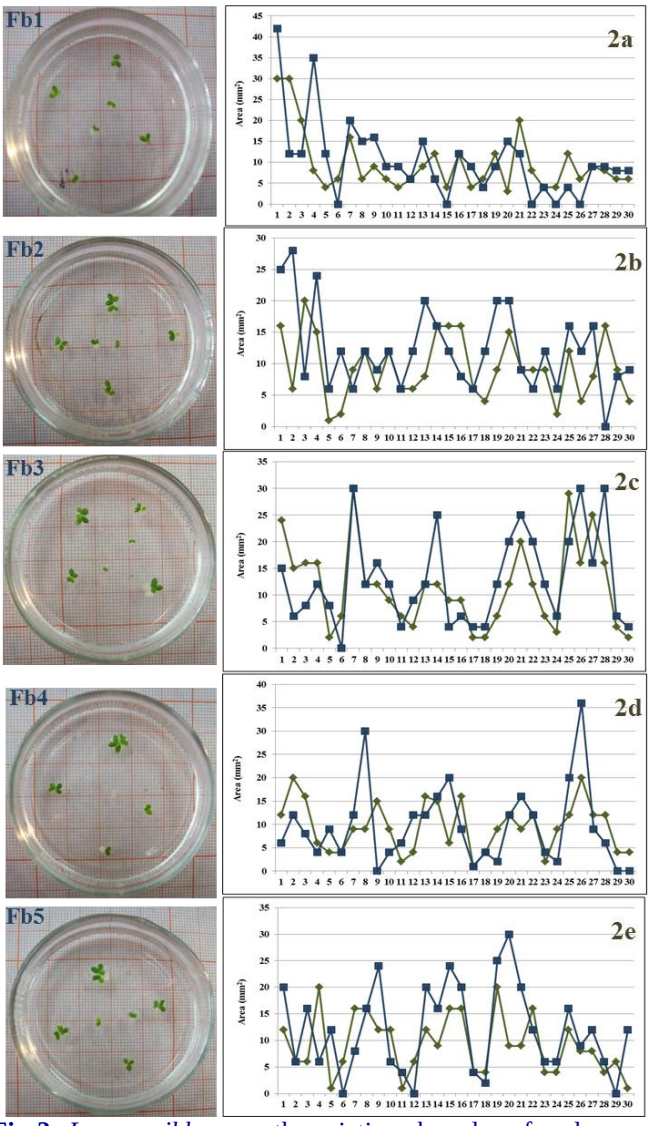


**Fig 2:** *Lemna gibba* growth variations based on fronds areas inoculated with: **2a)** *Bacillus* sp. strain Fb1; **2b)** *Bacillus* sp. strain Fb2; **2c)** *Bacillus* sp. strain Fb3; **2d)** *Bacillus* sp. strain Fb4; **2e)** *Bacillus* sp. strain Fb5 (values are mean values + SD from thirty replicates; without significant difference between experiments: $p \leq 0.001$).

Even it was between 40 to 70 % of the effect on *Lemna gibba* plants growth (without statistical difference, $p \leq 0.05$), a suitable promoting activity by the endophyte bacteria tested was obtained, and could be associated with their IAA production. It is important to mention here as Glick et al. (1995) and Ishizawa et al. (2017) noted that promotion of growth is one of the major functional markers of useful plant growth-promoting bacteria. Idris et al. (2004) reported that *Bacillus amyloliquefaciens* FZB42 showed a close correlation between plant growth promotion and auxin production in *Lemna minor* and the presence of tryptophan-like compounds induced the IAA synthesis in the endophyte bacteria that could be associated to the plants surface and colonize them. In this study, the Trp release by *Lemna gibba* plants could induce the association between them and selected endophyte bacteria.

Fig. 3 shows as a complement a principal component analysis (PCA) where Component 1 correlation values (95.65 %) indicates at first sign a particular positive association regarding only to IAA production and endophyte bacteria, between *Bacillus* sp. strain Fb4 and *Bacillus* sp. strain Fb5 with Basal (Control) and 5 m/L Trp conditions. Secondly; a particular relationship between total fronds number, *Bacillus* sp. strain Fb1, *Bacillus* sp. strain Fb2, *Bacillus* sp. strain Fb3 and IAA production in 2 mg/L Trp presence, where the highest average number of fronds were obtained at 10 days in the experimental conditions inoculated with *Bacillus* sp. strain Fb2 (19 fronds) followed by *Bacillus* sp. strain Fb1 (17 fronds) and *Bacillus* sp. strain Fb3 (15 fronds). These strains produced the highest IAA concentrations in presence of Trp 2 mg/L: Fb1 (29.43 µg/mL), Fb3 (26.5 µg/mL) and Fb2 (24.13 µg/mL). No association was obtained between *Lemna gibba* plants parameter tested: fresh weight, growth differences and frond areas at initial time (0 days) and final time (10 days) and endophyte bacteria tested (Component 2 correlation values: 2.93 %).

Matsuzawa et al. (2010); Yamaga et al. (2010); Xie et al. (2015); Quisehuatl-Tepexicuapan (2016) and Ishizawa et al. (2019b); reported a series of experiments performed to analyze the colonization dynamics of *Lemna minor* by three selected bacteria: *Aeromonas magnusonii* H3 and M3, and *A. excentricus* M6; strains isolated from the phytosphere of this plant species, where the growth-promoting effect of *A. ursingii* H3 was observed. Idris et al. (2007) reported that IAA-producing *Bacillus amyloliquefaciens* promotes the growth of *L. minor*. Even there has been reported not only by these authors, but also by Wang et al. (2014) and Ishizawa et al. (2019b), the frond area can properly estimate the gain biomass at small experimental conditions and changes in frond size could be an appropriated growth parameters. In this work, the EPG calculated by the fronds number produced and analyzed as indicator of the growth promoting action by the selected endophyte bacteria tested, was recommendable.

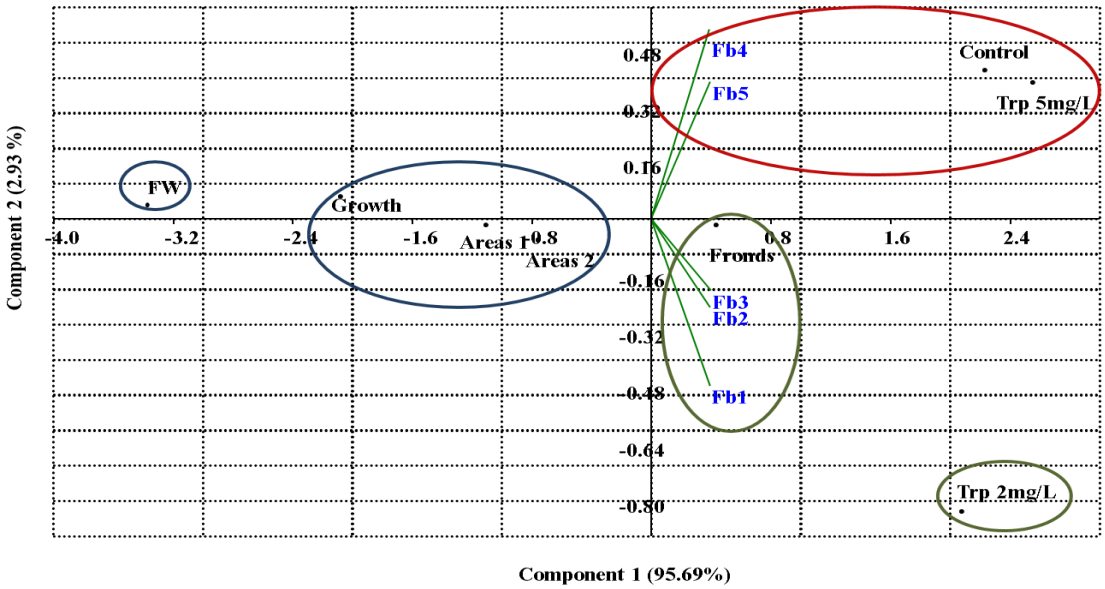


**Fig. 3:** Principal component analysis grouping the *Lemna gibba* all data set of experimental conditions from bioassays at 10 days and endophyte bacteria IAA production.

## Conclusions

This work contributes to the knowledge of the phytobacteria diversity in aquatic plants, particularly in Lemnaceae species, here the five endophyte bacteria were characterized as higher IAA producers and promoted the growth of *Lemna gibba* plants, suggesting these *Bacillus* strains as biostimulants in plant-microbe biossays.

## Conflict of interest statement

Authors declare that they have no conflict of interest.

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