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Characterization of indole acetic acid endophyte producers in authoctonus *Lemna* gibba plants from Xochimilco Lake

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Xochimilco's lacustrine zone is a network of channels that, along with the chinampas, conform a unique ecosystem which has served as source of aquatic resources. Duckweeds are small free-floating monocotyledon aquatic plants classified as macrophytes that serve as nutrient pumps and reduce eutrophication effects. Recently, there are number of new studies related to the aquatic plant-microbial interaction focused on the direct functional analysis that investigate plant microbe interactions at full biological hierarchy. The aim of this study was to compare the auxin *in vitro* production efficiency of the endophyte phytobacteria isolated from *Lemna gibba* L. plants collected nearby the Xochimilco aquatic agrosystem. There were 17 isolates obtained from the *L. gibba* plants collected in the dry season and 14 isolates for the rainy season. The environmental conditions and seasonal characteristics determined the number and identity of the isolated endophyte phytobacteria in *L. gibba* plants according to the several apparent differences in the water quality. This work contributes to the knowledge of the phytobacteria diversity in aquatic plants, particularly in Lemnaceae species; here the majority of the isolates have been characterized as higher indole acetic acid producers, recommended as candidates for their use as biofertilizers.

Key words: Plant growth-promoting bacteria, biofertilizers, *Lemna gibba*, Xochimilco.

INTRODUCTION

The Xochimilco's lake is located at the southern part of Mexico City basin and comprises a unique ecosystem which has served as source of aquatic resources, while its waters have been used for irrigation. The important of

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field cultures are named chinampas, artificial islands made by interweaving twigs and branches of trees filled with sediment from the lake's bottom (Quiroz et al., 1982; Juárez-Figueroa et al., 2003). López-López et al. (2006) and Gagné et al. (2002) mention that this lake had been used as a water supply by humans since the beginning of 1900, but began being negative influenced by the municipal effluent that have been discharged into it from Mexico City waste waters.

Duckweeds are one of the main groups considered as small (1 to 15 cm) free-floating macrophytes abundant in the Xochimilco's lake; that serve as nutrient pumps, reducing the eutrophication effects and providing oxygen from their photosynthetic activity; these plants are often seen growing on still nutrient rich fresh and slightly brackish waters (Hasan and Chakrabarti, 2009). Recently, there are number of new studies related to the aquatic plant-microbial interaction, particularly regarding to the duckweeds *Spirodela polyrrhiza* (Rahman et al., 2007), *Lemna minor* (Hou et al., 2007; Uysal and Taner, 2009), and *L. gibba*; focusing on the direct functional analysis that investigate plant microbe interactions at full biological hierarchy, starting with the genomic, transcriptomic, and proteomic analysis of plant-associated bacteria (Farinati et al., 2009; Stout and Nüslein, 2010). There were reports of 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); examples of this kind of studies are the works of Stout and Nüsslein (2005) whose compared the bacterial communities associated with the roots of *L. minor* plants; Yamaga et al. (2010) reported the response of phenol-degrading bacteria in the rhizosphere of *Lemna aoukikusa* to understand their beneficial symbiotic interactions. Sharma et al. (2013) mention that certain compounds produced by bacteria promote plant growth and Rajkumar and Freitas (2008) suggested that indole-3-acetic acid (IAA) increase plant biomass, this effect is produced by some plant-beneficial bacteria due to their bacterial production of plant hor-mones such as indole-3-acetic acid (IAA), cytokinins and gibberellins (Idris et al., 2007). Yamaga et al. (2010) and Suzuki et al. (2014) reported the characterization of plant growth promoting bacteria associated with aquatic plants; the authors isolated the phytobacteria *Acinetobacter calcoaceticus* P23 isolated from *L. aoukikusa*, and reported that this bacteria is a plant growth promoting bacteria associated with *L. minor*, *S. polyrhiza*, and *Wolffia arrhiza*. Their works demonstrated that this strain promotes the growth of *L. aoukikusa* to a 2-fold increase in growth rate (Yamaga et al., 2010). The authors reported that the bacteria colonized the plant surfaces and increase the chlorophyll content of *L. minor* and suggest that this isolated P23 has the potential to be a good biofertilizer. Idris et al. (2004) reported the results of a miniaturized biotest that analyzes the phytostimulatory effects by the isolated phytobacteria *Bacillus amyloliquefaciens* FZB42 on clones of *L. minor*; the authors showed that this Gram positive bacteria was able to produce significant amounts of IAA and its production increase after the addition of Trp (Idris et al., 2007). Koga et al. (1991) reported that the stimulation of IAA synthesis by tryptophan was described previously for gram-negative plant associated bacteria and Patten and Glick (2002) also employed a mutant of the gram-negative plant-beneficial bacterium *Pseudomonas putida*, with low IAA production that were added to *L. minor* fronds showing that plant-growth-promoting effects decreased. These authors demonstrated a close correla-tion of plant growth promotion and auxin production like the response did by *B. amyloliquefaciens* FZB42. The aim of this study was to compare the auxin *in vitro* production efficiency of the endophyte phytobacteria isolated from *L. gibba* plants collected nearby the Xochimilco aquatic agrosystem.

MATERIALS AND METHODS

Isolation and selection of endophyte phytobacteria from *L. gibba* plants

Three distinguish zones were selected in the Lake of Xochimilco, interconnected by a system of water channels according to their land use and environmental conditions (Lopez-López et al., 2006), namely: A) Chinampa zone ("CH", an agricultural zone adjacent to the water channels), B) Tourist zone ("T", a located zone of markets and boat rides on trajineras in water channels) and C) Urban zone ("U", where domestic waters are discharged into the water channels). Plants of *L. gibba* L. were collected from each water channel zone in two seasons: dry season [May, 2013 (MA)] and rainy season [August, 2013 (AG), in each selected channel there were established three sites along them, taken in each site samples of *L. gibba* plants (100 g) with a phytoplankton net (60 cm x 25 µ). The samples were deposited in Ziploc bags and transported in cold (4°C) to the laboratory. The endophyte phytobacteria were isolated according to Yamaga et al. (2010) suggestions; 10 g of *L. gibba* plant biomass was collected. 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 homogenate was transferred 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}); 0.2 mL was taken from each sample and placed on plates containing Nutrient Agar (NA) medium. The plates were incubated at 28°C in the dark for 24 h and the endophyte phytobacteria were selected and isolated according to their different colony morphology and maintained and preserved on NA medium plates, for their conventional bacterial test. The phytobacteria isolates were identified by the determination of gene 16S rRNA sequences. Colony PCR was performed from live cell cultured on 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 Basic Logical Alignment Search Tool analysis

(BLAST) at NCBI).

Measurement of the IAA production by the endophyte phytobacteria of *L. gibba*

The selected endophyte phytobacteria isolated from *L. gibba* plants were analyzed by their induction and efficiency of Indole Acetic Acid (IAA) production with the addition of the amino acid Tryptophan, according to the methods of Sheng and Xia (2006) and Zaidi et al. (2006), employing Salkowski reagent (Bric et al., 1991). Auxin production by the selected isolated strains was analyzed in the presence and absence of L-Tryptophan and determined by colorimetry (Melo et al., 2011). The assays were done taking 4.9 mL of sterile nutritive broth media, added to culture tubes (10 x 15 cm) without (control) and supplemented with L-Trp at final concentrations of 1, 2 and 5 mg/L. The culture tubes were inoculated with 0.2 mL of each rhizobacteria inoculum of 5 x 10^7 cells/mL in sterile distilled water. The culture tubes were incubated at 28°C for 120 h. After the incubation, the cultures were centrifuged at 3,500 rpm,at 25°C for 45 min 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 were performed by triplicate.

Statistical analysis

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. A numerical comparative analysis of the IAA production with and without the addition of L-Trp of the phytobacteria isolated from each zone was done; a distance matrix built using the conventional standard distance coefficient and a phenogram was resolved using the unweighted pair group method of arithmetic averages (UPGMA) method, and finally a correlation coefficient of Pearson was obtained using the version 2.11T Numerical Taxonomy and Multivariate Analysis System (NTSyS-PC) software.

RESULTS AND DISCUSSION

Isolated endophyte phytobacteria from *L. gibba* plants

Table 1 lists the isolated endophyte phytobacteria from *L. gibba* plants for each collected zone and season, and were identified based on its 16S rDNA sequence homology analysis. There were 17 isolates from the *L. gibba* plants collected in the dry season: six isolates from plants collected in urban zone, three isolates from plants collected in tourist zone and eight isolates from plants collected in chinampa zone.

For the rainy season, there were 14 isolates: nine isolates from plants collected in tourist zone, one isolate from plants collected in urban zone and four isolates from plants collected in chinampa zone. The environmental conditions and seasonal characteristics determined the number and identity of the isolated endophyte phytobacteria in *L. gibba* plants with several apparent differences in the water quality among the selected three zones. The environmental behavior as indicated by López-López et al. (2006), may be the result of a high nutrient enrichment in all three areas and the presence of microalgal blooms in the urban and tourist zones; the apparent differences in the studied zones also seems to be the result of the local wastewater inputs, run off and leached from the different zones of Lake Xochimilco studied which are a consequence of the land use and the level of man-made disturbance in adjacent land areas.

Another reason of the diversity and distribution of endophyte phytobacteria of *L. gibba* plants, could be the relative phosphate concen-trations in the channels of the selected zones; as Martínez-Cruz et al. (2006) mention, the dries months (April and May) presented high phosphate concentrations by the effect of water evaporation and in June to early November (rainy season), the phosphate content diminished due to the dilution of it by rain falls.

Richardson (1985) believed that emergent macrophytes were capable of higher phosphate absorption, due to rhizosphere activity and Martínez-Cruz et al. (2006) believe that chemical precipitation of reactive phosphate can also occur and thus prevent higher total phosphate removal. Martin and Gerald (1994) found that phosphorus absorption by plants occurs slowly and only for soluble phosphate compounds. Lalke-Porczyk and Donderski (2003) mention the number of epiphytic bacteria and heterotrophic epiphytic bacteria displayed a distinct seasonal variability; Niewolak (1974) and Olah (1974) explain the summer maximum of the number of bacteria on certain plants by citing the increased amount of organic substances secreted by the plants and the increase in the temperature of the water. On the other hand, the fall in the total number of bacteria observed in summer in macrophytes may be caused due to the excretion of antibacterial substances by plants or by algae inhabiting them, or due to the excessive exposure of plant surfaces to sunlight (Lalke-Porczyk and Donderski, 2003).

Efficiency of IAA produced by the selected endophyte phytobacteria from *L. gibba* plants

The results of the evaluation of IAA production by the isolates are presented in Figures 1 and 2. The isolated endophyte phytobacteria were screened for their ability to produce the auxin IAA, without and with different concentrations of tryptophan (0, 1, 2 and 5 mg/L) as inducer and precursor of it.

The IAA production of the isolates, considered as basal production without the addition of the amino acid was less in the endophyte phytobacteria of *L. gibba* plants collected in dry season (between 0.64 to 43.4 µg/mL), compared to the isolates from *L. gibba* plants collected in rainy season (between 24.45 to 53.98 µg/mL); apart from one of the isolates of *L. gibba*

Table 1. Auxin production by the endophyte phytobacteria isolated from *Lemna gibba* plants collected from two seasons.

Dry Season					Rainy season				
Identified endophyte phytobacteria	**Zone**	**Gram behavior**	**Identity (%)**	**IAA producer**	**Identified endophyte phytobacteria**	**Zone**	**Gram behavior**	**Identity (%)**	**IAA Producer**
Bacillus subtilis U-MA-1	A	Bacilli Gram +	98	L	*Stenotrophomonas* spp. TU-AG-1	B	Bacilli gram -	100	H
Bacillus spp. U-MA-2	A	Bacilli Gram +	96	L	*Pseudomonas* spp. TU-AG-3	B	Bacilli gram -	100	H
Bacillus spp. U-MA-3	A	Bacilli Gram +	97	H	*Serratia* spp. TU-AG-5	B	Bacilli gram -	99	H
Bacillus pumilus U-MA-4	A	Bacilli Gram +	96	H	*Serratia* spp. TU-AG-6	B	Bacilli gram -	99	H
Bacillus stratosphericus U-MA-5	A	Bacilli Gram +	96	H	*Serratia* spp. TU-AG-8	B	Bacilli gram -	98	H
Bacillus stratosphericus U-MA-6	A	Bacilli Gram +	96	H	*Stenotrophomonas* spp.TU-AG-9	B	Bacilli gram -	97	H
Enterobacter spp. TU-MA-7	B	Cocci Gram +	96	H	*Serratia* spp.TU-AG-10	B	Bacilli gram -	99	H
Paenibacillus spp. TU-MA-9	B	Bacilli Gram +	9	L	*Serratia* spp.TU-AG-11	B	Bacilli gram -	100	H
Bacillus simplex TU-MA-10	B	Bacilli Gram -	95	L	*Exiguobacterium* spp.TU-AG-12	B	Bacilli gram -	97	H
Achromobacter spp. CH-MA-13	C	Bacilli Gram +	95	L	*Stenotrophomonas* spp. U-AG-17	A	Bacilli gram -	100	H
Deinococcus spp. CH-MA-15	C	Bacilli Gram -	92	L	*Serratia* spp. CH-AG-19	C	Bacilli gram -	85	H
Enterococcus faecium CH-MA-16	C	Bacilli Gram -	95	L	*Serratia* spp. CH-AG-20	C	Bacilli gram +	97	H
Achromobacter spp. CH-MA-17	C	Bacilli Gram -	97	H	*Serratia* spp. CH-AG-21	C	Bacilli gram -	99	H
Pseudomonas spp. CH-MA-19	C	Bacilli Gram -	96	H	*Serratia* spp. CH-AG-22	C	Bacilli gram -	95	H
*Bacillus pumilus*CH-MA-21	C	Cocci Gram +	96	L					
Rahnella aquatilis CH-MA-23	C	Bacilli Gram -	95	H					
Enterobacter spp. CH-MA-24	C	Bacilli Gram -	94	H					

A, Urban zone; B) Tourist zone; C, Chinampa zone.

plants collected in dry season from the chinampa zone *Enterobacter* spp. CH-MA-24, with 76.84 µg/mL of IAA produced. In general, the increase of IAA production by the isolates as the concentration of Trp increased, was present only in three isolates of *L. gibba* plants collected in dry season and in six isolated plants collected in rainy season; again this response were more evident in the isolates from the plants collected from this season with an increase of IAA production in average from 40 to 55 µg/mL. According to the classification of Khalid et al. (2004) for the *in vitro* production of IAA by bacteria; categorized in three principal groups: lower producers (L= 1 to 10 µg/mL IAA), medium producers (M= 11 to 20 µg/mL IAA) and higher producers (H= 21 to 30 µg/mL IAA), the isolated endophyte phytobacteria based on their IAA basal production could be classified as Table 1 show.

Figure 3, shows the phenogram with the associated groups according to the selection of the higher IAA producers without and with the addition of Trp; this figure two groups forming at first: Group I made only by *Enterobacter* spp. strain CH-MA-24 with the highest production of IAA and the rest of the phytobacteria comprise the Group II: with Group IIa organized by the selected phytobacteria with a good IAA production with no distinction amongst their origin and season of collected *L. gibba* plants and the other endophyte phytobacteria forming Group IIb classified as relative medium and lower IAA producers. Among the principal genera identified, the endophyte phytobacteria isolated from the *L. gibba* plants collected in dry season, there were mainly eight isolates that belong to the *Bacillus* genera, six of them from plants collected in urban zone, one from the plants collected in tourist zone and one from the plants collected in chinampa zone and

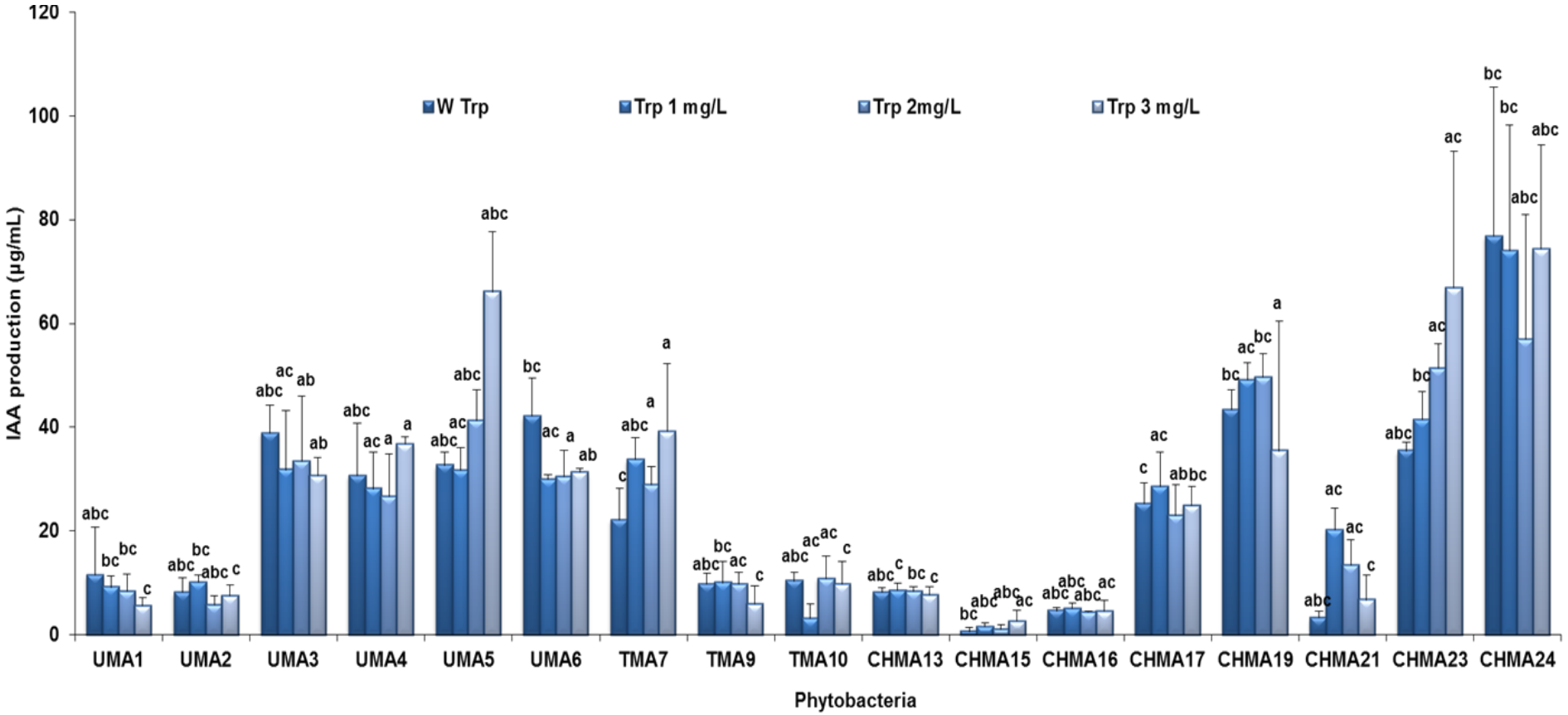

Figure 1. IAA production of the endophyte phytobacteria of *Lemma* gibba plants collected in rainy season, where, WTrp is without Tryptophan and Trp is concentration: 1, 2, 3 mg/L. Values are mean values + SD. from three replicates for IAA production. The different lower-case letters shows the significant difference between experiments ($p<0.001$).

diverse genera like: *Enterobacter, Achromobacter*, *Paenibacillus, Deinococcus, Enterococcus, Rahnella* and *Pseudomonas*. Nine of the fourteen identified endophyte phytobacteria isolated from the *L. gibba* plants collected in the rainy season, belong to the *Stenotrophomonas* genus and the rest of the isolates to the genus: *Exiguobacterium* and *Pseudomonas.* It is important to mention that all the isolates of *L. gibba* plants collected from the chinampa zone in the rainy season were *Serratia* bacteria with a diverse morphology. The isolated genera from *L. gibba* plants were new, compared with the findings by Stout et al. (2010) and Stout and Nüsslein (2005); these authors reported members of the genera *Flavobacterium* isolated from Environmental Protection Agency (EPA) *L. minor* plants and the response of one plant growth promoting bacteria that plays a role in the elongation of root zones improving heavy metal phytoremediation.

Regarding the ecological context, this work

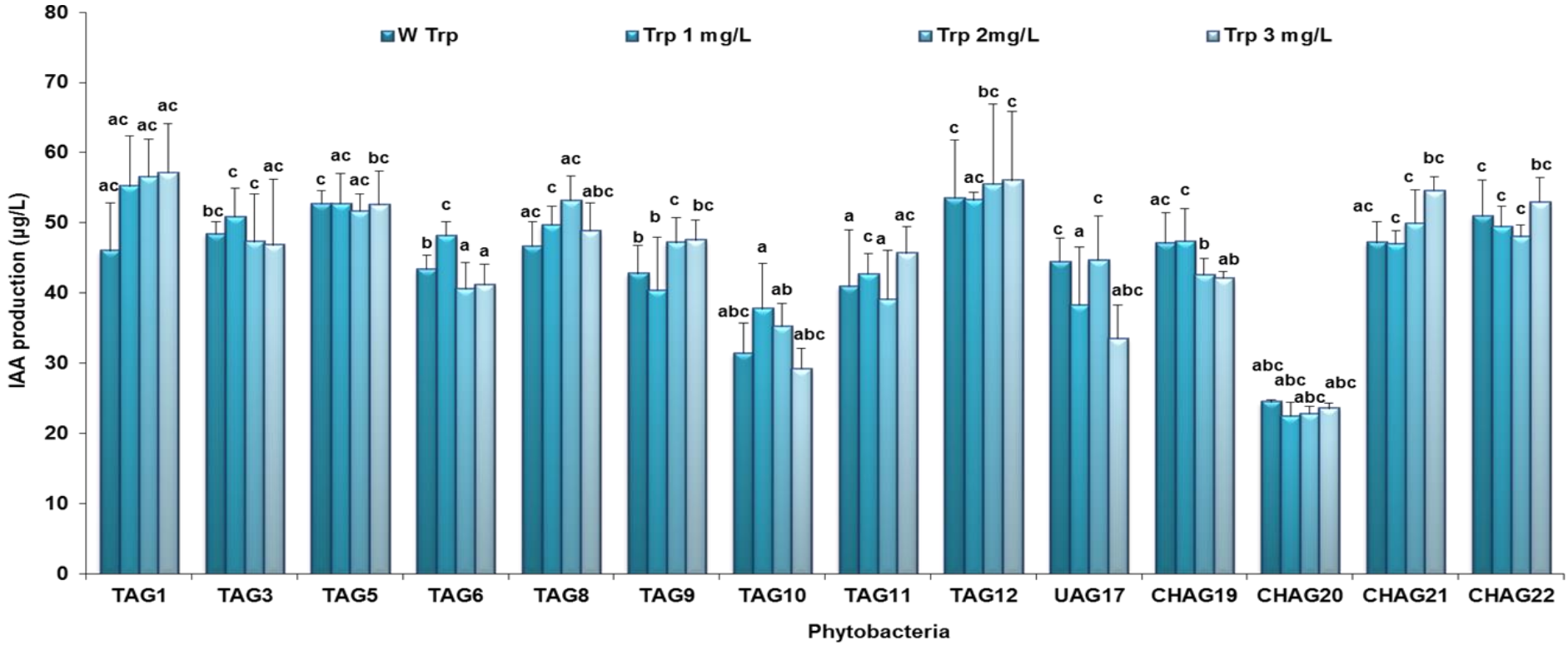

Figure 2. IAA production of the endophyte phytobacteria of *Lemma* gibba plants collected in rainy season, where, WTrp is without Tryptophan and Trp is concentration: 1, 2, 3 mg/L. Mean values + SD. from three replicates for IAA production. The different lower-case letters shows the significant difference between experiments ($p<0.001$).

agrees with the commentaries of Idris et al. (2004) regarding the nature of the IAA production obtained by the isolates identified in the plants of *L. gibba* collected in the channels of the Xochimilco Lake, because the IAA production was induced with the addition of Trp to the cultures. The authors' mention that, it is possible that the presence of tryptophan-like compounds liberated in plant exudates could stimulate the IAA synthesis in the plant growth promoting bacteria that colonize the structures of plant surface. The authors particularly mention that they reisolated *B. amyloliquefaciens* directly from *L. minor* plants, indicating that this bacterium lives in the vicinity of the plant's surface and could uptake the excreted plant compounds. Kamilova et al. (2006) mention that one of these compounds may be the amino acid. Thus, they suggested that the production of IAA stimulated in *B. amyloliquefaciens* FZB42 by the presence of Trp leads a promotion of plant growth giving close relationship between it and *L. minor* plants.

Conclusions

This work contributes to the knowledge of the phytobacteria potential in aquatic plants, particularly in Lemnaceae species; here the majority of the isolates have been characterized as

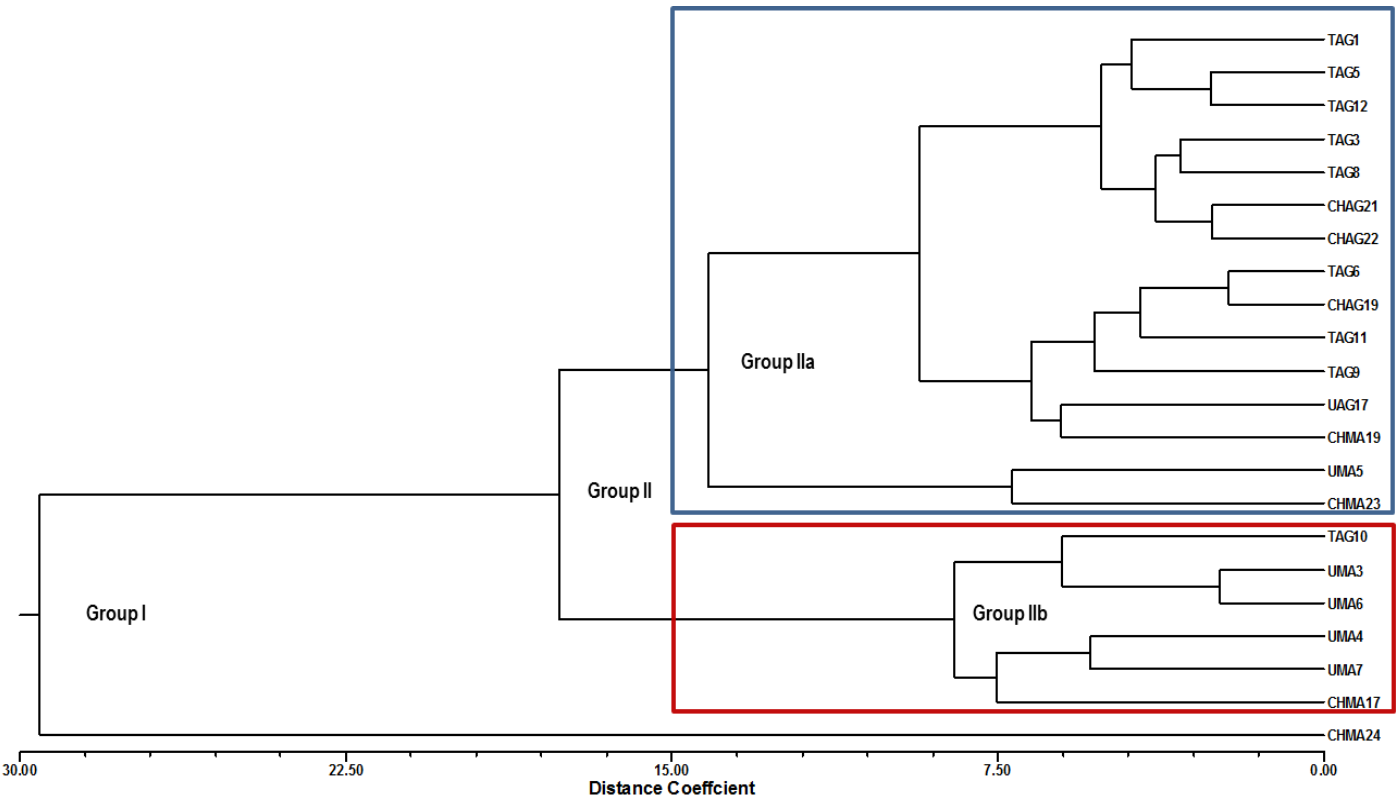

Figure 3. Phenogram of the isolated endophyte phytobacteria from *Lemma gibba* related to their IAA production (r=0.85).

higher IAA producers; recommended to candidates for their use as biofertilizers. The authors suggest further works with these isolates that involves plant-microbe systems as biossays to demonstrate the ecological role that these endophyte phytobacteria have in association with *L. gibba* plants, for the conservation of aquatic ecosystems.

Conflict of interests

The authors did not declare any conflict of interest.

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