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Indigenous bacteria, an excellent reservoir of functional plant growth promoters for enhancing duckweed biomass yield on site

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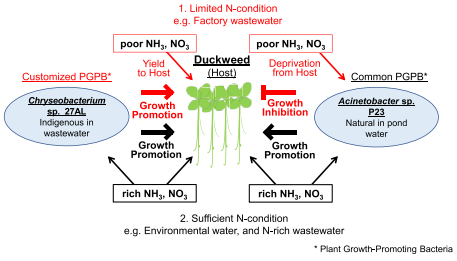
HIGHLIGHTS

- The unbalanced minerals in wastewater affected duckweed growth conditions and the activity of PGPB co-existing with duckweed.
- Common PGPB for the duckweed was inactive in factory wastewater containing excess SO_4^{2-} and PO_4^{-}, and little NH_4^{+}/NO_3^{-}.
- Novel PGPB derived from wastewater could maintain duckweed growth promotion ability due to its unique nitrogen metabolism.
- This study successfully expands PGPB technology to produce duckweed biomass effectively in wastewater with unbalanced minerals.

GRAPHICAL ABSTRACT



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ABSTRACT

The advantages of aquatic biomass production using wastewater as a cost-free fertilizer have recently been highlighted. Here, we report a successful study in which duckweed, *Lemna gibba*, biomass production in a food factory effluent containing low nitrogen and high salts was enhanced by employing customized plant growth-promoting bacteria (PGPB). Two common PGPB strains previously obtained from natural pond water, *Acinetobacter calcoaceticus* P23 and *Pseudomonas fulva* Ps6, hardly promoted the growth of duckweed; on the contrary, they inhibited its growth in treated factory wastewater, far different water conditions. Then, we asked if some indigenous wastewater bacteria could promote the growth of duckweed. We found that *Chryseobacterium* strains, a group of bacteria with limited nitrogen metabolism, were dominantly selected as effective PGPB. Moreover, we demonstrated that nitrogen limitation is the crucial environmental factor that induces the plant growth-inhibiting behavior of *A. calcoaceticus* P23 through competition for mineral nutrients with the host duckweed. This study uncovered points to be considered in PGPB technology to achieve efficient production of duckweed biomass in a factory effluent with unbalanced content of mineral nutrients.

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1. Introduction

Duckweed is the smallest flowering plant that can grow on the surface of still or slow-moving water bodies. It is known for its rapid, asexual reproductive cycle, enabling it to double its biomass every two or three days under optimal conditions (Stomp, 2005). Studies on duckweed have rapidly expanded from basic biology to bioengineering because of its high value as a useful bioresource (Appenroth et al., 2015). Indeed, duckweed has been shown to display a high starch content (maximum ca. 50%) with a low lignin content, which make it an advantageous fermentation substrate for both bioethanol and methane production (Xu et al., 2011; Toyama et al., 2018). Additionally, duckweed proteins (maximum ca. 40%) contain the WHO-recommended amino acid ratios and essential amino acids that are important for human and animal nutrition (Goopy and Murray, 2003; Appenroth et al., 2017). Owing to the strong benefits expected in a range of industries, the production of duckweed biomass has received significant attention in recent times.

Duckweed can be easily cultivated in pond water, sewage effluents, or industrial wastewater (WW) with no land irrigation because of its ability to take up dissolved pollutant minerals as cost-free fertilizer coupled with useful biomass production (Cheng and Stomp, 2009). In fact, Mohedano et al. (2012) showed significant performance of duckweed in nutrient-rich swine waste, with removal of 98.0% of the TKN (Total Kjeldahl Nitrogen) and 98.8% of the TP (Total Phosphorous), and a production of 68 t/(ha·year) of dry biomass containing 35% crude protein. Although the conventional duckweed-wastewater treatment system is slow and requires a large surface area, recent technologies have been used to improve the efficiency of this system. One of the relevant methods is the acceleration of duckweed growth by inoculation of plant growth-promoting bacteria (PGPB) (Yamaga et al., 2010; Toyama et al., 2017; Ishizawa et al., 2020).

PGPB have long been used to improve the yield of crops with less fertilization and pesticides. In fact, PGPB accelerate the growth of host plants either directly, by producing plant growth hormones, facilitating the uptake of minerals, and relieving environmental stresses, or indirectly, acting as biocontrol agents against pathogens (Glick, 2012). In particular, *Acinetobacter calcoaceticus* P23 and *Pseudomonas fulva* Ps6 have been identified as growth-promoting bacteria for some duckweed species, including *Lemna aequinoctialis* Welw (Former name: *Lemna aoukikusa*) and *Lemna minor* (Yamaga et al., 2010; Suzuki et al., 2014; Yamakawa et al., 2018). Notably, the PGPB-reinforced duckweed *L. minor* accelerated biomass production by 1.7−2.4-fold compared to natural duckweed in a secondary sewage effluent and in river water as well as displayed improved nutrient removal and CO_2 fixation (Toyama et al., 2017; Ishizawa et al., 2020). However, the interaction between PGPB and host plant varies depending on the environmental conditions and the plant species (Glick, 2012). For instance, *Azospirillum brasilense* has been reported to increase the shoot and root length of a cordon plant linearly as the nutrients declined, but in nutrient-rich soil this PGPB exerted no effect on cordon growth (Carrillo-Garcia et al., 2000). Another report showed that an engineered PGPB with IAA-overproduction increased the root weight of blackcurrant cuttings but inhibited root development of sour cherry, probably due to the different sensitivity of the latter to IAA (Dubeikovsky et al., 1993; Glick, 2012). Thus, environmental factors and host plant species should be considered to maximize PGPB activities for practical use.

Here, we attempted to enhance duckweed biomass production utilizing *Lemna gibba* in food factory WW by the application of PGPB. Treated WW was used in this study as the medium for cultivation of duckweed. Before discharge to the river, the treated WW is usually kept for a couple of weeks in a large buffering pond, in which duckweed can be easily cultivated without costly modification of WW treatment facilities. The effects of PGPB application were initially examined for previously isolated PGPB, i.e., *A. calcoaceticus* P23 and *P. fulva* Ps6. Surprisingly, the results showed that neither P23 nor Ps6 did improve the growth of the duckweed *L. gibba* in the treated WW, while P23 showed growth inhibition against *L. gibba*. Then, we wondered if some of the domestic bacteria in the factory WW exhibited growth promotion activity towards *L. gibba*. We found that bacteria belonging to a special group were selected as PGPB. Moreover, we identified the key factors responsible for the growth inhibition activity of P23 in the treated WW. The findings of this study provide new knowledge for the selection of suitable PGPB for efficient duckweed biomass production using practical food factory WW.

2. Materials and methods

2.1. Plant materials

We compared the growth and tolerance of aseptic duckweed strains in the food factory treated WW used in this study. *Lemna gibba* (G3 strain; RDSC serial number: 362; ID: DWC128) was selected as the best candidate among *Landoltia punctata*, *Lemna aequinoctialis*, *Lemna minor*, *Lemna turionifera*, *Spirodela polyrhiza*, *Wolffia microscopica*, *Wolffiella lingulata*, and others (data not shown). Duckweed was cultivated in a plant growth chamber (MLR352, Panasonic Corp., Osaka, Japan) at a temperature of 28 °C, an illuminance of 108 μmol/(m^2·s), a photoperiod of 16 h, and ca. 50% humidity. The sterility of the duckweed stock was routinely confirmed by the absence of bacterial colony formation on R2A agar plates incubated for 1 week at 30 °C.

2.2. Wastewater

Treated WW samples, namely A-wastewater (A-WW) and K-wastewater (K-WW), were collected from the final sedimentation tanks of two food factories. Water samples were sterilized using a membrane filter with a pore size of 0.22 μm (Sartolab, Sartorius AG, Göttingen, Germany) before use for the experiments of gnotobiotic duckweed culture. Unsterilized WW was used to evaluate the effects of indigenous bacterial community and novel PGPB isolates. The anion content of WW was analyzed by ion chromatography (IC-2010, Tosoh Corp., Tokyo, Japan) with a superIC- AZ column (Tosoh) and an eluent of 1.9 mM $NaHCO_3$ + 3.2 mM Na_2CO_3 at a flow rate of 0.8 mL/min and a temperature of 40 °C. Metal elements were analyzed using an ICP emission spectrometer (ICPE-9000, Shimadzu Corp., Kyoto, Japan) at the Hokkaido University Global Facility Center. Ammonium and COD were quantified using the PACKTEST kit (KYORITSU Chemical-Check Lab, Tokyo, Japan) according to the manufacturer's instructions. The pH was measured using the Docu-pH + pH-meter (Sartorius).

2.3. Growth media

Hoagland medium or NF medium was used to grow duckweed aseptically or gnotobiotically. The Hoagland medium contained 0.36 mM KNO_3, 1.68 mM K_2SO_4, 0.99 mM $CaCl_2·2H_2O$, 0.42 mM $MgSO_4·7H_2O$, 0.03 mM $NaH_2PO_4·2H_2O$, 0.012 mM $FeSO_4·7H_2O$, 0.02 mM H_3BO_3, 0.002 mM $MnCl_2·4H_2O$, 0.0003 mM $ZnSO_4·7H_2O$, 0.0001 mM $CuSO_4·5H_2O$, and 0.001 mM H_2MoO_4 (Yamaga et al., 2010). The pH was adjusted to 7.0 with NaOH. The NF medium contained 2.7 mM $CaCl_2·2H_2O$, 1.2 mM $MgSO_4·7H_2O$, 1.0 mM KH_2PO_4, 5 mM KNO_3, 0.02 mM $FeSO_4·7H_2O$, 0.05 mM Na_2-EDTA,

0.02 mM $MnCl_2 \cdot 4H_2O$, 0.05 mM H_3BO_3, 0.001 mM $ZnSO_4 \cdot 7H_2O$, 0.0003 mM $CuSO_4 \cdot 5H_2O$, and 0.0005 mM MoO_3 (Muranaka et al., 2015). The pH was adjusted to 5.0 with KOH.

Bacteria were cultured in either LB medium or R2A medium. The LB medium contained 5 g/L Bacto Yeast extract (BD Difco Laboratories, Franklin Lakes, NJ, USA), 10 g/L Bacto Tryptone (Difco), and 5 g/L NaCl. The pH of the medium was adjusted to 7.2 with NaOH. The R2A medium contained 0.5 g/L each of Bacto Proteose peptone No. 3 (Difco), Bacto Yeast extract, casamino acid, glucose, and soluble starch, 0.3 g/L each of KH_2PO_4 and sodium pyruvate, and 0.05 g of $MgSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 7.2 with NaOH. LB and R2A media were solidified by adding 1.5% agar when necessary.

2.4. Evaluation of the effect of the indigenous bacterial community from WW on L. gibba growth

Ten fronds, leaf-like structures, of aseptic *L. gibba* were cultivated for 14 days in filter-sterilized WW or non-sterilized WW. Duckweed growth was estimated by counting the total number of fronds and recording the biomass (dry weight) after the cultivation period.

2.5. Isolation of bacteria from WW capable of colonizing L. gibba

Aseptic *L. gibba* fronds were transferred to flasks containing 50 mL of non-sterilized A-WW or K-WW and cultivated for three days in a plant growth chamber, thereby allowing bacterial adhesion followed by colonization. After cultivation, 10 duckweed fronds were collected and homogenized to release bacteria from *L. gibba* in 1 mL of sterilized phosphate buffer saline using a Bio-Masher II (Nippi Inc., Tokyo, Japan). The homogenized sample was diluted by a 10^{-1}, 10^{-2}, and 10^{-3} factor using sterilized MilliQ water. The diluted homogenates were spread onto three types of solid media, LB, R2A, and one-fifth-diluted R2A**,** and then incubated at 30 °C for two to three days. All morphologically distinct colony-forming bacteria were isolated and stored at −80 °C in liquid medium containing 15% glycerol.

2.6. Examination of the effect of bacteria on L. gibba growth

The effect of bacteria on duckweed growth was examined under two conditions: 1) using a 12-well plate for preliminary selection of PGPB from WW in 4 mL of standard medium (NF medium); or 2) using a 100-mL flask or plant culture dish (SPL Life Sciences, Gyeonggi-do, South Korea) containing 50 mL of WW samples, Hoagland medium, or modified Hoagland medium.

The 12-well-plate method was performed as follows: A bacterial colony was inoculated with an inoculation loop into 4 mL of liquid LB medium in a test tube and shaken for 1–2 days, depending on the growth rate, at 30 °C and 100 strokes per minute (Personal-11 SD, Taitec, Tokyo, Japan). After growth, bacterial cells were harvested by centrifugation, washed, and resuspended in 1 mL of NF medium. Inoculation of duckweed was performed by placing aseptic *L. gibba* on 4 mL of NF medium containing bacterial cells with a final OD_{600} of 0.3 for 24 h. After bacterial inoculation, two fronds of gnotobiotic *L. gibba* were transferred to a new 12-well plate containing 4 mL of NF medium and cultivated in a plant growth chamber. The growth of *L. gibba* was measured by counting the total number of fronds after the cultivation period.

The 100-mL flask or plant culture dish method was performed as follows: A bacterial colony was inoculated with an inoculation loop into 20 mL of LB medium in a 100-mL flask and shaken for 1 day at 30 °C and 100 strokes per minute. Bacterial cells were harvested by centrifugation, washed twice with Hoagland medium, and resuspended in Hoagland medium. Bacterial inoculation was performed by placing aseptic *L. gibba* on 50 mL of Hoagland medium containing bacterial cells at a final OD_{600} of 0.3 for 24 h in a 100-mL flask or plant culture dish. After bacterial inoculation, gnotobiotic *L. gibba* was transferred to 50 mL of fresh bacteria-free WW, Hoagland medium, or modified Hoagland medium, and duckweed growth was estimated by counting the total number of fronds and measuring the biomass (dry weight) after the cultivation period.

2.7. Identification of selected PGPB by 16S rRNA sequence analysis

Bacterial DNA was extracted using the InstaGene DNA Purification Matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The DNA was used as a template for PCR amplification using the set of primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′) and the KOD-Plus-Neo DNA polymerase with a standard protocol (Toyobo, Kyoto, Japan). Amplicons were purified with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The resulting sequences were compared to those included in the GenBank nucleotide sequence database with the NCBI Nucleotide BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for taxonomic identification. The nucleotide sequences of the 16S rRNA genes of stains 27AL and 29AL were deposited in DDBJ/GenBank/EMBL under accession numbers LC567048 and LC567049, respectively.

2.8. Quantification of plant-colonizing bacterial cells

Ten duckweed fronds and roots were rinsed twice with sterilized water to remove weakly attached bacteria. The duckweed samples were transferred into 1.5-mL plastic tubes containing 1 mL of sterilized water and homogenized as described above. The homogenized samples were diluted, spread onto LB agar plates, and incubated at 30 °C for 24 h. The number of bacterial colonies was counted, and the average number of plant-colonizing bacterial cells was expressed in colony-forming units (CFU)/plant.

2.9. Analysis of general PGP factors produced by bacteria

The production of IAA and related compounds was assessed according to Ishizawa et al. (2017) with modifications. Briefly, bacteria were cultured for 24 h at 30 °C in 2 mL of LB medium with or without tryptophan (200 μg/mL), and 1 mL of each culture was centrifuged to recover the culture supernatant. Two hundred μL of Salkowski reagent (29.16 mL of 60% $HClO_4$, 1 mL of 0.5 M $FeCl_2$, and 19.84 mL of MilliQ water) was added to 1 mL of half-diluted supernatant. The mixture was incubated in the dark for 25 min and the absorbance was measured at 530 nm. The relative productivity of IAA and related compounds of these isolates, in presence or absence of tryptophan, was determined using a standard curve that was constructed using different concentrations of IAA (5–100 μg/mL). In addition, siderophore production and phosphate solubilization activity were tested on solid agar media according to Yamakawa et al. (2018).

2.10. Examination of bacterial nitrogen metabolism

The pathways of nitrogen metabolism of bacterial strains were retrieved from the KEGG database (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg/pathway.html). The

ability of bacteria to utilize each nitrogen compound was tested by cell growth assays in basal media. The composition of the basal salt (BS) medium is as follows: 0.41 g/L KH_2PO_4, 0.052 g/L K_2HPO_4, 0.05 g/L Na_2SO_4, 0.5 g/L $CaCl_2$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.005 g/L $FeSO_4 \cdot 7H_2O$, 0.0025 g/L $Na_2MoO_4 \cdot 2H_2O$, and 2 g/L succinic acid. The pH was adjusted to 7.0 with NaOH. BS medium was supplemented with different nitrogen compounds, including 1 g/L casamino acid (organic nitrogen), 1 g/L $NaNO_3$, or 1 g/L NH_4Cl. Single colony was inoculated with an inoculation loop into the BS medium with and without nitrogen and the growth was observed after 72 h of shaking at 30 °C.

2.11. Statistical analysis

Statistical analysis was conducted using SPSS software ver. 27.0 (IBM, Armonk, NY, USA). All results are reported as mean ± standard deviation (SD) with the values of three sample replicates per experiment. Significance ($P < 0.05$) was calculated using Student's t-test for experiment within two conditions or one-way ANOVA (followed by post-hoc test Tukey HSD if among groups value is significant) for experiment with more than two conditions.

3. Results and discussion

3.1. Mineral nutrient content and duckweed growth in WW and Hoagland medium

The mineral nutrient content of A-WW and K-WW was compared with that of the popular Hoagland medium (Table 1). A-WW and K-WW had similar content of ions and minerals, except for PO_4. Indeed, K-WW contained a significantly higher amount of PO_4 than A-WW and Hoagland medium. However, these WW samples both contained significantly large amount of Na but small amount of NO_3 compared to the Hoagland medium. Finally, A-WW and K-WW contained traces of NH_3. With respect to the growth in Hoagland medium, *L. gibba* growth was significantly reduced by both WW treatments (Fig. 1). In particular, chlorosis (emergence of white color) was observed in K-WW-grown fronds, probably due to excess PO_4 content (3.35 mM) (Supplementary data Fig. S1).

Table 1
Mineral contents of wastewater and Hoagland medium for duckweed.

Composition	Content (mM)		
	A-WW	K-WW	Hoagland
B	0.02^a	0.02^a	0.02^a
Ca	0.25^b	0.22^b	1.00^a
Cu	0.0005^a	0.0005^a	0.0002^a
Fe	0.0009^b	0.0003^b	0.012^a
K	0.39^b	0.26^c	3.72^a
Mg	0.16^b	0.10^b	0.42^a
Mn	0.0002^b	0.0005^b	0.002^a
Na*	48.72^a	46.11^a	0.03^b
Zn	0.004^a	0.004^a	0.0003^b
NH_3	0.012^a	0.042^b	–
Cl	5.35^b	11.27^a	2.00^c
$NO_3^{\dagger}$	0.006^b	0.005^b	0.357^a
SO_4	10.03^a	4.34^b	2.11^c
PO_4**	0.02^b	3.35^a	0.03^b
COD	20 mg/L	13–20 mg/L	–
pH	8.4	8.2	7

*,† the mineral significantly large or small amounts in both wastewater compared to Hoagland medium, respectively.
** the mineral significantly large amount only in K-WW
Different alphabets indicate significant differences in each mineral (using one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).
Relative standard deviation was less than 10% or 45% for the minerals value higher or less than 0.1 mM, respectively.

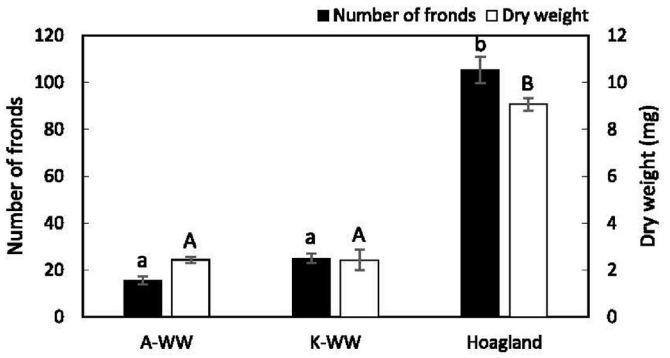

Fig. 1. Comparison of the growth of *L. gibba* in sterilized wastewater and Hoagland medium based on the number of fronds (closed bars) and dry weight (open bars) after 14 days of cultivation. The initial number of fronds was two. Values are mean ± SD ($n = 3$). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

3.2. Examination of the activity of A. calcoaceticus P23 and P. fulva Ps6 in WW

We tested whether the P23 and Ps6 bacterial strains displayed growth-promoting activities towards *L. gibba* in filter-sterilized A-WW and K-WW. These strains were previously isolated from the surface of duckweed naturally growing in a pond of the Hokkaido University Botanic Garden (Yamaga et al., 2010; Yamakawa et al., 2018). Their activities were different depending on the duckweed species (Supplementary data Fig. S2). P23 could also promote the growth of *Lemna aequinoctialis* to a higher extent than that of *L. minor* (Toyama et al., 2017). Therefore, we first examined the PGP activities of these PGPB towards *L. gibba* in Hoagland medium (Fig. 2A). The growth of *L. gibba* was increased by 1.6- and 1.7-fold by P23 and Ps6, respectively. Next, PGP activities were examined in A-WW and K-WW (Fig. 2B and C). Ps6 showed PGP activity in A-WW (1.25-fold growth increase). However, neither P23 nor Ps6 promoted the growth of *L. gibba* in K-WW; on the contrary, P23 showed a growth inhibition effect on *L. gibba* in both A-WW (0.79-fold) and K-WW (0.75-fold), according to the dry weight.

These results indicated that the PGP activities of P23 and Ps6 are not universal but depend on the plant species, such as *L. minor* or *L. gibba*, and the water conditions, whether optimal medium or nutrient-biased WW. Nevertheless, it is worth noting that P23 showed significant PGP activity in the secondary effluent of a municipal sewage treatment system (Toyama et al., 2017; Ishizawa et al., 2020). This water contained 4.27–6.01 mg/L NH_4–N, 0.07–0.66 mg/L NO_2–N, 7.72–8.63 mg/L NO_3–N, and 0.98–1.84 mg/L PO_4–P at pH 7.5, but no excess Na, Cl, or SO_4, and thus presented more favorable mineral conditions for duckweed growth than A-WW and K-WW.

3.3. Preliminary examination of PGP activities of indigenous bacteria in A-WW and K-WW

After testing known PGPB, we were interested in seeking novel PGPB from the food factory effluents where A-WW and K-WW had been collected. First, we evaluated how the total microbial community of non-sterile WW affected the growth of *L. gibba*. After 14 days of cultivation, duckweed placed in non-sterilized A-WW and K-WW showed significantly increased dry weight than in sterilized WW by 1.4- and 1.3-fold, respectively (Fig. 3A). Moreover, the

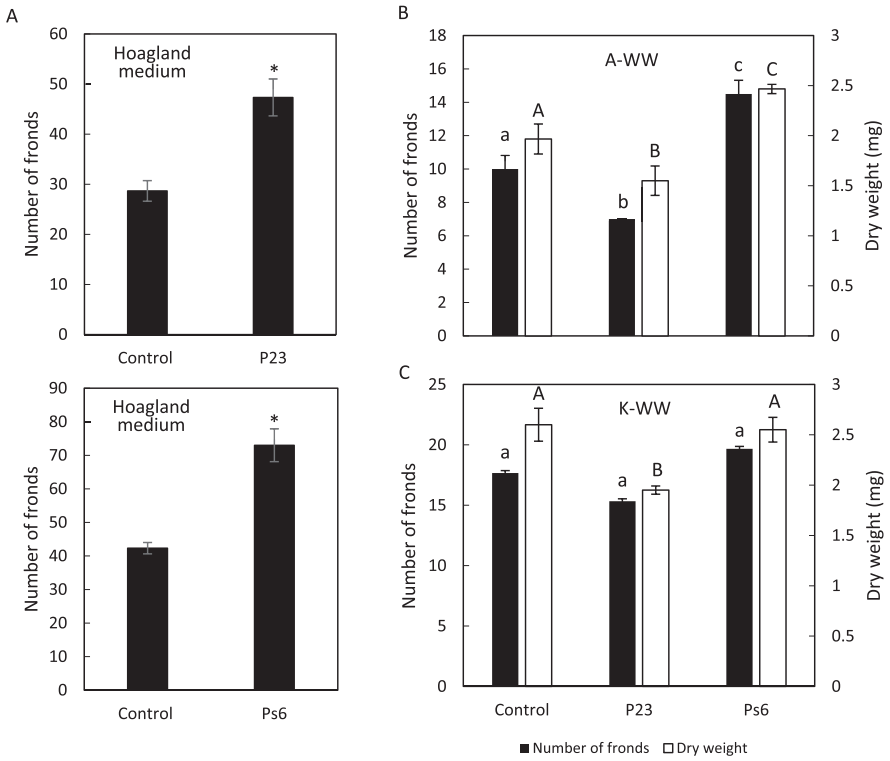

Fig. 2. The effect of PGPB, P23 and Ps6, on the growth of *L. gibba* in sterilized A) Hoagland, B) A-WW, and C) K-WW based on the number of fronds (closed bars) and dry weight (open bars) after 10 days of cultivation. "Control" is aseptic duckweed with no bacteria. The initial number of fronds was two in all experiments. Values are mean ± SD (n = 3). Asterisks in A indicate the significant differences between values with and without PGPB (Control) (Student's t-test, $P < 0.05$). Different alphabets between treatments in B and C indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

fronds of duckweed growing in non-sterilized A-WW were greener and larger (Fig. 3B). These results strongly suggest the possibility that A-WW and K-WW naturally harbor potential PGPB that can promote or restore the growth of duckweed in WW conditions.

3.4. Isolation of PGPB from WW

Isolation of effective bacteria from WW was conducted by first selecting the bacteria that have the ability to adhere to and colonize

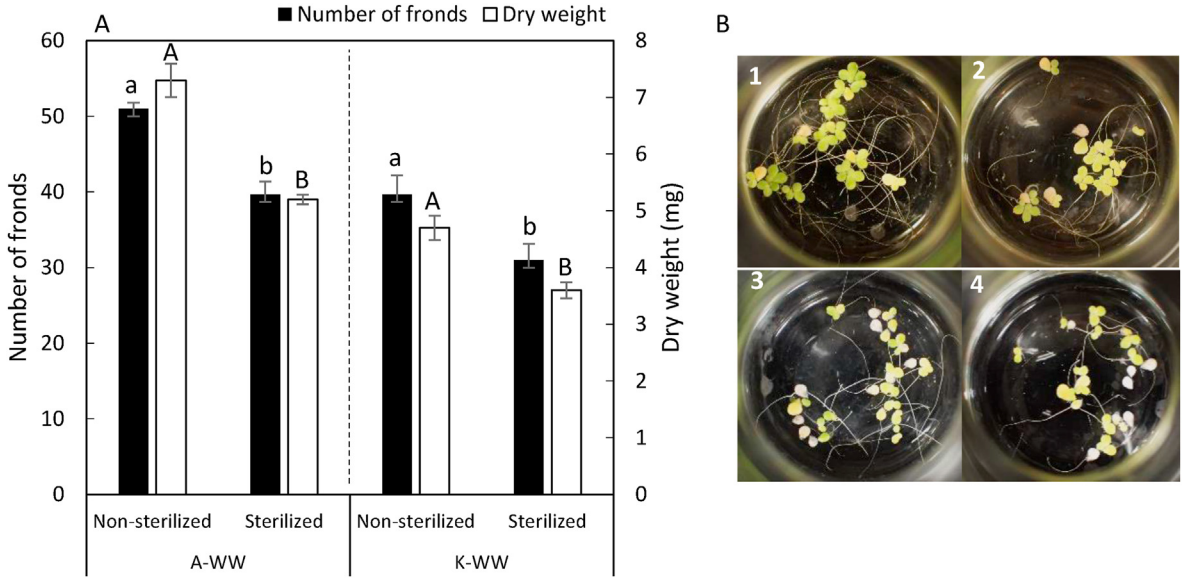

Fig. 3. A) Effect of indigenous microbial community in A-WW and K-WW on *L. gibba* growth based on the number of fronds (closed bars) and dry weight (open bars) after 14 days of cultivation. B) Photo image of *L. gibba* after 14 days of cultivation in 1) non-sterilized A-WW, 2) sterilized A-WW, 3) non-sterilized K-WW, 4) sterilized K-WW. The initial number of fronds was ten. Values are mean ± error (n = 2). Different alphabets indicate the significant differences between values of duckweed growth in non-sterilized and sterilized conditions (Student's t-test, $P < 0.05$).

the surface of duckweed. In fact, many agriculturally useful symbiotic bacteria such as *Rhizobium, Agrobacterium, Pseudomonas, Azospirillum*, and others, have been reported to attach to the plant surface (Wheatley and Poole, 2018). More importantly, the colonization of the host plant during water flow is an essential trait of PGPB of aquatic plants (Yamakawa et al., 2018). Therefore, aseptic *L. gibba* was cultivated in non-sterilized A-WW and K-WW for three days to allow indigenous bacteria to adhere to the surface of duckweed. After cultivation, bacterial strains colonizing duckweed were isolated. After subsequent selection, seven (20AL, 24AL, 25AL, 26AL, 27AL, 28AL, and 29AL) and ten (3 KL, 4 KL, 5 KL, 6 KL, 7 KL, 15 KL, 16 KL, 17 KL, 18 KL, and 19 KL) candidate bacterial strains were obtained from the duckweed grown in A-WW and K-WW, respectively, and used for further experiments. Finally, two bacterial strains, namely 27AL and 29AL from A-WW, showed notable PGP activity in NF medium compared to other isolates (Fig. 4A and B).

Furthermore, we investigated the PGP ability of strains 27AL and 29AL in A-WW and K-WW conditions. These strains significantly improved the growth of duckweed, as shown by the increased frond number and dry weight after 10 days compared to the bacteria-free control and to P23-inoculated duckweed (Fig. 4C). Therefore, it was clear that the indigenous WW PGPB 27AL and 29AL are more effective than environmental water-derived PGPB in enhancing duckweed biomass production under factory WW conditions.

Based on the 16S rRNA gene sequences, both 27AL and 29AL belong to genus *Chryseobacterium* and the closest species is *Chryseobacterium taichungense*, with identity scores of 98.92% and 98.63%, respectively. *Chryseobacterium* strains have been reported in a variety of environments, including fresh water, sewage, and WW (Kämpfer et al., 2003; Bernardet et al., 2006). For example, *C. taichungense* was isolated from a tar-contaminated soil in Taiwan (Shen et al., 2005). Some *Chryseobacterium* strains have also been reported to exert PGP activity. For instance, *Chryseobacterium gleum* alleviated salt stress and enhanced the growth of bread wheat, *Triticum aestivum* L., by producing ACC deaminase, IAA, siderophores, ammonia, HCN, and fungal cell wall hydrolyzing enzymes (Bhise et al., 2017). Moreover, inoculation with *Chryseobacterium palustre* and *Chryseobacterium humi* improved the growth of corn, *Zea mays*, and its biomass production (Marques et al., 2010). It has been suggested that *C. indologenes* AM2 can fix nitrogen upon detection of PCR-amplified DNA fragments using a set of primers for the *nifH* gene (Dhole et al., 2017); however, no *nifH* gene has yet been found in the genome of *C. indologenes*. On the other hand, the examination of general PGP factors of *Chryseobacterium* sp. 27AL and 29AL showed that they both can produce IAA and siderophore compounds (Supplemental data, Table S1). However, no nitrogen-fixing activity was detected for 27AL and 29AL by acetylene reduction assay (data not shown). To the best of our knowledge, this is the first report on the isolation of duckweed PGPB from factory WW.

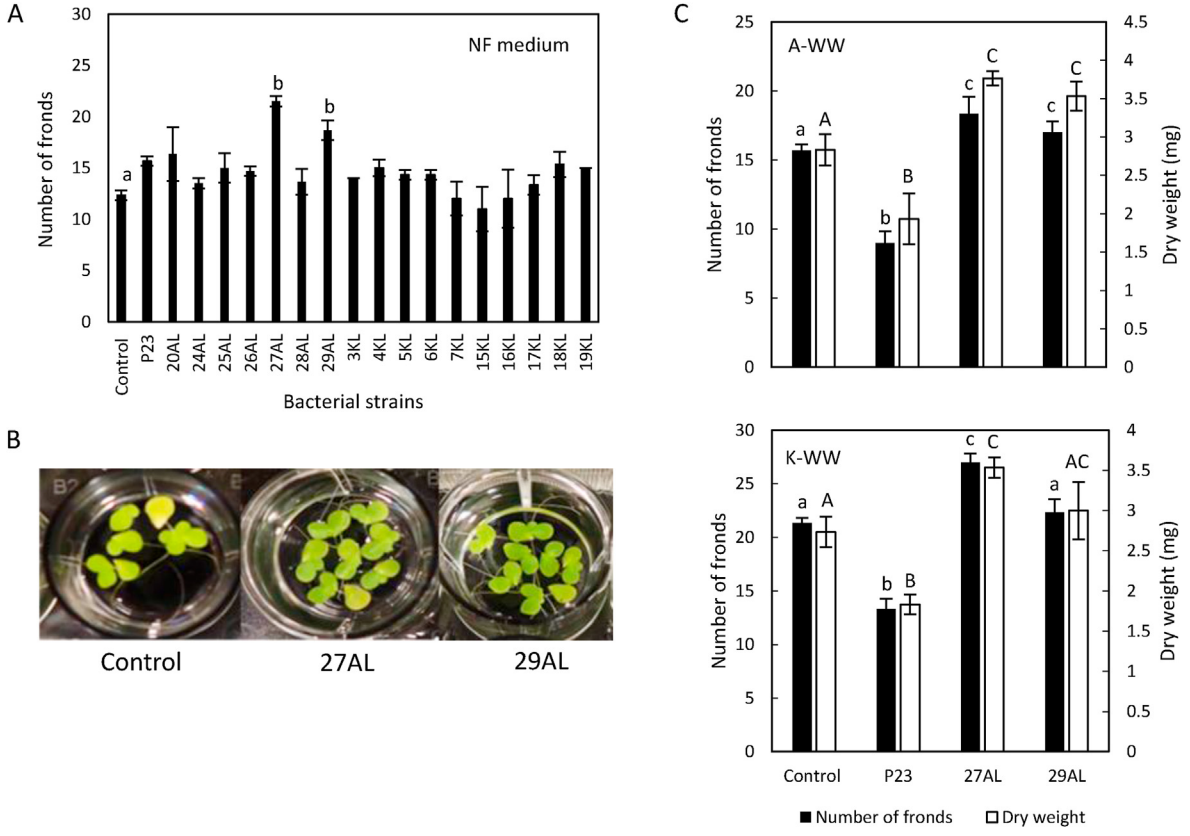

Fig. 4. A) Effect of bacterial strains isolated from WW on *L. gibba* growth compared to previously isolated PGPB (P23) and control (no bacteria) based on the number of fronds after 10 days of cultivation in NF medium. The symbols "AL" and "KL" represent the bacteria isolated from A-WW (A) or K-WW (K) that are capable of adhering on the surface of *L. gibba* (L). B) Photo image of *L. gibba* after 10 days of cultivation in NF medium colonized with the best two PGPB, 27AL and 29AL, and no bacteria control. C) Effects of P23, 27AL, and 29AL in sterilized A-WW and K-WW on the *L. gibba* growth compared to control based on the number of fronds (closed bar) and dry weight (open bar) after 10 days of cultivation. The initial number of fronds was two in all experiments. Values are mean ± SD (n = 3). Different alphabets between treatments in A (only given on selected bacteria and control) and C indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

3.5. Restrictive metabolic pathways of nitrogen in Chryseobacterium bacteria

According to the analysis of mineral nutrients described above, both A-WW and K-WW have very low nitrogen contents compared to the plant medium Hoagland, which was assumed to be a growth-limiting factor for duckweed (Table 1). Nitrogen is an essential mineral for the growth and reproduction of living organisms, including duckweed and bacteria (Fang et al., 2007). Consistently, supplementation of ammonium (NH_4) or nitrate (NO_3) to A-WW restored duckweed growth (Supplemental data, Fig. S3). Therefore, we hypothesized that the mutualistic interaction between duckweed and PGPB interfered with the competition for nutrients including nitrogen sources. Indeed, the competition between land plants and soil microorganisms for inorganic and organic nitrogen has been reported even at relatively fertile sites (Kaye and Hart, 1997; Jones et al., 2018).

Therefore, we wondered whether *Chryseobacterium* has some specific trait for avoiding nitrogen competition with a host plant. We then analyzed and compared the nitrogen metabolic pathways retrieved from the KEGG database of three bacterial species, *A. calcoaceticus* CA16, *P. fulva* 12-X, and *Chryseobacterium indologenes* FDAARGOS_337, that are the same or closely related to the PGPB strains P23, Ps6, and the newly isolated 27AL/29AL, respectively. Strains 27AL and 29AL shared about 96% identities with the 16S rRNA gene of *C. indologenes*. Based on the KEGG pathway map (Supplemental data, Fig. S4), *Chryseobacterium* has apparently limited nitrogen pathway compared to bacteria of other genera. To confirm this finding, we carefully searched for relevant genes in the *C. indologenes* genome and found putative NarK/NasA family nitrate transporter genes (TLX26322 and TLX26323) and a nitrite reductase gene (TLX26356); however, no nitrate reductase gene was detected. On the other hand, neither nitrite nor nitrate reduction activities have been reported for most *Chryseobacterium* strains, including *C. taichungense* (Shen et al., 2005). Thus, limited use of nitrogen compounds seems generally shared across the genus *Chryseobacterium*.

To verify this hypothesis, we conducted growth experiments of 27AL in BS medium with different nitrogen sources (Supplemental data, Fig. S5). As a control we used *A. calcoaceticus* P23, which is suggested to exploit a broad range of nitrogen sources. This assay revealed that 27AL grew normally in BS medium with casamino acids (organic nitrogen) but failed to grow in media with either NO_3 or NH_4 as sole nitrogen sources. On the other hand, P23 could grow in media containing both organic and inorganic nitrogen compounds. These results strongly suggested that 27AL does not compete with duckweed for inorganic nitrogen sources, which is an advantageous trait for PGPB application under nitrogen-limiting conditions.

3.6. Factors affecting PGP behavior of A. calcoaceticus P23

P23 exerted no duckweed growth-promoting effect, but rather inhibited the growth of *L. gibba* in A-WW and K-WW conditions as shown in Fig. 2B and C. However, as suggested above, nitrogen limitation in WW may be one of the key factors causing this inhibition effect of P23 due to competition with the host plant for nutrients. Therefore, we prepared various Hoagland media with mineral compositions mimicking K-WW in order to identify factors affecting the PGP activity of P23. We chose to mimic K-WW because both PGPB (either P23 or Ps6) could not clearly show PGP activity in this condition. Among the ions and minerals of WW, we focused on NH_4, NO_3, Na, Cl, SO_4, and PO_4 because of their significant excess or depletion compared to the Hoagland medium as well as their biological importance for duckweed growth. Therefore, the

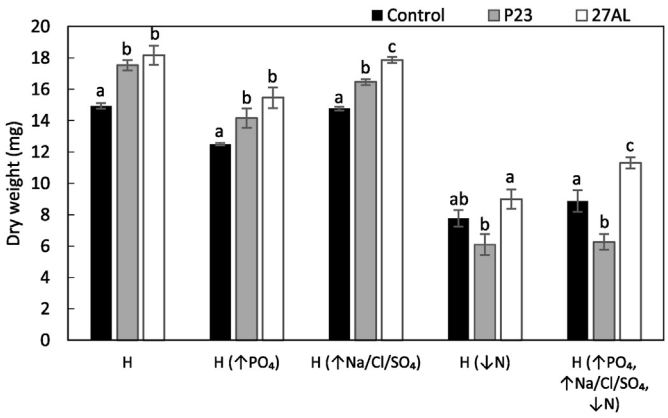

Fig. 5. Effect of different amount minerals on P23 (grey bars) and 27AL (open bars) activities against *L. gibba* growth compared to aseptic duckweed control (closed bars) after 14 days of cultivation in modified Hoagland medium mimicking K-WW. The initial number of fronds inoculated was ten. Values are mean ± SD (n = 3). Different alphabets in each treatment indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

Hoagland medium was modified as shown in Supplemental data, Table S2 and used for duckweed growth experiments (Fig. 5). We found that high PO_4, Na, Cl, or SO_4 conditions did not significantly affect the PGP activities of P23 and the newly isolated 27AL. On the other hand, low nitrogen (NH_4 or NO_3) conditions generally resulted in reduced growth of duckweed by switching the behavior of P23 from duckweed growth promotion to inhibition. In contrast, 27AL promoted duckweed growth under all conditions, including low nitrogen. Moreover, the phenomena of duckweed growth inhibition by P23 and promotion by 27AL were slight but most significantly observed in the combination of low NH_4/NO_3 and high PO_4/Na/Cl/SO_4, which is the condition most similar to K-WW.

Furthermore, we observed the effects of NH_4/NO_3, PO_4, Na, Cl, and SO_4 on bacterial colonization by quantifying average colony-forming units (CFU) per plant in each modified Hoagland medium. Notably, the initial CFU/plant value of P23 (7.3×10^5) was more than three times that of 27AL (2.2×10^5) suggesting a higher activity of adhesion and growth on duckweed in Hoagland medium. However, the CFU/plant value of P23 after 14 days of growth was dramatically decreased in normal Hoagland medium, from 7.3×10^5 to 6×10^2. Conversely, the CFU/plant value of P23 after 14 days of growth was highest (7×10^4) in the combined low NH_4/NO_3 and high PO_4/Na/Cl/SO_4 condition, in which P23 most severely inhibited duckweed growth (Fig. 5). Understandably, the more P23 cells colonized the duckweed, the more they competed with it for nitrogen. On the other hand, the CFU/plant value of 27AL was not significantly different between normal Hoagland medium (4.3×10^3) and low NH_4/NO_3 and high PO_4/Na/Cl/SO_4 conditions (1.1×10^3). In summary, these results showed that different mineral conditions can affect the colonization of bacteria on duckweed, with beneficial or detrimental effects depending on nutrient availability in the environment. However, the reason why P23 colonized more in combined low NH_4/NO_3 and high PO_4/Na/Cl/SO_4 conditions is still unclear. We observed that P23 also behaved similarly against another duckweed, *L. minor* (data not shown).

4. Conclusions

PGPB are currently expected to become a new nature-based technology for increasing duckweed biomass production. Here we call attention to the use of PGPB, especially in factory WW with uneven nutritional conditions. Highly active PGPB obtained from

the environmental water in which duckweed naturally grows do not have a universal function for plant growth promoting. Nevertheless, we showed for the first time that among the indigenous bacteria that naturally grow in factory WW unrelated to duckweed habitat there exist bacteria that can promote duckweed growth in the water condition. Overall, in order to practically produce duckweed biomass in different water environments, it is important to select and utilize PGPB adapted to each environment.

Credit author statement

Yeni Khairina: Writing-original manuscript, Conceptualization, Methodology, Investigation, Validation. Rahul Jog: Experimental guidance, discussion, Validation. Chanita Boonmak: Project administration, discussion, Validation. Tadashi Toyama: Project administration, discussion, Validation. Tokitaka Oyama: Providing plant resources, Project administration, discussion, Validation. Masaaki Morikawa: Funding acquisition, Project administration, Conceptualization, Supervision, Writing – review & editing manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.129247.

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Supplemental data

Table S1. Production of general PGP factors of *A. calcoaceticus* P23 and *Chryseobacterium* sp. 27AL and 29AL

Bacterial strain	IAA	Phosphate solubilization	Siderophore
P23	–	++	+
27AL	+ (81.73 µg /mL)	–	+
29AL	+ (83.76 µg /mL)	–	+

IAA production: The value in parenthesis is the mount of IAA produced in LB medium supplemented with tryptophan.
Phosphate solubilization: Formation of clear zone around the colony on Pikovskaya agar plate.
Siderophore production: Formation of orange color zone around the colony on CAS agar plate.

Table S2. Modification of Hoagland medium to examine the effect of significantly large and small amount minerals on the PGPB activities for duckweed

Minerals	Treatment (mineral content, mM)					K-WW
	Hoagland **: H**	Hoagland with high PO_4^- **: H(↑PO_4^-)**	Hoagland with high Na/Cl/SO_4 **: H(↑Na/Cl/SO_4)**	Hoagland with low N **: H(↓N)**	Hoagland with high PO_4, high Na/Cl/SO_4, low N **: H(↑PO_4↑Na/Cl/SO_4↓N)**	
Na	0.03	3.35†	46.11	0.03	49.46†	46.11
SO_4	2.11	2.11	13.63†	2.11	13.63†	4.34
Cl	2.00	2.00	25.04†	2.00	25.04†	11.27
NO_3	0.36	0.36	0.36	0.005	0.005	0.005
PO_4	0.03	3.35	0.03	0.03	3.35	3.35
NH_3	-	-	-	0.04	0.04	0.04

Underlined values indicate the minerals modified to Hoagland medium.
† Values changed due to unavoidable effect of counterions.

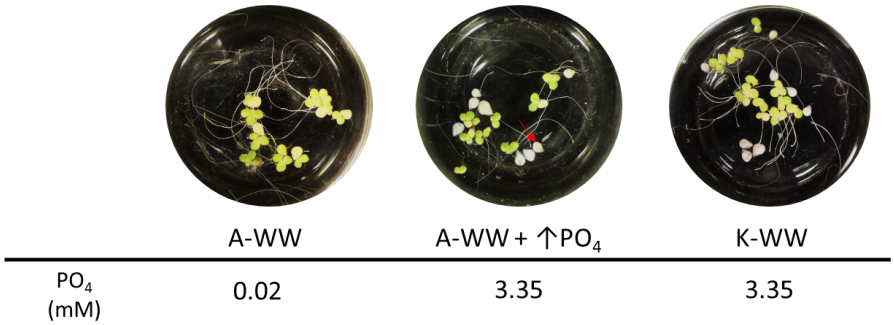


Fig. S1. Effects of different PO_4 concentration on *L. gibba*. Experiments were conducted by growing *L. gibba* for 10 days in A-WW, K-WW, and modified A-WW that was adjusted PO_4 concentration similar to K-WW. Change of the fronds color from green to white (chlorosis) was observed upon growing under high PO_4 (3.35 mM) condition.

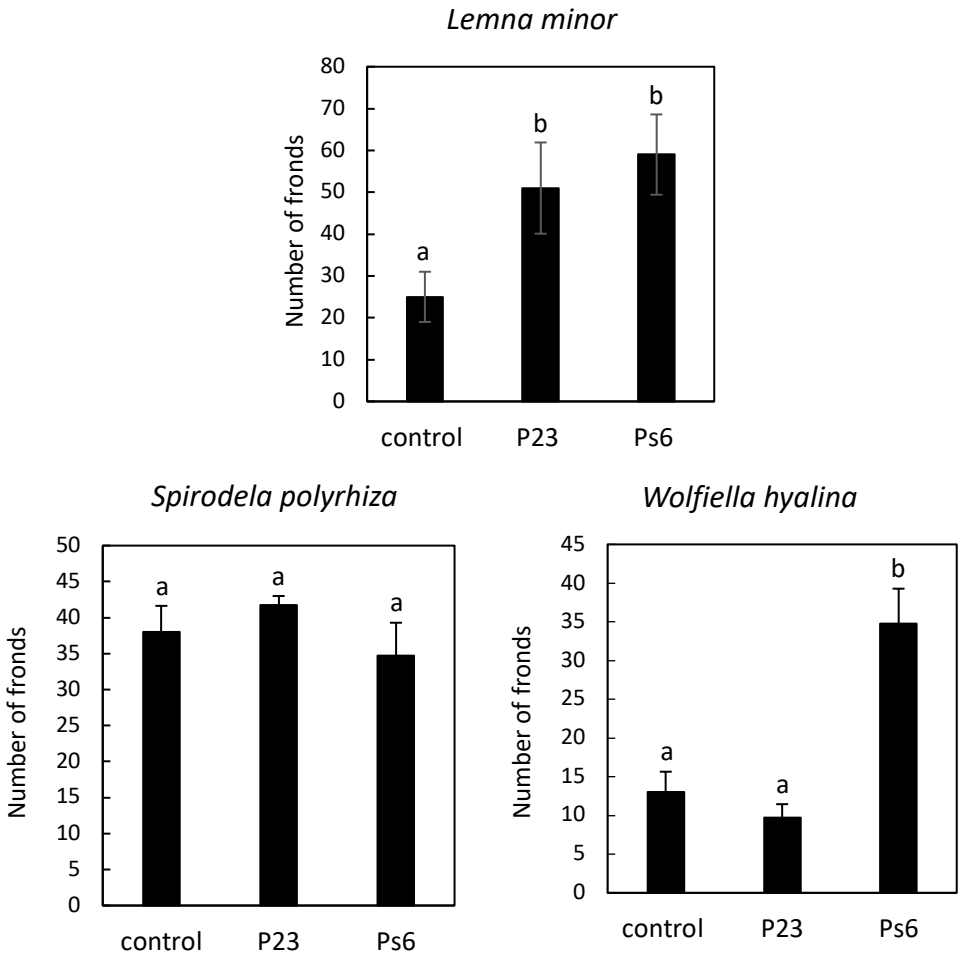


Fig. S2. Effects of previously isolated PGPB (P23 and Ps6) on the growth of different duckweed species after 10 days in Hoagland medium. The initial number of fronds inoculated was two in all experiments. Values are mean ± SD (n = 3). The results show that the duckweed growth-promoting activity of each PGPB is various depending on the duckweed species. Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

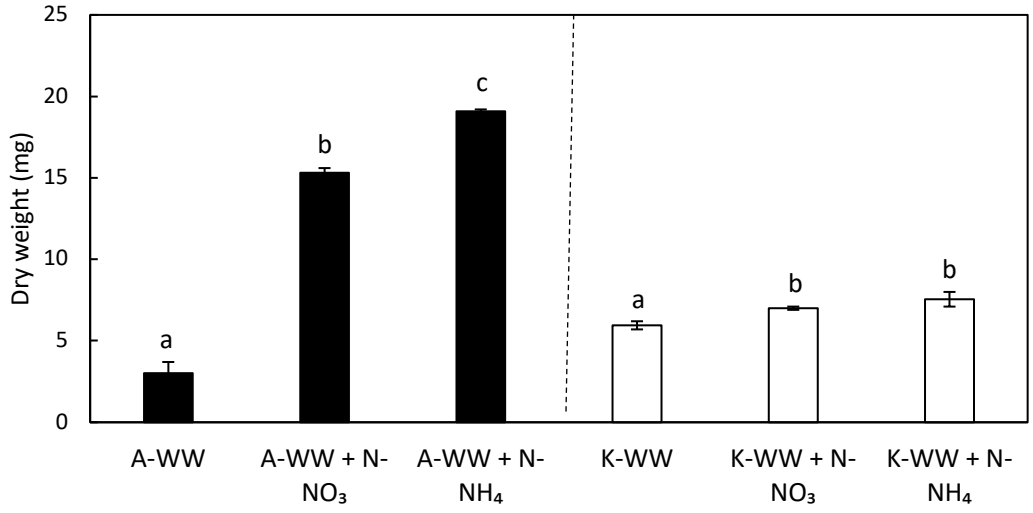


Fig. S3. The growth of *L. gibba* in A-WW and K-WW supplemented with or without 10 mg/L of N-NH_4 (as NH_4Cl) or N-NO_3 (as KNO_3). Total dry weight was compared after 20 days of cultivation. Initial number of fronds inoculated was two in all experiments. Values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

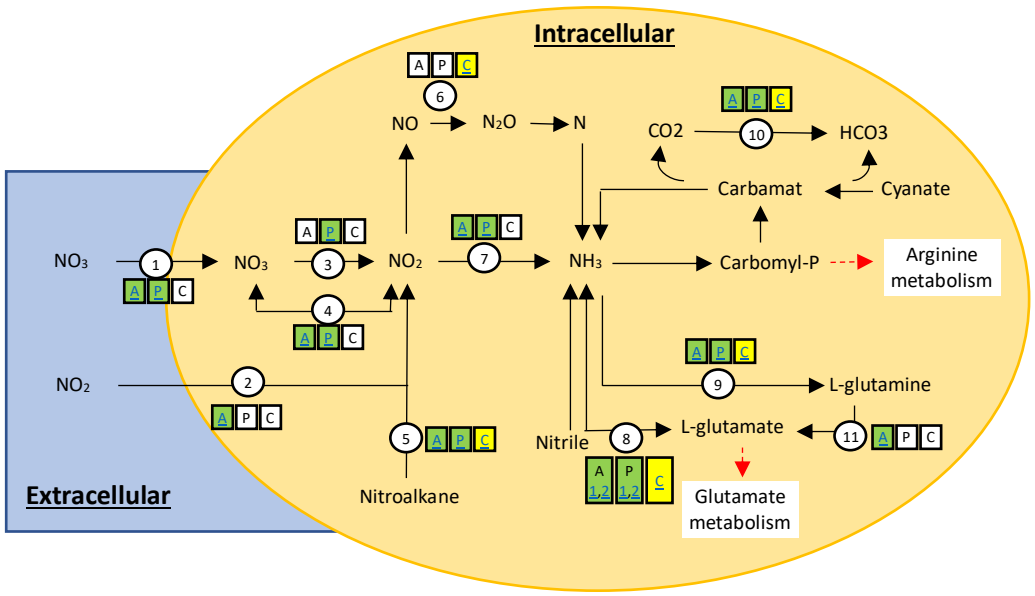
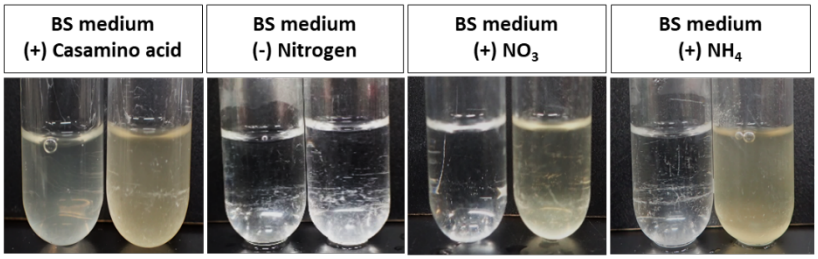


Fig. S4. Nitrogen metabolic pathway retrieved from KEGG database. The green color describes the existence of the gene/enzyme for A) *Acinetobacter calcoaceticus*; P) *Pseudomonas fulva*; while yellow color describes the existence gene in C) *Chryseobacterium indolegense*, if the bacteria don't have the gene, white color is applied. Numbers 1 to 11 are the genes for: 1) MFS transporter, NNP family, nitrate transporter; 2) MFS transporter, NNP family, nitrite transporter; 3) Nitrate reductase; 4) assimilatory nitrate reductase catalytic; 5) nitronate monooxygenase; 6) nitric-oxide reductase; 7) nitrite reductase; 8) glutamate dehydrogenase; 9) glutamine synthetase; 10) carbonic anhydrase; 11) glutamate synthase, respectively.



Strain	27AL	P23	27AL	P23	27AL	P23	27AL	P23
OD	0.333	1.020	0.002	0.001	0.003	0.250	0.002	0.850

Fig. S5. Growth tests of *Chryseobacterium* sp. 27AL and *Acinetobacter* sp. P23 in BS medium; 1) with casamino acid (1 g/L) as a positive control; 2) no nitrogen (negative control); 3) with $NaNO_3$ (1 g/L); with NH_4Cl (1 g/L). Cultures were shaken for 3 days at 30°C and measured OD_{600}. White lines and spots are scratches of glass test tubes.