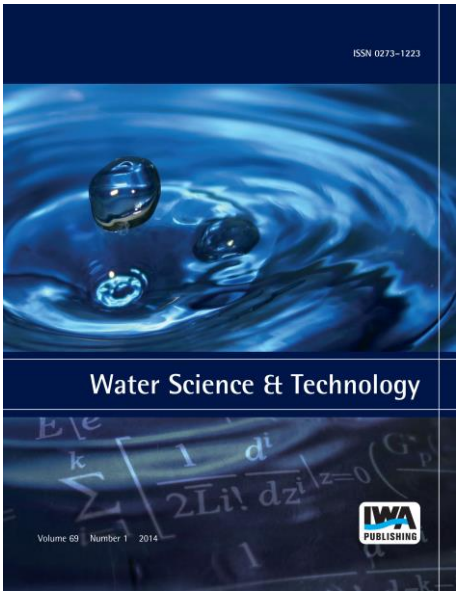

Enhanced biomass production of duckweeds by inoculating a plant growth-promoting bacterium, *Acinetobacter calcoaceticus* P23, in sterile medium and non-sterile environmental waters

T. Toyama, M. Kuroda, Y. Ogata, Y. Hachiya, A. Quach, K. Tokura, Y. Tanaka, K. Mori, M. Morikawa and M. Ike

ABSTRACT

Duckweed offers the promise of a co-benefit culture combining water purification with biomass production. *Acinetobacter calcoaceticus* P23 is a plant growth-promoting bacterium isolated from a duckweed, *Lemna aequinoctialis*. This study quantified its growth-promoting effect on three duckweeds (*L. aoukikusa*, *L. minor*, and *Spirodela polyrhiza*) in sterile Hoagland solution and evaluated its usefulness in duckweed culture under non-sterile conditions. P23 promoted growth of three duckweeds in sterile Hoagland solution at low to high nutrient concentrations (1.25–10 mg NO_3-N/L and 0.25–2.0 mg PO_4-P/L). It increased the biomass production of *L. aequinoctialis* 3.8–4.3-fold, of *L. minor* 2.3–3.3-fold, and of *S. polyrhiza* 1.4–1.5-fold after 7 days compared with noninoculated controls. P23 also increased the biomass production of *L. minor* 2.4-fold in pond water and 1.7-fold in secondary effluent of a sewage treatment plant under non-sterile conditions at laboratory-scale experiments. P23 rescued *L. minor* from growth inhibition caused by microorganisms indigenous to the pond water. The results demonstrate that the use of P23 in duckweed culture can improve the efficiency of duckweed biomass production, and a positive effect of P23 on duckweed-based wastewater treatment can be assumed.

Key words | *Acinetobacter calcoaceticus*, biomass production, duckweed, plant growth-promoting bacteria

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INTRODUCTION

Duckweeds are the smallest and fastest-growing aquatic plants, classified in the Araceae subfamily Lemnoideae, which includes five genera: *Lemna*, *Landoltia*, *Spirodela*, *Wolffia*, and *Wolffiella* (Landolt 1986). They are useful agents for removing nitrogen (N) and phosphorus (P) from municipal (Dalu & Ndamba 2003; Ran *et al.* 2004), livestock (Cheng *et al.* 2002; Xu & Shen 2011), and industrial (Ozengin & Elmaci 2007) wastewaters because of their high growth rate and high nutrient uptake capabilities. They are also used to clean up eutrophied water bodies (Ansari & Khan 2008, 2009). Wastewater treatment and purification of polluted waters using duckweeds offers successful, cost-effective, low-energy, and environmentally friendly options around the world.

In addition, duckweeds have recently attracted attention as a good alternative feedstock for biofuel production owing to their high growth rate and high starch accumulation

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capabilities (Cui & Cheng 2014). Conversion of duckweed biomass to ethanol (Xu *et al.* 2011, 2012; Soda *et al.* 2015), butanol (Su *et al.* 2014), hydrogen gas (Xu & Deshusses 2015), bio-oil (Muradov *et al.* 2010; Duan *et al.* 2013) and liquid fuels (e.g., gasoline, diesel, and kerosene) (Baliban *et al.* 2013) has been demonstrated by using biological, pyrolysis, hydrothermal, or thermochemical processes. Duckweeds are considered to have several advantages over terrestrial energy crops: they can take up nutrients directly from water, do not need extra fertilization or irrigation, can be easily grown and harvested, and do not compete with food crop production and agricultural land use. Therefore, they have high potential as an energy crop, especially when they are grown in a co-benefit system that combines water purification with biomass production (Xu *et al.* 2011, 2012). Enhancement of duckweed growth is critical to achieving a highly efficient co-benefit system. Proposed strategies include selecting fast-growing species (Zhao *et al.* 2015; Ziegler *et al.* 2015) and optimizing growth conditions such as water depth, duckweed coverage ratio, and harvest period (Zhao *et al.* 2014a). However, different strategies to further enhance duckweed growth are highly desired.

The use of plant growth-promoting bacteria (PGPB; Bashan & Holguin 1998) may offer a rational way to further increase biomass yields. A number of PGPB have been found to improve growth and yields of terrestrial agricultural crops. Significantly increased crop productivity, with 1.1- to 2.6-fold increases in yields, has been reported in greenhouse and field trials (Lucy *et al.* 2004; Adesemoye & Kloepper 2009; Bhattacharyya & Jha 2012; Pérez-Montaño *et al.* 2014). In contrast, PGPB in aquatic plants had not been studied until we isolated *Acinetobacter calcoaceticus* P23 from a duckweed species, *Lemna aequinoctialis* (Yamaga *et al.* 2010). We initially isolated P23 as a phenol-degrading bacterium and then found that it doubled the growth rate of *L. aequinoctialis* in axenic Hoagland solution both with and without phenol. If PGPB can be used in duckweed culture, in combination with the above approaches for optimizing duckweed production, they should greatly enhance biomass productivity. However, little information is yet available on the capacity of PGPB in duckweed culture. To apply PGPB to practical duckweed culture, it is necessary to evaluate their host-plant specificity and effects on plant growth under different conditions. In particular, it is important to confirm that they can indeed promote plant growth in non-sterile environmental waters, including wastewater.

Our objectives were therefore to quantify the growth-promoting effect of P23 on different duckweeds under various conditions in order to clarify its general usefulness in duckweed culture. We evaluated the effects of P23 on the growth of three major duckweed species, *L. aequinoctialis*, *L. minor*, and *Spirodela polyrhiza*. We quantified the growth-promoting effects in sterile Hoagland nutrient solution at various nutrient concentrations, verified the effects in real environmental water, and finally confirmed the effects in real secondary effluent from a sewage treatment plant.

MATERIALS AND METHODS

Plant materials

Bacteria-free *L. aequinoctialis*, *L. minor*, and *S. polyrhiza* were prepared by washing in 0.5% sodium hypochlorite for 3 min, then in 70% ethanol for 1 min, and finally in sterilized water three times for 1 min. Plants were aseptically cultured in flasks containing sterile Hoagland solution (36.1 mg/L KNO_3, 293 mg/L K_2SO_4, 3.87 mg/L NaH_2PO_4, 103 mg/L $MgSO_4{\cdot}7H_2O$, 147 mg/L $CaCl_2{\cdot}H_2O$, 3.33 mg/L $FeSO_4{\cdot}7H_2O$, 0.95 mg/L H_3BO_3, 0.39 mg/L $MnCl_2{\cdot}4H_2O$, 0.03 mg/L $CuSO_4{\cdot}5H_2O$, 0.08 mg/L $ZnSO_4{\cdot}7H_2O$, and 0.254 mg/L $H_2MoO_4{\cdot}4H_2O$; pH 7.0) in a growth chamber ($28 \pm 1\ ^\circ C$, fluorescent lamps at a photosynthetic photon flux density of 80 $\mu mol/m^2/s$, 16 L:8 D photoperiod).

Acinetobacter calcoaceticus P23 culture and preparation of cell suspensions

Strain P23 was grown in LB medium (10 g/L Bacto Peptone, 5 g/L Bacto Yeast extract, 5 g/L NaCl; pH 7.2) overnight at $28\ ^\circ C$, with shaking at 150 rpm. Cells were harvested by two cycles of centrifugation (10,000 $\times g$, room temperature, 5 min) and washing for 30 s in sterilized Hoagland solution, and then were resuspended in the same medium. The P23 cell suspension, whose optical density at 600 nm (OD_{600}) was first adjusted to a known value, was inoculated as described below. For determination of cell density, the cells were dried for 3 h at $90\ ^\circ C$ and weighed. $OD_{600} = 1$ was equivalent to 0.496 mg dry weight/mL.

Pond water and secondary effluent from a sewage treatment plant

Water collected from Inukai Pond (Suita, Osaka, Japan) was first passed through an Isopore membrane filter (pore size, 10 μm; polycarbonate; Merck Millipore) to remove

microalgae so as to avoid overgrowth during experiments. It had a pH of 7.3; undetectable NH_4-N, NO_2-N, and PO_4-P; and 0.01 mg/L NO_3-N. After incubation for 7 days at 28 °C on 0.1× LB agar plates, it was determined to have 8.0×10^3 CFU/mL of total heterotrophic bacteria. Secondary effluent was collected from a municipal sewage treatment plant in Kofu city (Yamanashi, Japan). It had a pH of 7.5, 4.27 mg/L NH_4-N, 0.66 mg/L NO_2-N, 7.72 mg/L NO_3-N and 0.98 mg/L PO_4-P, and 8.5×10^6 CFU/mL of total heterotrophic bacteria.

Co-culture of duckweed with P23 cells in Hoagland solution under sterile conditions at various nutrient concentrations

Ten fronds of each duckweed species were placed in 300-mL flasks ($n = 2$ per species per nutrient concentration, including control) containing 100 mL of sterile Hoagland solution. We then inoculated P23 cells into the flasks at a final density of 0.15 mg dry weight/mL. The Hoagland solution concentrations ranged from 0.25× to 2×; this corresponded to nutrient concentrations ranging from 1.25 mg NO_3-N/L + 0.25 mg PO_4-P/L to 10 mg NO_3-N/L +2.0 mg PO_4-P/L. All of the flasks were incubated without shaking in the chamber for 7 days as described above. The fronds were counted daily. After 7 days, all plants in each flask were collected, dried for 3 h at 90 °C, and weighed.

Co-culture of duckweed with P23 cells in pond water under sterile and non-sterile conditions

We examined the growth-promoting activity of P23 with *L. minor*, which is one of the most widespread duckweed species in the world (Holm *et al.* 1997). To examine the effects of indigenous microorganisms in pond water on duckweed growth and the ability of P23 to promote growth, we used both sterile and non-sterile pond water. Sterile pond water was prepared by filtration (pore size 0.2 μm). Both water samples were then supplemented with KNO_3 (1.25 mg NO_3-N/L) and NaH_2PO_4 (0.25 mg PO_4-P/L). Ten fronds of *L. minor* were transferred into 200-mL flasks containing 100 mL of the water samples ($n = 3$), followed by inoculation with P23 cell suspension at 0.15 mg dry weight/mL. All flasks were incubated without shaking in the chamber as described above for 15 days. The number of fronds was counted daily. After 15 days, all plants in each flask were collected, dried, and weighed.

To further determine the effect of cell density on the ability of P23 to promote duckweed growth under non-sterile conditions, we inoculated P23 cell suspensions at 0, 0.025, 0.05, and 0.15 mg dry weight/mL into flasks containing 10 fronds of *L. minor* in 100 mL of non-sterile pond water ($n = 3$ per cell density). All flasks were incubated without shaking in the chamber for 12 days. The fronds were counted daily. After 12 days, all plants in each flask were collected, dried, and weighed.

Co-culture of duckweed with P23 cells in secondary effluent under non-sterile conditions

To verify the ability of P23 to promote duckweed growth in real sewage effluent, we placed 10 fronds in 500-mL flasks ($n = 3$) containing 200 mL of secondary effluent with or without P23 cells at a final density of 0.15 mg dry weight/mL. All flasks were incubated without shaking in the chamber as described above for 7 days. The fronds were counted daily, and the dry weights of all plants in each flask were measured after 7 days. These three co-culture experiments in Hoagland solution, pond water, or secondary effluent are summarized in Figure 1.

Statistical analysis

Each value used in the statistical analysis represents the results of two or three samples ($n = 2$ or 3 replicates) per experiment. All results are expressed as mean ± standard deviation (SD). Significance ($P < 0.05$) was assessed using the *t*-test in IBM SPSS Statistics v. 22.0.

RESULTS AND DISCUSSION

Growth promotion of *L. aequinoctialis*, *L. minor*, and *S. polyrhiza* by P23 cells under sterile conditions at various nutrient concentrations

We grew each of the three duckweed species with P23 cells (0.15 mg dry weight/mL) in sterile Hoagland solution at different nutrient concentrations for 7 days. The results are summarized in Figures 2–5 and Table 1. At all nutrient concentrations, all three duckweed species grew more rapidly in the presence of P23 cells than in the absence. The increase in the number of fronds (final number minus initial 10) was 2.9–3.3-fold in *L. aequinoctialis*, 2.7–3.8-fold in *L. minor*, and 1.2–1.4-fold in *S. polyrhiza* compared with the non-inoculated controls (Table 1). Biomass production (increase in dry weight) was similarly increased 3.8–4.3-fold,

I. Co-culture of duckweed with P23 cells in Hoagland solution under sterile conditions at various nutrient concentration

- Duckweeds (3 species): *L. aequinoctialis*, *L. minor*, and *S. polyrhiza*
- Hoagland solution (5 different concentrations): 0.25 ×, 0.5 ×, 1.0 ×, 1.5 ×, and 2.0 ×
  Nutrient concentration in 1.0 × Hoagland = 5 mg NO_3-N/L and 1 mg PO_4-P/L
- P23 (with/without P23): 0 and 0.15 mg dry weight/mL

II. Co-culture of duckweed with P23 cells in pond water under sterile and non-sterile conditions

- Duckweed (1 species): *L. minor*
- Pond water : sterile and non-sterile
  Nutrient concentrations in pond water = 1.25 mg NO_3-N/L and 0.25 mg PO_4-P/L
- P23 (4 different cell densities): 0, 0.025, 0.05, and 0.15 mg dry weight/mL

III. Co-culture of duckweed with P23 cells in secondary effluent under non-sterile condition

- Duckweed (1 species): *L. minor*
- Secondary effluent: non-sterile
  Nutrient concentrations = 4.27 mg-NH_4-N/L, 0.66 mg NO_2-N/L, 7.72 mg NO_3-N/L, and 0.98 mg PO_4-P/L
- P23 (with/without P23): 0 and 0.15 mg dry weight/mL

Figure 1 | Experimental scheme.

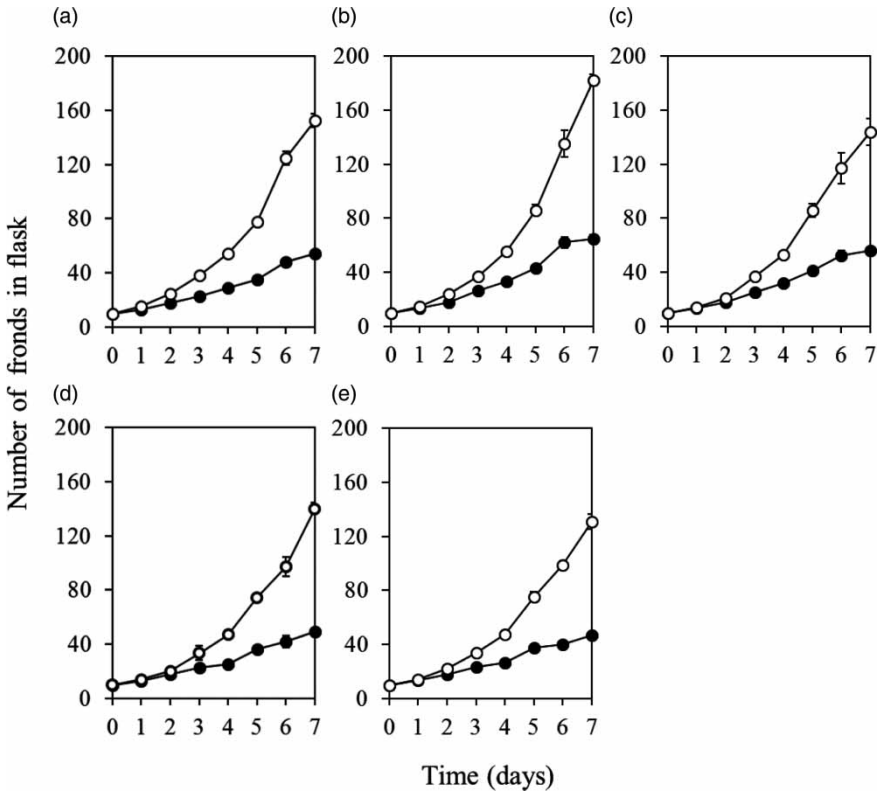

Figure 2 | Changes in the number of *L. aequinoctialis* fronds in culture with P23 (○, 0.15 mg dry weight/mL) and without P23 (●) in Hoagland solution (1× =5.0 mg NO_3-N/L + 1.0 mg PO_4-P/L) at different nutrient concentrations: (a) 0.25×; (b) 0.50×; (c) 1.0×; (d) 1.5×; (e) 2.0×. Values are mean ± SD ($n = 2$).

2.3–3.3-fold, and 1.4–1.5-fold, respectively (Table 1). The nutrient concentration of the medium did not greatly affect the growth-promoting activity of P23 in any species.

The fact that P23 could promote the growth of all three duckweed species indicates its versatile potential to accelerate biomass production. Various duckweed species have

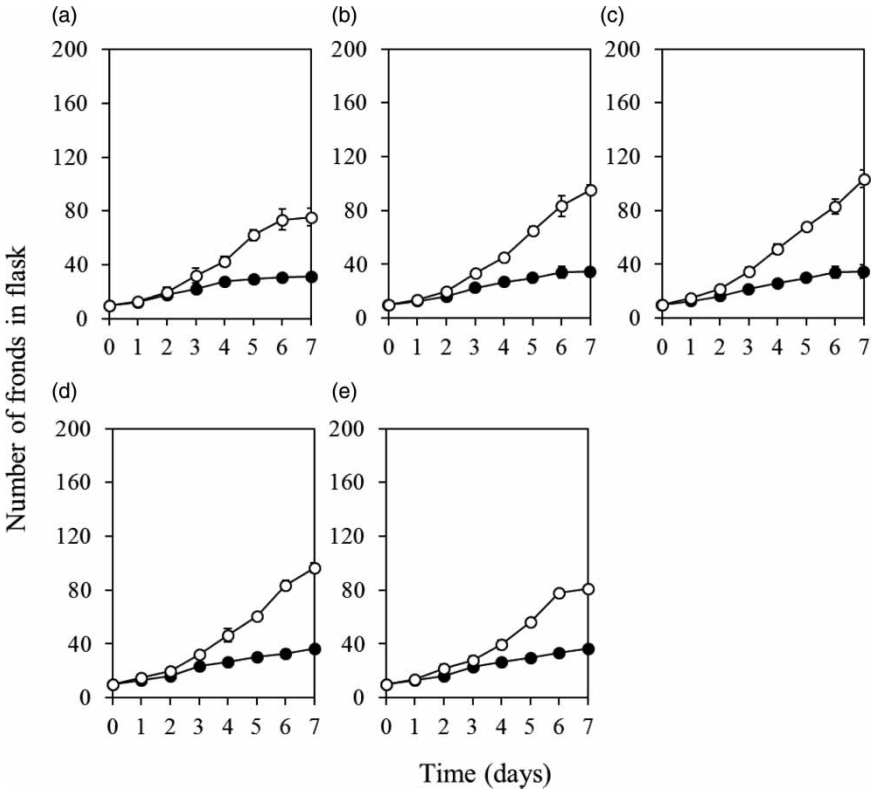

Figure 3 | Changes in the number of *L. minor* fronds in culture with P23 (○, 0.15 mg dry weight/mL) and without P23 (●) in Hoagland solution (1× = 5.0 mg NO_3-N/L + 1.0 mg PO_4-P/L) at different nutrient concentrations: (a) 0.25×; (b) 0.50×; (c) 1.0×; (d) 1.5×; (e) 2.0×. Values are mean ± SD ($n = 2$).

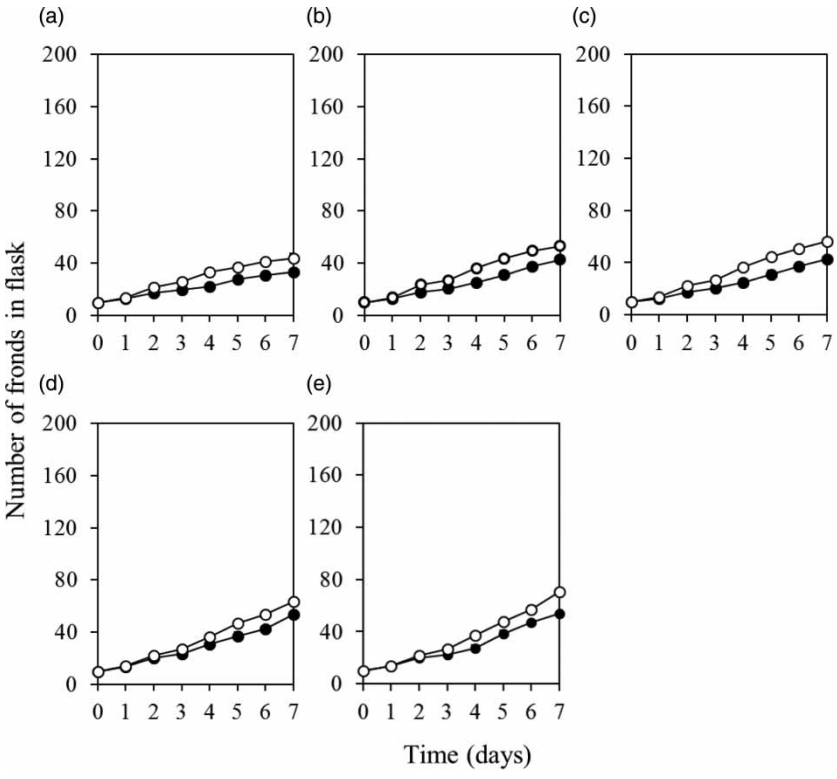

Figure 4 | Changes in the number of *S. polyrhiza* fronds in culture with P23 (○, 0.15 mg dry weight/mL) and without P23 (●) in Hoagland solution (1× = 5.0 mg NO_3-N/L + 1.0 mg PO_4-P/L) at different nutrient concentrations: (a) 0.25×; (b) 0.50×; (c) 1.0×; (d) 1.5×; (e) 2.0×. Values are mean ± SD ($n = 2$).

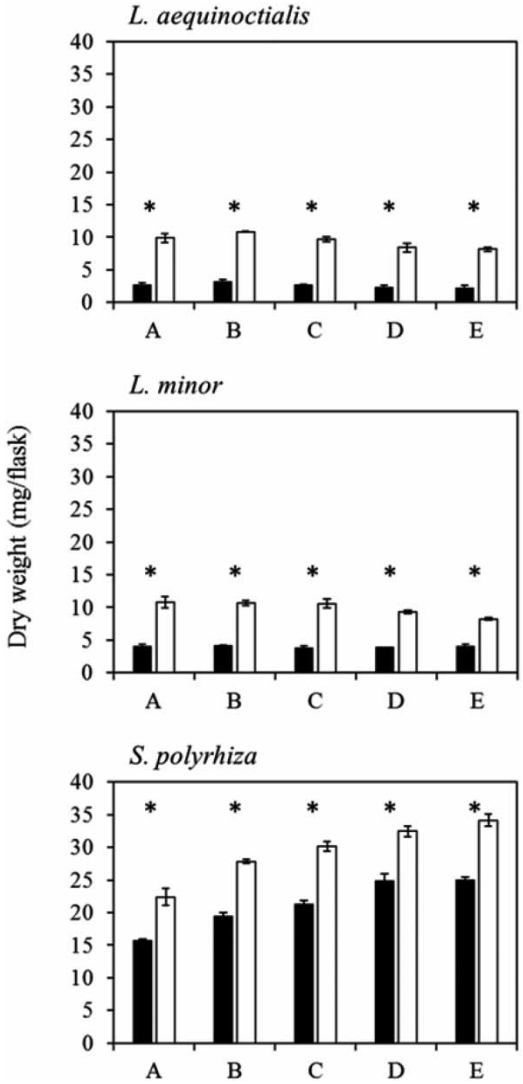

Figure 5 | Dry weight of duckweed biomass after 7 days in culture with P23 (□, 0.15 mg dry weight/mL) and without P23 (■) in Hoagland solution (1× = 5.0 mg NO_3-N/L + 1.0 mg PO_4-P/L) at different nutrient concentrations: (A) 0.25×; (B) 0.50×; (C) 1.00×; (D) 1.5×; (E) 2.0×. Initial dry weights of 10 fronds per flask: *L. aequinoctialis*, 0.45 ± 0.07 mg; *L. minor*, 0.85 ± 0.09 mg; *S. polyrhiza*, 3.4 ± 0.14 mg. Values are mean ± SD ($n = 2$). *Significant difference ($P < 0.05$) between values with and without P23 within a treatment.

been used in wastewater treatment, water purification, and biomass production according to wastewater, temperature, and purpose, because growth rate, nutrient removal ability, optimal growth conditions, and chemical composition differ among species (Landolt 1986; Lemon *et al.* 2001; Zhao *et al.* 2014a, 2014b; Soda *et al.* 2015; Ziegler *et al.* 2015). For example, *L. minor* and *S. polyrhiza* are suitable for culture at lower and higher temperatures, respectively, because they grow at 5 to 31 °C and 15 to above 35 °C, respectively (Landolt 1986). *S. polyrhiza* is suitable for saline wastewater treatment because of its high salt tolerance (Sree *et al.* 2015). In addition, mixed cultures of duckweed species have higher nutrient removal and biomass production rates than single cultures (Zhao *et al.* 2014b). Therefore, it will be useful to examine a variety of species to achieve a high-efficiency culture system. In this context, the broad-spectrum growth-promoting effect of P23 is of great practical significance.

Although P23 promoted the growth of all three species, it promoted the growth of *L. aequinoctialis* and *L. minor* more than that of *S. polyrhiza*. The ability of P23 to promote the growth of *L. aequinoctialis* was the highest. The finding may result from a certain host specificity. Also, the ability of P23 to promote the growth of *L. minor* more than that of *S. polyrhiza* may relate to the evolutionarily closer relationship of *L. minor* and *L. aequinoctialis* (Les *et al.* 2002).

In terrestrial soils, N and P often limit plant growth. N-fixing PGPB and P-solubilizing PGPB thus play significant roles by providing nutrients that support plant growth. Their effects are strongly influenced by nutrient conditions (Adesemoye & Kloepper 2009; Bhattacharyya & Jha 2012), with much stronger effects in nutrient-deficient soils than in nutrient-rich soils (Egamberdiyeva 2007). In contrast, P23 promoted duckweed growth over a wide range of nutrient concentrations in this study, suggesting that the nature and mechanisms of duckweed growth promotion by P23 might differ from that of soil PGPB. Thus, growth-promoting mechanisms specific to aquatic plants must be explored in future studies.

Enhancement of *L. minor* growth by P23 cells in pond water and secondary effluent

In the absence of P23, the increases in frond number and biomass production of *L. minor* were significantly lower ($P < 0.05$) in non-sterile pond water than in sterile pond water (Figure 6, Table 2). This result indicates that indigenous microorganisms in the pond water inhibited the growth of *L. minor*.

In the presence of P23 in the sterile pond water, the increased frond number and biomass production of *L. minor* were increased 1.3- and 1.9-fold, respectively, relative to its absence (Figure 6, Table 2). This enhanced biomass production must result from the direct growth promotion effect of P23, as observed in sterile Hoagland solution. Although the growth of *L. minor* in the absence of P23 was less in non-sterile pond water than in sterile pond water, it is noteworthy that P23 increased the frond number and biomass production even in non-sterile pond water 3.8- and 2.4-fold, respectively (Figure 6,

Table 1 | Effects of P23 on the growth of *L. aequinoctialis*, *L. minor*, and *S. polyrhiza* in sterile Hoagland solution at indicated nutrient concentrations

Duckweed species	Hoagland solution	Treatment	Increase in number of fronds per flask during 7 days[a] (and ratio of P23 to control)	Biomass production (increase in dry weight; mg) of plants per flask during 7 days[a] (and ratio of P23 to control)
L. aequinoctialis	0.25×	P23	143 ± 5.0 (3.2)*	9.45 ± 0.71 (4.2)*
		Control	44.5 ± 0.7	2.25 ± 0.28
	0.5×	P23	172 ± 4.2 (3.1)*	10.4 ± 0.07 (3.8)*
		Control	55.0 ± 2.8	2.75 ± 0.28
	1.0×	P23	134 ± 9.9 (2.9)*	9.20 ± 0.35 (4.1)*
		Control	46.0 ± 1.4	2.25 ± 0
	1.5×	P23	130 ± 4.2 (3.3)*	7.95 ± 0.71 (4.3)*
		Control	39.0 ± 2.8	1.85 ± 0.28
	2.0×	P23	121 ± 5.7 (3.3)*	7.70 ± 0.35 (4.3)*
		Control	36.5 ± 0.7	1.80 ± 0.35
L. minor	0.25×	P23	65.5 ± 6.3 (3.0)*	9.95 ± 0.85 (3.1)*
		Control	21.5 ± 0.7	3.25 ± 0.28
	0.5×	P23	85.5 ± 3.5 (3.5)*	9.80 ± 0.35 (3.0)*
		Control	24.5 ± 3.5	3.30 ± 0.07
	1.0×	P23	93.5 ± 6.4 (3.8)*	9.75 ± 0.71 (3.3)*
		Control	24.5 ± 5.0	3.00 ± 0.21
	1.5×	P23	86.5 ± 3.5 (3.3)*	8.45 ± 0.28 (2.8)*
		Control	26.5 ± 2.1	3.05 ± 0
	2.0×	P23	71.0 ± 2.8 (2.7)*	7.40 ± 0.21 (2.3)*
		Control	26.5 ± 0.7	3.25 ± 0.28
S. polyrhiza	0.25×	P23	34 ± 1.4 (1.4)*	19.0 ± 1.2 (1.5)*
		Control	23.5 ± 3.5	12.4 ± 0.1
	0.5×	P23	43.0 ± 1.4 (1.3)*	24.5 ± 0.4 (1.5)*
		Control	32.5 ± 0.7	16.1 ± 0.5
	1.0×	P23	46.5 ± 2.1 (1.4)*	26.8 ± 0.8 (1.5)*
		Control	32.5 ± 0.7	17.9 ± 0.6
	1.5×	P23	53.5 ± 2.1 (1.2)*	29.1 ± 0.8 (1.4)*
		Control	43.5 ± 2.1	21.5 ± 1.0
	2.0×	P23	60.5 ± 2.1 (1.4)*	30.8 ± 0.9 (1.4)*
		Control	44.0 ± 1.4	21.7 ± 0.4

Values are means ± SD. Values in parentheses are the ratio of the P23 value to the control (no P23) value.
[a]Final value minus initial value.
*Significant difference ($P < 0.05$) between values with and without P23 within a treatment.

Table 2). This highly enhanced biomass production is attributable to synergism between P23's direct growth promotion effect and its indirect effect of rescue from growth inhibition by indigenous microorganisms. It is interesting that P23's growth promotion effects were greater in non-sterile pond water (3.8-fold effect on frond number increase and 2.4-fold effect on biomass production) than in sterile pond water (1.3- and 1.9-fold, respectively), indicating that P23 could be especially useful in practical culture systems. On the other hand, the size of *L. minor* grown in the sterile pond water with P23 was a little bit larger than that in the non-sterile pond water with P23. The highest biomass production of *L. minor* was reached in the sterile pond water with P23 (Figure 6, Table 2).

Terrestrial plant PGPB often fail to perform in field conditions (Lucy *et al.* 2004). For example, Cu-tolerant PGPB highly promoted the growth of plants in sterilized tailings, but only weakly promoted it in non-sterile conditions (Liu *et al.* 2014). The report suggested that terrestrial plant PGPB cannot fully express their growth-promoting effect under non-sterile conditions owing to competition with other indigenous microorganisms (Liu *et al.* 2014). It is notable that P23 significantly promoted the growth of *L. minor* in real pond water, which harbors complex indigenous microbial communities. This trait of the duckweed–P23 association in water confers an advantage over the terrestrial plant–PGPB association in soil.

To determine the effective cell density of P23 to promote duckweed growth under non-sterile conditions, we grew

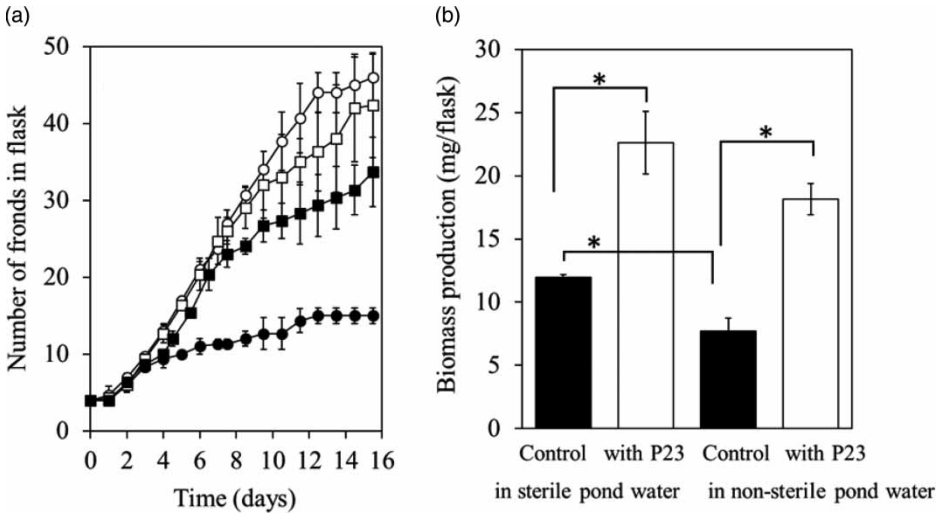

Figure 6 | Effects of P23 (0.05 mg dry weight/mL) on the growth of *L. minor* in sterile or non-sterile pond water for 15 days. (a) Changes in number of fronds growing in sterile (■, □) or non-sterile (●, ○) pond water without (■, ●, control) or with P23 cells (□, ○). (b) Biomass production (final minus initial dry weight) of *L. minor* in sterile or non-sterile pond water with or without P23 cells. Values are mean ± SD ($n = 3$). *Significant difference ($P < 0.05$) between dry weights.

Table 2 | Effects of P23 on the growth of *L. minor* in sterile or non-sterile pond water

Pond water	Treatment	Increase in number of fronds per flask during 15 days[a] (and ratio of P23 to control)	Biomass production (increase in dry weight; mg) of plants per flask during 15 days[a] (and ratio of P23 to control)
Sterile	P23	38 ± 6.8 (1.3)*	22.6 ± 2.5 (1.9)*
	Control	30 ± 4.5	12.0 ± 0.2
Non-sterile	P23	42 ± 3.0 (3.8)*	18.2 ± 1.2 (2.4)*
	Control	11 ± 1.07	7.7 ± 1.0

Values are means ± SD. Values in parentheses are the ratio of the P23 value to the control (no P23) value.
[a]Final value minus initial value.
*Significant difference ($P < 0.05$) between values with and without P23 within a treatment.

L. minor in pond water at various densities of P23 cells. Although there was no significant effect on growth at a cell density of 0.025 mg/mL, the effect of P23 became significant ($P < 0.05$) at higher cell densities (Figure 7, Table 3). The increased frond number and biomass production were increased 2.0- and 1.4-fold, respectively, in the presence of P23 cells at 0.05 mg/mL, and 4.3- and 1.8-fold at 0.15 mg/mL. Thus, the initial inoculation density of P23 cells would be important for the significant promotion of duckweed growth in this system.

Finally, P23 at 0.15 mg/mL significantly promoted the growth of *L. minor* even in real secondary sewage effluent (Figure 8, Table 4). After 7 days' culture, the frond number and biomass production were increased about 1.7-fold by P23 inoculation relative to the control. These results demonstrate that P23 can enhance duckweed biomass production even in real-world secondary sewage effluent.

It is notable that P23 is capable of exerting synergistic effects of growth promotion and rescue from growth inhibition under non-sterile conditions, increasing biomass production of *L. minor* 2.4- and 1.7-fold in real pond water and secondary sewage effluent, respectively. This growth-promoting effect was equivalent to or higher than that of terrestrial PGPB in crops (1.1- to 2.6-fold increases in yields at greenhouse and field trials) (Lucy *et al.* 2004; Adesemoye & Kloepper 2009; Bhattacharyya & Jha 2012; Pérez-Montaño *et al.* 2014). Moreover, P23 enhanced biomass production more effectively than previous approaches of optimizing water depth (1.5-fold), duckweed coverage ratio (1.6-fold), and harvest period (1.1-fold) (Zhao *et al.* 2014a). Thus, aquatic PGPB technology offers hope for enhancing duckweed growth to achieve effective biomass production coupled with effective purification of eutrophic environmental water and sewage. In the next steps, it will be necessary to evaluate the sustainable effect and utilization

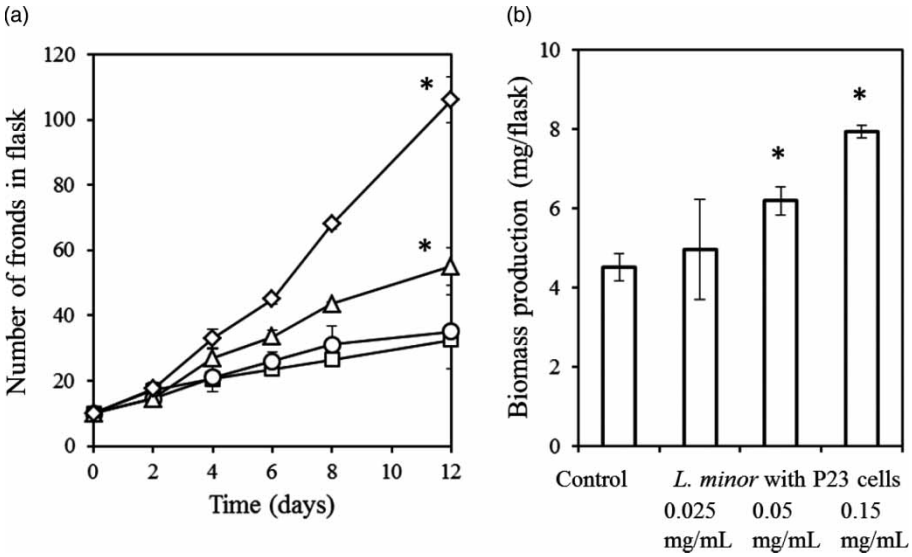

Figure 7 | Effects of P23 on the growth of *L. minor* in non-sterile pond water for 12 days. (a) Changes in the number of fronds growing with P23 cells at densities of 0 (□, control), 0.025 (○), 0.05 (Δ), or 0.15 (◇) mg dry weight/mL. (b) Biomass production (final minus initial dry weight) of *L. minor* in pond water with P23 cell densities of 0 (control), 0.025, 0.05, or 0.15 mg dry weight/mL during 12 days. Values are mean ± SD ($n = 3$). *Significant difference ($P < 0.05$) from control.

Table 3 | Effects of different cell densities of P23 on the growth of *L. minor* in non-sterile pond water

Cell density of P23 (mg dry weight/L)	Increase in number of fronds per flask during 12 days[a] (and ratio of P23 to control)	Biomass production (increase in dry weight; mg) of plants per flask during 12 days[a] (and ratio of P23 to control)
0 (control)	22 ± 2.5	4.5 ± 0.4
0.025	24 ± 9.6 (1.1)	5.0 ± 1.3 (1.1)
0.05	45 ± 4.8 (2.0)*	6.2 ± 0.4 (1.4)*
0.15	94 ± 6.7 (4.3)*	8.0 ± 0.1 (1.8)*

Values are means ± SD. Values in parentheses are the ratio of the P23 value to the control (no P23) value.
[a]Final value minus initial value.
*Significant difference ($P < 0.05$) between values with and without P23 within a treatment.

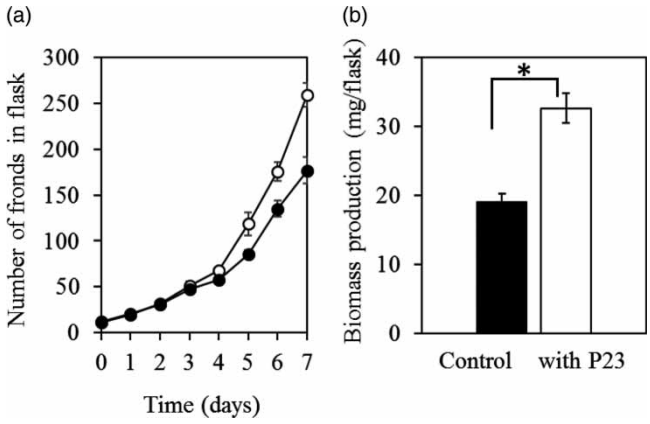

Figure 8 | Effects of P23 (0.15 mg dry weight/mL) on the growth of *L. minor* in secondary effluent for 7 days. (a) Changes in the number of fronds growing without (●) or with P23 cells (○). (b) Biomass production (final minus initial dry weight) of *L. minor* during 7 days. Values are mean ± SD ($n = 3$). *Significant difference ($P < 0.05$) between treatments.

of P23 in pilot-scale experiments to construct a practical PGPB–duckweed system.

CONCLUSION

Acinetobacter calcoaceticus P23, a plant growth-promoting bacterium originally isolated from *L. aequinoctialis*, significantly promoted the growth of three duckweed species (*L. aequinoctialis*, *L. minor*, and *S. polyrhiza*) and accelerated their biomass production, not only in sterilized Hoagland solution at a range of nutrient concentrations, but also in non-sterile pond water and secondary sewage

Table 4 | Effects of P23 on the growth of *L. minor* in secondary effluent

Treatment	Increase in number of fronds per flask during 7 days[a] (and ratio of P23 to control)	Biomass production (increase in dry weight; mg) of plants per flask during 7 days[a] (and ratio of P23 to control)
P23	266 ± 15 (1.7)*	32.6 ± 2.1 (1.7)*
Control	159 ± 15	19.0 ± 1.3

Values are means ± SD. Values in parentheses are the ratio of the P23 value to the control (no P23) value.
[a]Final value minus initial value.
*Significant difference ($P < 0.05$) between values with and without P23 within a treatment.

effluent. This excellent biomass productivity enhanced by P23 can be ascribed to synergistic effects of direct growth promotion and rescue from growth inhibition by indigenous microorganisms. Overall, these results strongly suggest that aquatic PGPB such as P23 offer the promise of effective biomass production in duckweed culture and seem to be beneficial for duckweed-based wastewater treatment and water purification.

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