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NOTE

Plant growth-promoting bacterium *Acinetobacter calcoaceticus* P23 increases the chlorophyll content of the monocot *Lemna minor* (duckweed) and the dicot *Lactuca sativa* (lettuce)

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***Acinetobacter calcoaceticus* P23 is a plant growth-promoting bacterium that was isolated from the surface of duckweed (*Lemna aoukikusa*). The bacterium was observed to colonize on the plant surfaces and increase the chlorophyll content of not only the monocotyledon *Lemna minor* but also the dicotyledon *Lactuca sativa* in a hydroponic culture. This effect on the *Lactuca sativa* was significant in nutrient-poor (×1/100 dilution of H2 medium) and not nutrient-rich (×1 or ×1/10 dilutions of H2 medium) conditions. Strain P23 has the potential to play a part in the future development of fertilizers and energy-saving hydroponic agricultural technologies.**

[**Key words:** Plant growth-promoting bacteria; *Acinetobacter calcoaceticus* P23; *Lemna minor*; *Lactuca sativa*; Chlorophyll content]

Food shortage is becoming a critical global issue for humans, and plants are essential primary producers in the biospheric food chain. Because biological elements such as nitrogen and phosphorous are only available in limited amounts on earth, it is clear that the reduction, reuse, and recycling of fertilizers is an important technological trend for saving energy, which is key to sustainable agriculture (1). Future agricultural production may also require vegetables to be grown in barren areas such as deserts and the polar regions, and even in extraterrestrial space. Hydroponic farming of plants and vegetables could be one of the best technologies for achieving this aim (2). Indoor hydroponic farming technology has enabled the cultivation of plants anywhere and at any time. However, hydroponic cultivation has disadvantages, including high initial set-up costs as well as the high costs of the regular energy inputs required under controlled culture conditions. These costs limit the practical application of the hydroponic farming technology to advanced countries such as Japan. To ensure a sustainable food supply, reductions in the use of fertilizers, in the total energy required for food production, and in the costs associated with hydroponic farming, are required.

Replacing chemical fertilizers with biofertilizers that mimic the natural ecosystem is expected to reduce the total energy input. Biofertilizers mainly consists of beneficial plant-associated microorganisms such as plant growth-promoting bacteria (PGPB) (3). The application of beneficial microbes was initiated in agricultural practice more than 60 years ago, and there is increasing evidence that these beneficial microbial populations can also enhance plant resistance to adverse environmental stresses such as water and nutrient deficiency, heavy metal contamination, and plant pathogens (4–6).

Acinetobacter calcoaceticus P23 was isolated from a duckweed species (*Lemna aoukikusa*) in the Hokkaido University Botanical Gardens (7). The bacterium is a PGPB associated with the Lemnaceae family, which includes species such as *Lemna aoukikusa*, *Lemna minor*, *Spirodela polyrhiza*, and *Wolffia arrhiza* (Dr. T. Toyama, personal communication). It has previously been shown to degrade hydrocarbons including aromatic phenols and aliphatic alkanes, and to promote the growth of *Lemna aoukikusa* (leading to a 2-fold increase in growth rate) (7). It is presently unclear whether the growth-promoting effects of strain P23 are limited to *Lemna aoukikusa*. Therefore, we determined whether strain P23 has a growth-promoting effect on plants other than duckweed, such as vegetables in a hydroponic culture system.

Strain P23 was grown in L broth (5 g/L Bacto Yeast extract, 5 g/L NaCl, and 10 g/L Bacto Tryptone; pH 7.2; BD Difco, NJ, USA) overnight at 30°C. *Escherichia coli* DH5α was grown in L broth at 37°C and used as a reference for comparison. *Lemna minor* (sourced from Hokkaido University Botanical Gardens) and vegetable seeds were surface-sterilized in 0.5% hypochloric acid and 0.02% Triton X-100 for 3–5 min, then washed five times with sterilized water. While majority of *Lemna minor* were killed after this treatment, one frond of bacteria-free *Lemna minor* survived and grew normally. Hoagland medium 1 (H1) (pH 7.0) was used for *Lemna minor* culture. The medium contains 36.1 mg/L KNO_3, 293 mg/L K_2SO_4, 147 mg/L $CaCl_2 \cdot 2H_2O$, 103 mg/L $MgSO_4 \cdot 7H_2O$,

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5.03 mg/L $NaH_2PO_4 \cdot 2H_2O$, 3.33 mg/L $FeSO_4 \cdot 7H_2O$, 0.95 mg/L H_3BO_3, 0.39 mg/L $MnCl_2 \cdot 4H_2O$, 0.08 mg/L $ZnSO_4 \cdot 7H_2O$, 0.39 mg/L $MnCl_2 \cdot 4H_2O$, 0.03 mg/L $CuSO_4 \cdot 5H_2O$, and 0.23 mg/L H_2MoO_4. The culture conditions for the plants were as follows: 25°C, 70% humidity, 5000 lux, and 16-h light/8-h dark cycle, unless indicated otherwise. Bacterial cells were washed twice by centrifugation and suspended with the appropriate medium at a concentration of $OD_{600} = 0.3$. This bacterial suspension was used for inoculating the plants. In order to minimize nutrient leakage from dead bacterial cells, we transferred the hydroponic plants in their bacterial suspension medium to a fresh bacteria-free medium at the start of the experiments. This enabled us to evaluate the effects of only the bacteria that attached to the plants. Plant growth was measured by the increase in wet and dry (48 h after drying at 60°C) weight. Chlorophylls were extracted by 1 mL of cold ethanol saturated with $Ca(CO_3)_2$ using 0.5 g glass beads (ϕ 0.1 mm) and a multibead shocker [MB755U(S), Yasui Kikai, Japan] at 2500 rpm for 60 s (4°C). Chlorophylls present in the centrifuged clear extracts were quantified by measuring photometric absorption at 649 nm and 665 nm (8). The chlorophyll content was determined by mg chlorophylls/100 g wet weight of frond or leaf specimens.

We first compared the growth and chlorophyll content of *Lemna minor* with and without bacteria. Ten fronds of sterilized *Lemna minor* were incubated for 72 h in 50 mL H1 medium with and without strain P23. Further, two fronds with colonized bacteria were rinsed in sterilized water and transferred to a 100-mL flask containing 50 mL of fresh H1 medium and cultured for a further 10 days. Three sets of the flask cultures were prepared for each. The growth of *Lemna minor* plants inoculated with strain P23 (Lemna/P23, 60.5 mg wet weight [SD = 5.75 mg], 3.34 mg dry weight [SD = 0.182 mg], 164 fronds) was 2-fold that of sterilized *Lemna minor* plants only (31.3 mg wet weight [SD = 15.4 mg], 1.69 mg dry weight [SD = 0.848 mg], 102 fronds) (Fig. 1A–C), indicating that the strain P23 has apparently the growth-promoting effect on *Lemna minor*. We noticed that the green color of the frond of Lemna/P23 was darker than that of sterilized *Lemna minor* plants. When we compared their chlorophyll content (chlorophylls/wet weight), we confirmed that it was higher in the Lemna/P23 plant (103.1 mg/100 g) than in the sterilized plants (62.1 mg/100 g) (Fig. 1A, D). These findings suggest that an increase in photosynthetic activity associated with the increase in chlorophyll content is one of the reasons for the growth-promoting effect of strain P23 in *Lemna minor*. It may be of importance to note that the ratio of chlorophyll *a* to chlorophyll *b* was not significantly different between the uncolonized *Lemna* ($a/b = 2.36$) and the colonized Lemna/P23 ($a/b = 2.23$) plants.

Further, we examined the effects of strain P23 on several vegetable plants in a hydroponic culture. *Escherichia coli* DH5α was also used as a reference for comparison. The vegetables evaluated in this study were *Brassica rapa* var. *chinensis* (pak choi; Sakata Seed, Japan), *Raphanus sativus* L. cv. *Raphanus sativus* (radish; Nihon Nousan Shubyou, Japan), and *Lactuca sativa* L. cv. Great Lakes (lettuce; Atariya Noen, Japan). Sterilized seeds were germinated on solid Hoagland medium 2 (H2) (pH 6.0) containing 606.6 mg/L KNO_3, 944.6 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 246.5 mg/L $MgSO_4 \cdot 7H_2O$, 230.1 mg/L $NH_4H_2PO_4$, 9 mg/L Fe·EDTA, 3.7 mg/L KCl, 1.54 mg/L H_3BO_3, 0.57 mg/L $ZnSO_4 \cdot 7H_2O$, 0.5 mg/L $MnSO_4 \cdot 5H_2O$, 0.12 mg/L $CuSO_4 \cdot 5H_2O$, 0.08 mg/L H_2MoO_4, and 1.5% agar. Ten young seedlings with three or four leaves were then transferred to hydroponic culture boxes (15 cm × 15 cm × 10 cm; Hipack S-38, ENTEC, Japan) containing 1.5 L H2 medium at different dilutions (×1 H2, ×1/10 H2, ×1/100 H2) with and without bacteria. After incubating the young seedlings of the vegetable plants with and without strains P23 and DH5α for one week to allow colonization, ten seedlings were selected from each of the following conditions: seedlings only,

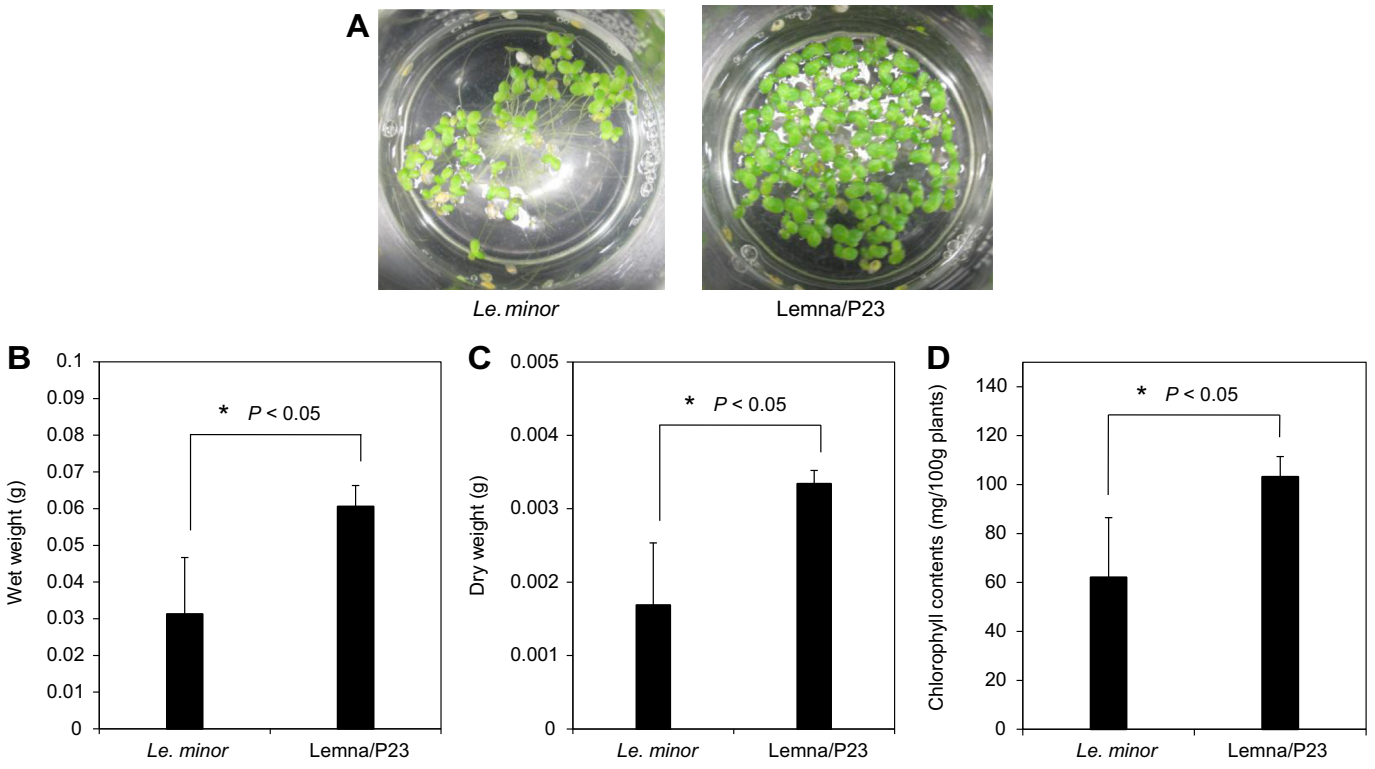

FIG. 1. Effects of PGPB *A. calcoaceticus* P23 on the growth and chlorophyll content of *Lemna minor*. (A) Bird's-eye view of the culture flasks on the seventh day after transferring the plants to bacteria-free fresh H1. (B) Wet weight of all plants present in a flask for each combination. (C) Dry weight of all plants in a flask. (D) Chlorophyll content of four randomly chosen fronds. Values are the average of three independent experiments and error bars indicate standard deviation (SD). Statistical significance was analyzed by Student's *t*-test, and the results were shown above the bars.

seedlings/P23, seedlings/DH5α; these were rinsed with water, transferred to a new medium, and cultured for another one week. Strain P23 had no effect on the growth of *B. chinensis* (pak choi) and *R. sativus* (radish) in terms of growth rate, body shape, and leaf color (data not shown). On the other hand, when *Lactuca sativa* (lettuce) was cultivated with strain P23 (Lactuca/P23) for seven days, its growth rate improved and the green color of its leaves had intensified (Fig. 2A). The effect of strain P23 was significant in nutrient-poor conditions (×1/100 dilution of H2 medium) rather than nutrient-rich conditions (×1 or ×1/10 H2). It is possible that strain P23 rescues the plant from stress, induced by nutrient depletion. The average of chlorophyll content (chlorophylls/wet weight) of the leaf specimens from independent three plants of *Lactuca sativa* was 70 mg/100 g in ×1/100 diluted H2. In contrast, it was decreased in Lactuca/DH5α (58 mg/100 g), but clearly increased in Lactuca/P23 (93 mg/100 g) (Fig. 2B), indicating that the strain P23 has effects of increasing the chlorophyll content on not only a monocotyledon plant *Lemna minor* but also a dicotyledon plant *Lactuca sativa*. Growth was marginally better in the Lactuca/P23 plants than *Lactuca sativa* but the difference was not significant (data not shown). Colonization of P23 on the root surface of *Lactuca sativa* (green color in Fig. 3) was verified by fluorescent microscopy

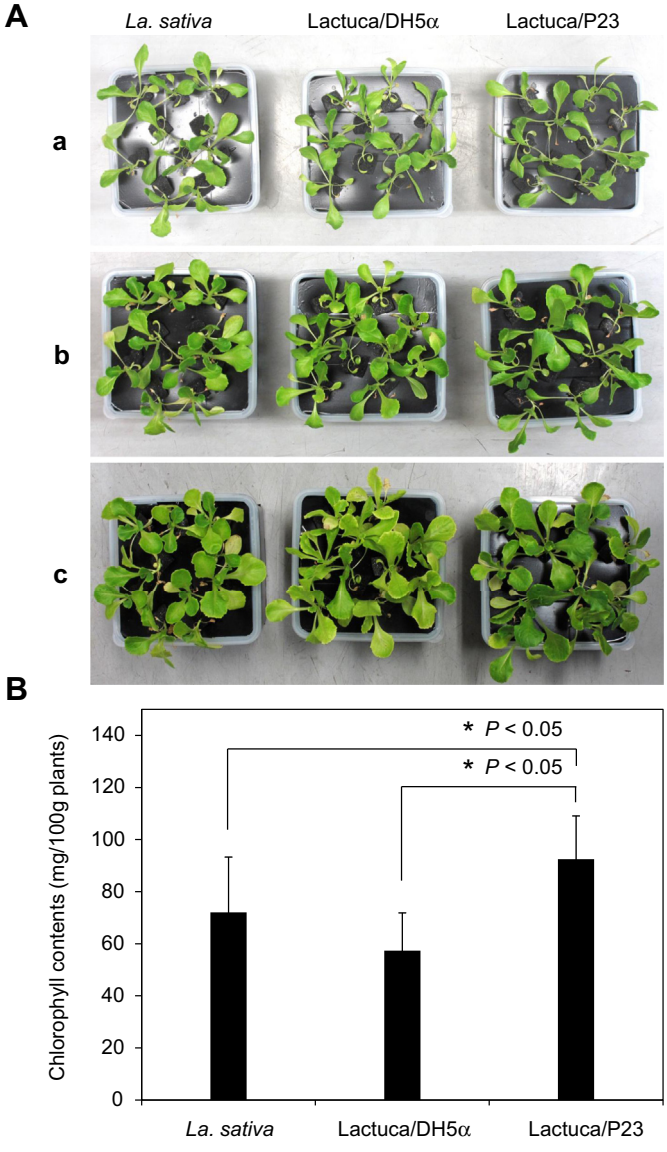

FIG. 2. Effects of PGPB *A. calcoaceticus* P23 on the growth and chlorophyll content of *Lactuca sativa*. (A) Bird's-eye view of the hydroponic culture boxes. (a) *Lactuca sativa* seedlings were pre-grown in ×1/100 H2 for 21 days. (b) Samples in panel a were grown for seven days in ×1/100 H2 with bacterial suspension. (c) Samples in panel b were grown for a further seven days after being transferred to bacteria-free ×1/100 H2. (B) Chlorophyll content of the plants. The eighth leaf from the bottom was punched to cut out four circular specimens (5 mm in each diameter) and used for chlorophyll extraction. Values are the average of three independent experiments and error bars indicate standard deviation (SD) values. Statistical significance was analyzed by Student's *t*-test, and the results were shown above the bars.

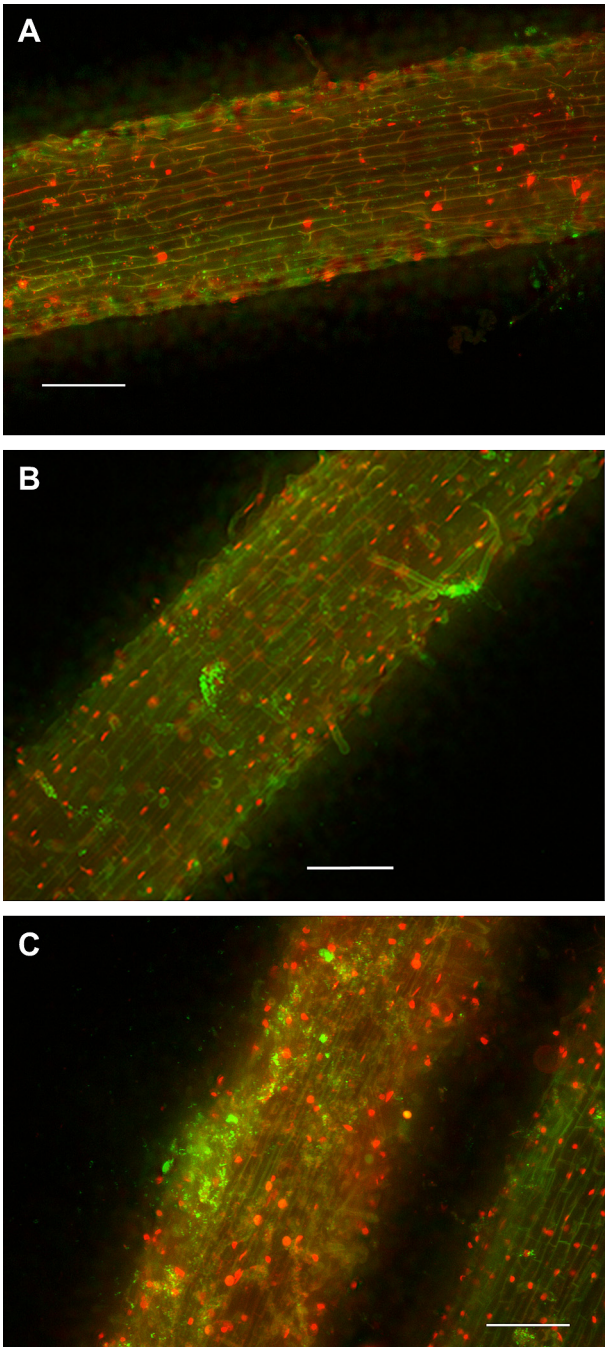

FIG. 3. Fluorescent microscopic observation of the roots of *Lactuca sativa*. (A) *Lactuca sativa* cultivated alone for seven days after transfer. (B) Lactuca/DH5α cultivated for seven days after transfer in bacteria-free ×1/100 H2. (C) Lactuca/P23 cultivated for seven days after transfer in bacteria-free ×1/100 H2. The green clusters on the root surfaces in panel C show the colonies of strain P23. The red spots are chloroplasts showing the autofluorescence of chlorophylls. The bar indicates a size of 0.1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

using a LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, CA, USA) and BZ9000 (Keyence, Japan) (7,9) (Fig. 3). Colony formation by *E. coli* DH5α was not as clearly observed as that by strain P23. The amount of chlorophyll (red spots in Fig. 3) in the root cells of *Lactuca sativa* increased after colonization by strain P23. The plants could not be fully grown in ×1/100 H2 medium because of nutrient depletion. When the plants were transferred to ×1 H2 for further growth, differences in chlorophyll content between the plants exposed to strain P23 and those that were not became negligible.

It is not clear why only *Lactuca sativa* was affected by strain P23. However, our experimental results present that strain P23 has the ability to increase the chlorophyll content of phylogenetically distant plants, in different classes in the phylum Magnoliophyta (monocotyledon class Liliopsida, order Arales, family Lemnaceae, genus *Lemna,* species *minor* and dicotyledon class Magnoliopsida, order Asterales, family Asteraceae, genus *Lactuca,* species *sativa*). Although no significant increase in the growth of *Lactuca sativa* was observed, the fact that strain P23 increased the chlorophyll content of the plant under very poor nutrient conditions suggests that the bacterium has the potential to contribute to the development of new fertilizers and/or to energy-saving hydroponic agricultural technologies. It should be verified in the future whether the plants of higher chlorophyll contents with P23 can grow under less lighting conditions than the plants without P23.

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