Effects of co-inoculation of two different plant growth-promoting bacteria on duckweed

Running title: Competition and cooperation of duckweed growth-promoting bacteria

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Abstract

Aseptic *Lemna minor* was soaked for 4 h in pond water where wild *L. minor* was naturally flourishing. Seven of the eight surface-colonizing bacterial strains were found capable of promoting the growth of *L. minor*. This high appearance of plant growth-promoting bacteria (PGPB) suggests that association of environmental bacteria is generally beneficial rather than harmful for host plants. One of the PGPB, *Pseudomonas* sp. Ps6, enhanced the growth of *L. minor* by 2–2.5-fold in 10 days. This activity was higher than that previously reported for *Acinetobacter calcoaceticus* P23, which enhanced growth of *L. minor* by 1.5–2-fold. Ps6 mostly adhered to and colonized the root rather than the frond, a leaf-like structure of duckweed where P23 preferentially adheres. It was expected that these two strains can share niches, coexist, and enhance the growth of duckweed additively upon co-inoculation. However, contrary to expectation, the growth of *L. minor* was enhanced by only 2.3-fold by co-inoculation of these two bacteria. P23 lowered the initial adhesion of Ps6 cells by 98.2% on the fronds and by 79.5% on the roots. However, initial adhesion of P23 cells to the roots increased dramatically, by 47.2-fold, following co-inoculation with Ps6. However, the number of P23 cells decreased dramatically to 0.7% on the root and to 3.6% on the frond after 10 days, whereas Ps6 cells increased by 12.5-fold on the frond and kept 69% on the root, thereby eventually restoring the population on the plant surfaces. Because duckweed is the fastest growing vascular plant and it is easy to grow an aseptic and axenic plant, the duckweed/bacteria co-culture system will be a model platform for studying multiple interactions among host plants and the associated bacteria.

Keywords: *Lemna minor*; plant growth-promoting bacteria, *Acinetobacter*, *Pseudomonas*, three-way symbiosis

Abbreviation: PGPB, plant growth-promoting bacteria

Introduction

Duckweed, a group of small floating aquatic plants of sub-family *Lemnonidae*, grow fast, via clonal proliferation, and is becoming one of the model organisms for studies on plants (Appenroth et al. 2016; Chang et al. 2016; Okada et al. 2017). Its genome analyses have recently

advanced a lot (https://www.lemna.org/). Duckweed is also highlighted as a future biomass resource that does not compete with food crops (Toyama et al. 2017a). It can accumulate protein up to approximately 30% and starch to approximately 45%, with less lignin content, depending on the conditions (Cheng et al. 2009). In addition to reducing carbon dioxide emission with their photosynthetic activities, duckweed grows in wastewater and sewage, thereby acting as an environmentally low-impact water purification system (Körner et al. 2003). Owing to its rapid growth capacity, biomass production yield of duckweed is 3–10-times higher than that of corn and wheat per unit area (National Agricultural Statistics Service 2011; Xu et al. 2012). Therefore, duckweed is a potential livestock feed, biofuel resource, as well as a raw material for starch-based green chemistry.

Every plant harbors complex indigenous and exogenous microbial communities in positive or negative symbiosis. Bacteria that promote plant growth and health are called plant growth-promoting bacteria (PGPB), whereas those that inhibit growth are known as plant growth-inhibiting bacteria (PGIB) (Ishizawa et al. 2017a). Designing and stabilizing the microbial community structure with dominant PGPB and recessive PGIB population would be an ultimate biotechnological tool for nature-friendly and cost-effective industrial production of plant biomass (Adesemoye et al. 2009). However, collection and analyses of plant-associated microorganisms, including PGPB and PGIB, for duckweed have been largely delayed (Berg et al. 2016; Appenroth et al. 2016; Ishizawa et al. 2017a). Compared with the soil environment, plant-associated microorganisms in aquatic environments are necessary to adhere and colonize plant bodies to avoid draining off by the running water. Quick adhesion and stable colonization are expected to be important traits of aquatic PGPB, except for endophytes. *Acinetobacter calcoaceticus* P23, initially isolated as a phenol-degrading bacterium from the surface layer of wild *Lemna aoukikusa* (*L. aequinoctialis*), is one of the first duckweed PGPB described in literature (Yamaga et al. 2010). P23 adheres and colonizes the surface of plants as well as on plastic surfaces, showing excellent ability to form beneficial biofilms (Morikawa 2006). Moreover, P23 increases the chlorophyll content in lettuce, a dicotyledon, as well as in the monocotyledon plant, duckweed (Suzuki et al. 2014). Furthermore, it has been recently shown that P23 stimulates growth-promoting activity in duckweed in an environment where an unspecified number of indigenous microorganisms exist, such as in pond water and secondary effluent of a sewage treatment plant (Toyama et al. 2017b).

In this study, we first aimed to obtain a series of rapidly surface-colonizing PGPB for the common duckweed *L. minor*, which is widely distributed in freshwater areas and is one of the representative species in the genus *Lemna*. Aseptic *L. minor* was submerged for a short time in the water of the original pond. This method enabled us to select bacteria that can potentially establish symbiosis with *L. minor* at an early stage. It was found that seven of the eight early colonizing bacteria are PGPB and beneficial symbionts. Finally, we attempted to construct a three-way symbiosis by intermixing the most competent PGPB— Ps6 and P23—with the host plant, *L. minor*. To the best of our knowledge, this is the first report that quantitatively evaluates the interlocking of multiple PGPB on plant surfaces.

Materials and methods

Duckweed culture

L. minor RDSC #5512, native to a pond in Hokkaido University Botanical Garden, was previously sterilized by sodium hypochlorite treatment and maintained in the laboratory (Suzuki et al. 2014). Culture conditions of *L. minor* were 28°C, 60% humidity, 5000 lux (75 μmol m^{-2} s^{-1}) illumination, 16 h-light photoperiod in Hoagland medium. Hoagland medium contained 36.1 mg l^{-1} KNO_3, 293 mg l^{-1} K_2SO_4, 147 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 103 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 5.03 mg l^{-1} $NaH_2PO_4 \cdot 2H_2O$, 3.33 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 0.95 mg l^{-1} H_3BO_3, 0.39 mg l^{-1} $MnCl_2 \cdot 4H_2O$, 0.08 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.39 mg l^{-1} $MnCl_2 \cdot 4H_2O$, 0.03 mg l^{-1} $CuSO_4 \cdot 5H_2O$, and 0.23 mg l^{-1} H_2MoO_4. pH of the medium was adjusted to 7.0 by KOH. Sterility of the plant was routinely confirmed by no bacterial colony formation on LB agar plate for one week at 30°C.

Bacterial culture

LB medium (1 l) containing 5 g Bacto Yeast extract, 10 g Bacto Tryptone, 10 g NaCl (pH 7.2) was used for culturing bacteria. LB agar plate containing 15 g agar in 1 l of LB was used for isolation of bacteria. *A. calcoaceticus* P23 was previously isolated from the same pond (Yamaga et al. 2010).

Isolation of bacteria adhering to L. minor *acclimated to the pond water*

Fresh pond water was collected from Hokkaido University botanical garden, and acclimatization of aseptic duckweed was performed on the same day. First, 100 ml of pond water was placed in a

300-ml Erlenmeyer flask capped with Silicosen Kabuse-type (NEG, Hyogo, Japan), and 100 aseptic plants were floated on the water surface. After 4 h, when the number of adhering bacteria reached the maximum level, the plant surface was gently washed twice by transferring 50 plants with adhered bacteria to Hoagland medium at an appropriate amount in two sterilized Petri dishes. After washing out weakly-adhering bacteria, ten plant bodies of *L. minor* were transferred to a 1.5-ml plastic tube containing 400 µl of sterilized phosphate buffered saline. *L. minor* was crushed using a homogenizer (Nippi BioMasher II, Tokyo, Japan), and 600 µl of sterilized water was added to make the total volume to 1 ml. The homogenates were serially diluted from 10^{-2} to 10^{-7} and spread onto LB agar medium to isolate the adhered bacteria.

Re-adhesion of isolated bacteria to sterile L. minor

Colonies of isolated bacteria cultured on LB agar plate were inoculated in 20 ml of LB medium and pre-cultured by shaking at 30°C for 24 h. The culture was transferred to a sterilized 50-ml plastic tube and centrifuged (4,000 ×g, 15 min, 20°C). The cell pellet was suspended in an equal volume of Hoagland medium and centrifuged again for washing under the same conditions. The washing step was performed twice. The supernatant was decanted, and the cells were resuspended in 10 ml of sterilized Hoagland medium. The cell suspension was diluted with Hoagland medium in culture flasks so that the final OD_{600} was 0.3. Ten plant bodies of aseptic *L. minor*, with ten fronds and roots, were floated on 50 ml of cell suspension in 100-ml Erlenmeyer flasks for adhesion and incubated at 28°C for 4 h under light condition. The resultant axenic *L. minor* inoculated with each bacterial strain was gently surface washed by sterilized Hoagland medium and used for growth experiments.

Measurement of the number of adhering bacterial cells

Ten plant bodies that on which bacterial adhesion was allowed for 4 h or after 10 days of cultivation were rinsed twice with sterile water to remove the weakly-adhering bacteria. Next, the fronds and roots of the plant were cut using tweezers. Ten fronds and ten roots were separately transferred to 1.5-ml plastic tubes containing 400 µl of sterile water. Each plant part was crushed using a homogenizer, and 600 µl of sterilized water was added to make the total volume to 1 ml. A dilution series of up to 10^{-5} of homogenates was spread onto LB agar plate for culturing at 30°C. Culturing was continued for 3 days until colonies were formed. The number of colonies

was counted, and cfu (colony forming units) per frond and root was calculated, and this value was considered as the number of adhering bacteria.

Evaluation of growth-promoting activity of isolated bacteria on L. minor

Two plant bodies of *L. minor,* previously inoculated with/adhered to by each isolated bacterium were planted in a 100-ml Erlenmeyer flask containing 50 ml of Hoagland medium on day 0. Then, these were statically cultured for 10 days, and the number of fronds was measured. We set five flasks in quintuplicate for each experiment and eliminated two flasks that showed the highest and the lowest frond numbers, and an average of triplicates was considered for measurement.

Stability test of growth-promoting activity and colonization of P23 and Ps6

After 10 days of culturing, in the 1st cycle, two plant bodies were replanted in a 100-ml Erlenmeyer flask containing 50 ml of new Hoagland medium. After another 10 days, in the 2nd cycle, the number of fronds was measured. This operation was repeated once more, i.e., in the 3rd cycle, to evaluate the stability of the growth-promoting effect for a total of 30 days. The culture flasks were prepared in quintuplicate for each cycle, and an average of triplicates was adopted for measurement. In the same manner as described above, the number of colonizing bacteria on the 10th, 20th, and 30th day was measured and compared with that in the 0-day sample, which was immediately measured after 4 h of bacterial adhesion.

Analysis of 16S rRNA gene sequence of the isolated bacteria

Template DNA was prepared from each of the ten isolated bacteria using InstaGene DNA purification matrix (BioRad, Hercules, CA, USA). PCR was performed using a set of forward primer (5′-GTCCACGCCAACGATG-3′) and reverse primer (5′-GGCTACCCTTGTTACGACTT-3′), which correspond to the nucleotide positions 804 to 820 and 1510 to 1492, respectively, of most bacterial 16S rRNA genes. KOD-plus-Neo DNA polymerase was used according to the standard protocol recommended by the manufacturer (Toyobo, Kyoto, Japan). The nucleotide sequence was determined using BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The sequence data have been deposited to DDBJ/EMBL/GenBank under accession numbers, LC339924–339933. Each bacterium was identified by comparing

approximately 1,350 bases of the 16S rRNA gene sequences with the database using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EZtaxon (https://www.ezbiocloud.net). The evolutionary distances were computed using the Kimura 2-parameter method and are presented in the units of the number of base substitutions per site. Evolutionary analyses were conducted using MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (http://www.megasoftware.net; Molecular Biology and Evolution 33:1870-1874). A phylogenetic tree was constructed using the neighbor-joining (N-J) method.

Auxin (Indoleacetic acid; IAA) production activity

Each bacterial strain was cultured for 24 h at 30°C in 25 ml of liquid LB medium in a 100-ml Erlenmeyer flask. LB medium supplemented with 200 mg l^{-1} tryptophan was also tested for IAA production activity (Gordon and Weber 1951). After centrifugation (4,000 ×*g*, 15 min, 4°C), two drops of phosphoric acid were added to 2 ml of the culture supernatant and 4 ml of Salkowski's reagent (a mixture of 50 ml of 35% perchloric acid and 1 ml of 0.5 M $FeCl_3$). The optical density was measured at 530 nm. The relative auxin productivity of these isolates with and without tryptophan was determined using a standard curve that was constructed using different concentrations of IAA.

Siderophore production activity

Siderophore production activity was examined by a yellow halo formation around the colonies on CAS agar medium (Schwyn and Neilands 1987).

Phosphate solubilization activity

Phosphate solubilizing activity was recorded as observation of a clear phosphate solubilizing halo formed around the colonies on Pikovskaya's agar medium plate containing solid calcium phosphate (Sundra Rao and Sinha 1963).

Co-inoculation of P23 and Ps6 on L. minor

Each bacterial strain was pre-cultured at 30°C for 24 h in LB medium and washed twice with Hoagland medium. Cell suspension was inoculated in Hoagland medium in flasks at OD_{600} of 0.15 each. Ten plant bodies of *L. minor* were suspended in this bacterial cell mixture, followed by

standing culture for 4 h under light. The order of bacterial inoculation was also examined. For example, P23 was initially allowed to adhere for 4 h, and then the plants were transferred to a flask containing Ps6 cell suspension for another 4 h and *vice versa*. The culture flasks were prepared in quintuplicate, and an average of triplicates was adopted for measurement. Significant difference in the growth and shape of P23 and Ps6 colonies enabled us to count their cfu separately.

Results

Isolation of bacteria capable of adhering to L. minor

After acclimating sterile *L. minor* to the pond water for 4 h, approximately 70 bacterial strains were obtained as adhering bacteria whose colonies showed different morphology and color. Next, these colonies were sequentially subjected to the re-adhesion test with *L. minor*. Finally, ten strains with adhesion capacity equal to or higher than that of *A. calcoaceticus* P23 (5.2×10^5 cfu/plant) were selected. Approximately 1,350 bases of the 16S rRNA gene were analyzed for each strain, and their homology search by nucleotide Blast revealed the identity of the bacteria as *Delftia* sp. (De1), *Aeromonas* sp. (Ae2), *Pseudomonas* sp. (Ps3), *Sphingomonas* sp. (Sp4), *Pseudomonas* sp. (Ps5), *Pseudomonas* sp. (Ps6), *Pseudomonas* sp. (Ps7), *Pseudomonas* sp. (Ps8), *Pseudomonas* sp. (Ps9), and *Pseudomonas* sp. (Ps10) (Fig. 1). Ps3 was chosen and used for further experiments as a representative strain of the *P. alcaligenes* group.

It was found that each bacterium preferentially adhered to *L. minor* at different portions, either fronds or roots. P23 (0.52×10^6 cfu and 0.015×10^6 cfu for fronds and roots, respectively), Ae2 (0.18×10^6 cfu and 0.049×10^6 cfu for fronds and roots, respectively), Ps7 (5.2×10^6 cfu and 0.90×10^6 cfu for fronds and roots, respectively), Ps8 (1.1×10^6 cfu and 0.094×10^6 cfu for fronds and roots, respectively) mostly adhered to the fronds rather than to the roots. However, De1 (0.38×10^6 cfu and 1.069×10^6 cfu for fronds and roots, respectively), Ps3 (0.08×10^6 cfu and 4.31×10^6 cfu for fronds and roots, respectively), Sp4 (0.046×10^6 cfu and 0.27×10^6 cfu for fronds and roots, respectively), Ps 5 (0.092×10^6 cfu and 8.00×10^6 cfu for fronds and roots, respectively), Ps 6 (0.23×10^6 cfu and 8.27×10^6 cfu for fronds and roots, respectively), and Ps10 (0.57×10^6 cfu and 13.7×10^6 cfu for fronds and roots, respectively) adhered mostly to the roots than to the fronds (Fig. 2a).

Growth-promoting activity of each adhering bacteria

Aseptic *L. minor* was soaked for 4 h under light with each bacterial suspension in Hoagland medium for inoculation to prepare axenic *L. minor* (*L. minor*/bacterium symbiosis system). Two plants with two fronds of each axenic *L. minor* were gently surface washed and transferred to new Hoagland medium and cultured at 28°C and 16 h-light photoperiod condition. It was found that of the eight strains tested, except for Ps10, which had the highest number of adhering bacteria to *L. minor*, seven had significant growth-promoting activity (Fig. 2b). In particular, Ps6 showed the highest duckweed growth-promoting activity among the tested bacteria. Based on the results (Figs. 2 and 3), it was inferred that Ps6 was a bacterium having characteristics of both high adhering ability and high growth-promoting activity for *L. minor*. In addition, P23 adhered more to the fronds than that to the roots, whereas Ps6 had characteristics to adhere more to the roots than that to fronds. It was, thus, considered that Ps6 isolated in this study was an excellent PGPB having properties different from those of P23. The *L. minor*/P23 symbiosis system in which P23 colonized the plant, the *L. minor*/Ps6 symbiosis system in which Ps6 colonized the plant, and *L. minor*/P23/Ps6 three-way symbiosis system in which P23 and Ps6 simultaneously adhered and colonized the plant were used for further experiments.

Productivity of general plant growth-promoting factors

The isolated eight bacterial strains were subjected to the following tests: phosphate-solubilizing activity, auxin (IAA) production activity, and siderophore production activity (Table 1). *A. calcoaceticus* P23 had apparent phosphate solubilizing activity and produced a small amount of IAA (1.1 mg/g biomass). *Pseudomonas* sp. Ps6 showed a little higher IAA production activity (2.9 mg/g biomass), but phosphate solubilization and siderophore production were negligible. Notably, there are no factors that directly correlate to the degrees of plant growth-promoting activity against the duckweed *L. minor*. Quite recently, we examined the effect of external addition of several growth regulator compounds, including IAA (Utami et al. 2018). We could not observe growth-promoting activity of IAA on *L. minor* at any concentrations tested (0–50 μM). Thus, IAA does not seem to be a primary growth-promoting factor for *L. minor*.

Stability of the enhanced growth of L. minor *by* A. calcoaceticus *P23 and* Psudomonas *sp. Ps6 and their colonization*

Two plants with two fronds of *L. minor*/P23 and *L. minor*/Ps6 system were repeatedly grown for 10 days for three cycles, for a total of 30 days (Fig. 3a). The number of fronds was counted every 10 days before transfer. *L. minor*/P23 showed almost no decline in growth-promoting activity for 30 days. However, the growth-promoting activity of *L. minor*/Ps6 was very high for initial 10 days, but it decreased to approximately 60% on the 20^{th} day, which was almost the same as that of *L. minor*/P23. The activity did not decrease significantly until 30 days thereafter.

To investigate the difference in growth-promoting activity of the *L. minor*/P23 and *L. minor*/Ps6 systems in more detail, cfu of P23 and Ps6 were counted for each system (Fig. 3b). It was found that the number of colonizing bacteria in both P23 and Ps6 decreased significantly by the 10^{th} day, end of the 1^{st} cycle. P23 decreased from 2.0×10^5/plant to 3.2×10^4 (16%), and Ps6 decreased from 1.8×10^6/plant to 2.7×10^5/plant (15%). In addition, when comparing the change in the amount of adhesion after another 10 days, P23 further reduced to 2.1×10^4/plant (64%) on the 20^{th} day, end of the 2^{nd} cycle, but there was no subsequent decrease, and a slight increase was observed on the 30^{th} day, end of the 3^{rd} cycle. This recovery of population was significant on the root. However, Ps6 decreased further to 5.9×10^4 (22%) in the 2^{nd} cycle and did not clearly change in the 3^{rd} cycle.

Effect of co-inoculation of A. calcoaceticus *P23 and* Pseudomonas *sp. Ps6 on the growth of* L. minor

P23 showed significant phosphorus solubilizing activity and mainly adhered to the fronds of *L. minor*, whereas Ps6 had little phosphorous solubilizing and higher IAA production activities, adhered primarily on the roots, and exerted high growth-promoting activity for duckweed. Thus, we sought to determine if additive growth promotion was possible by simultaneously adhering of two kinds of PGPB with different characteristics, viz., P23 and Ps6. It was observed that plant growth-promoting activity of P23 and Ps6 was not simply additive (Fig. 4). The growth yield of axenic *L. minor* was slightly lower upon co-inoculation of P23 and Ps6 (32.8 fronds) than that of single inoculation with Ps6 (34.3 fronds). This tendency was also observed when the order of inoculation was changed, i.e., first P23 followed by Ps6 and *vice versa* (data not shown).

Competition and cooperation of P23 and Ps6 on the surface of L. minor

A probable reason for not observing an additive effect in *L. minor* growth was a change in the cfu

of P23 and Ps6 during the 10-day co-cultivation on *L. minor* (Fig. 5a). In the single inoculation experiment, the initial cfu of P23 on a frond and root was 1.9×10^5 and 0.9×10^4, respectively. However, cfu reduced to 4.0×10^4 (21%) on the frond, whereas cfu on the root was unchanged, although the level was low (0.9×10^4). With respect to Ps6, cfu values for both the frond and root were similarly reduced by 85%–80%. When P23 and Ps6 were co-inoculated on *L. minor*, initial cfu of P23 increased dramatically (by 47.2-fold) on the root (4.3×10^5) compared with single inoculation (0.9×10^4). However, initial cfu of Ps6 decreased on both the frond and root, particularly on the frond (from 11×10^4 to 0.2×10^4; 1.8%). It is also significant that cfu of P23 on the root reduced to 0.3×10^4 (0.7%), whereas Ps6 on the frond increased to 2.5×10^4 (12.5-fold). It is evident that Ps6 was initially vulnerable to P23 but eventually revived the population on duckweed surface.

Discussion

In this study, we collected culturable bacteria from pond water where *L. minor* grew naturally, which could adhere to and colonize *L. minor* at an early stage. Eight different bacteria were finally obtained after eliminating the duplicated species Ps5 and Ps9. Interestingly, seven of the eight strains promoted the growth of *Lemna*. This high appearance of PGPB suggests that the association of environmental bacteria is generally beneficial, rather than harmful, for the host plants. We recently tested the effect of 15 randomly selected pond water samples on the growth of aseptic *L. minor* (Ishizawa et al. 2017a) and revealed that seven water samples showed growth-promoting and three showed growth-inhibiting activities; the remaining five showed no significant activities. Next, 10 and 12 surface-attached bacterial strains were isolated from *L. minor* previously grown for 7 days in the highest growth-promoting and the highest growth-inhibiting water, respectively. It was found that there were five PGPB and two PGIB in the former 10 strains and seven PGPB and four PGIB in the latter 12 strains. These data also support the idea that naturally more PGPB exist in pond waters than PGIB. However, our observations do not deny another attractive idea that, “Plants are naturally equipped with the ability to recruit beneficial microorganisms” (Rudrappa et al. 2008; Kessler et al. 2018).

The diversity of the early colonizing bacteria revealed that five of the eight early adhering bacteria were of the genus *Pseudomonas*, and the others were bacteria in the genera *Aeromonas*, *Delftia* (previously *Comamonas*), and *Sphingomonas*. Bacteria in the genus

Pseudomonas generally possess high adhesion ability and biofilm formation ability on inorganic and organic solid surfaces, and many studies on its biofilm formation mechanisms and application have been reported (Mikkelsen et al. 2011; Shimada et al. 2012; Valentini et al. 2016). Thus, it is hard to say that duckweed specifically chose *Pseudomonas* as the most preferable cell group (Preston 2004). However, *Pseudomonas* are closely associated to plants and are often used as a biocontrol agent. (Keel et al. 1992; Haas et al. 2000; Patil et al. 2016). High proportion of the genus *Pseudomonas* in *L. minor*-associated bacteria, including PGPB and PGIB, is suggested to be a common feature of soil and aquatic plants, as well as an indicator of the rich diversity of the *Pseudomonas* bacterial group. *Aeromonas hydrophila* P73 is a PGPB of soybean *Glycine max* (L.) (Zhang et al. 1997). *Delftia* (*Comamonas) acidovorans* RC41 isolated from wild raspberry has IAA production activity and promotes root elongation after kiwi fruit stem cutting (Erturk et al. 2010). A *Sphingomonas* strain isolated from the roots of greenhouse tropical orchids also exhibits IAA production activity, but it is widely present in the phyllosphere of soil plants and contributes to avoid infection by phytopathogenic bacterium *Pseudomonas syringae* (Tsavkelova et al. 2005; Innerebner et al. 2011). Another *Sphingomonas* strain has been reported in the phyllosphere of *Acacia caven* (Rivas et al. 2004).

The newly isolated *Pseudomonas* sp. Ps6 exhibited exceptional activity to promote *L. minor* growth by 2.4–2.7-fold than that of sterilized plants. The growth-promoting activity of a previous isolate, *A. calcoaceticus* P23, when tested under the same conditions was 1.5–2.0-fold of sterilized plants. It was found that Ps6 exhibited higher *L. minor* growth-promoting activity and surface-colonizing activity when compared with P23. In addition, P23 adhered more to the fronds than that to the roots, whereas Ps6 showed overwhelmingly more adhesion to and colonization of the roots than that of the fronds. Regarding the stability of bacterial colonization, the initial amount of Ps6 cells on *L. minor* exceeded 1×10^6 per plant, but on the 10^{th} day, it decreased drastically in both the roots and fronds. However, the growth-promoting activity was approximately 60% even after the 20^{th} day (Fig. 3), suggesting that there is an upper limit for the number of adhered cells that leads to the growth-promoting effect on *L. minor* or that the growth-promoting activity of *L. minor* stimulated by adhered bacteria persists for a certain period of time. This tendency was more conspicuous in P23, and there was no significant difference in the growth-promoting effect during the 1^{st}, 2^{nd}, and 3^{rd} cycles of 10-day cultures. Taken together, it was revealed that P23 is an excellent PGPB capable of continuing to promote duckweed

352 growth for a long period of time with a small number of adhered cells. When growth-promoting
353 activities were divided by the number of adhered cells at the end of the 2nd cycle, each unit cell
354 activity was 2.4-fold/2.13 × 10^4 cells for P23 and 2.6-fold/5.9 × 10^4 cells for Ps6. Another
355 explanation would be that a plant growth-promoting factor of P23 is more structurally or
356 functionally durable than that of Ps6.
357 It has been reported that IAA-producing *Bacillus amyloliquefaciens* promotes the
358 growth of *L. minor* (Idris et al. 2007). However, we have observed that external addition of IAA
359 did not clearly affect the growth of *L. minor* (Utami et al. 2018). P23 exhibited no IAA
360 production but weak siderophore production and relatively good phosphorus solubilizing
361 activities. Ps6 had only low IAA production activity and little phosphate solubilizing activity. It is
362 yet unclear whether these general plant growth-promoting factors for soil plants are also
363 functional for growth of aquatic plant bodies, including *L. minor*. Ps6 isolated in this study
364 mainly adhered to different parts of *L. minor* from those by P23 and, at the same time, probably
365 exhibited different growth-promoting mechanisms in a manner such that Ps6 was expected to be
366 able to coexist with P23 and promote growth of *L. minor* additively. Contrary to our expectation,
367 however, both the strains did not coexist, and the number of adhered P23 cells exceeded Ps6
368 temporarily on the root surface, but later, Ps6 was dominating on the 10th day (Fig. 4). The
369 molecular mechanisms of the above-mentioned cooperation and competition between P23 and
370 Ps6 remains to be clarified. Because it was observed that the colony expansion (swarming
371 motility) ability of each strain was neither inhibited nor promoted when Ps6 and P23 were
372 co-cultured in close proximity on a 0.3% soft agar LB culture plate, they did not directly affect
373 the cell growth of each other (data not shown). Moreover, scanning electron microscopic
374 observation of *Lemna*/P23/Ps6 three-way symbiosis system also suggested that these two bacteria
375 colonized locally on the root surfaces, and neither significant co-aggregation nor repulsion of
376 cells were observed (Fig. 5b). It has been reported that there formed commensalism between two
377 Gram-negative bacteria *Acinetobacter baumannii* and *Pseudomonas aeruginosa* share a similar
378 acyl homoserine lactone compound as the quorum-sensing molecule, and exhibit commensalism
379 and coexist without interfering with each other (Bhargava et al. 2012). It has also been reported
380 that *P. aeruginosa* strongly outcompetes *Agrobacterium tumefaciens in vitro*, but upon
381 co-inoculation of these two bacteria in a tobacco plant, *Nicotiana benthamiana*, Type VI
382 secretion DNase produced by *A. tumefaciens* counterattacks *P. aeruginosa* for niche colonization

(Ma et al. 2014).

Recently, we examined exhaustive co-inoculation of three PGPB and four PGIB in *L. minor* (Ishizawa et al. 2017a). As a result, no additive effect was observed for the combination of PGPB, but partial additive effect was observed for PGIB combination. Moreover, it has been found that compared with PGPB, PGIB induces production of higher amounts of $O_2^{\bullet-}$, H_2O_2, and malondialdehyde (MDA) in *L. minor*, although all bacteria consistently increase $O_2^{\bullet-}$ content by more than two times compared with that in aseptic control plants (Ishizawa et al. 2017b). The degree of oxidative stress seemed to be negatively correlated to the effect on plant growth. The additive effect of PGIB can be explained by this rule. Furthermore, a PGPB, *Aquitalea magnusonii* H3, can robustly exert growth-promoting activity in all combinations tested, whereas the activity of other PGPB is largely cancelled when coexisting with a PGIB.

Our knowledge on multiple-way symbiosis in plants and their associated microorganisms is still poor, and relevant studies have just been initiated. A rapidly growing aquatic plant, duckweed, is useful for studies on bacterial symbiosis because of the ease in preparation and cultivation of aseptic and axenic plants. Rational designing of microbial community on and in a plant body is a frontier research area, the findings of which can lead to sustainable enhancement in biomass and crop production.

Conclusions

Here, we demonstrated, by single inoculation experiments, that most of the early adhering bacteria in the original environment are beneficial for the growth of host plants. They adhere to either the fronds or roots with some specificity. Co-inoculation of two PGPB with different specificity revealed that they mutually interfered; *Acinetobacter* (P23) initially overwhelmed and occupied the surface of *L. minor*, but later, *Pseudomonas* (Ps6) revived and seemed to exclude P23. It is suggested that the duckweed/bacteria co-culture system is a useful tool to understand unknown interactions among plants and bacteria.

Author contribution statement

YY, RJ, and MM conceived and designed the research. YY performed most of the experiments with the help of RJ. YY and MM interpreted the data and wrote the manuscript. RJ revised the manuscript.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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TABLE 1

	Phosphate	Siderophore	IAA (mg/g biomass)
De1	−	−	+++ (35.0)
Ae2	+	+	++ (16.8)
Ps3	−	−	+ (7.8)
Sp4	+	−	− (0)
Ps6	+/−	−	+ (2.9)
Ps7	++	++	+/− (0.5)
Ps8	−	−	+ (6.6)
Ps10	−	−	+ (4.9)
P23	+++	+	+/− (1.1)

FIGURE LEGENDS

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences using the N-J method. The nucleotide sequences were obtained from De1 (LC339924), Ae2 (LC339925), Ps3 (LC339926), Sp4 (LC339927), Ps5 (LC339928), Ps6 (LC339929), Ps7 (LC339930), Ps8 (LC339931), Ps9 (LC339932), Ps10 (LC339933), *Delftia lacustris* DSM 21246^{T} (EU888308.1), *Aeromonas hydrophila* ATCC 7966^{T} (X60404.2), *Pseudomonas alcaligenes* ATCC 14909^{T} (Z76653.1), *Pseudomonas fulva* NRIC 0180^{T} (AB060136.1), P*seudomonas nitroreducens* ATCC 33634^{T} (AM088473.1), and *Sphingomonas azotifigens* NBRC 15497^{T} (AB217471.1).

Fig. 2. a. Adhering activities of the bacteria to aseptic *L. minor*. Closed and open bars indicate the average number of cells adhered to one frond and one root, respectively. Adhered cells after 4 h of incubation with aseptic *L. minor* from ten fronds or ten roots were dispersed using a homogenizer in a tube, and colony-forming units (cfu) were counted. **b.** Duckweed growth-promoting activity of bacteria. Two plant bodies, two fronds, and two roots of axenic *L. minor* were grown in a flask, and the number of fronds was counted after 10 days. Mean ± SD are shown (n = 3, three independent flasks).

Fig. 3. a. Persistence of enhanced growth capacity of *Lemna*/P23 and *Lemna*/Ps6 systems. "1st", "2nd", and "3rd" indicate a cultivation cycle of 10 days each starting from two axenic plants. Mean ± SD are shown (n = 3, three independent flasks). **b.** Stability of P23 and Ps6 in colonization of *L. minor*. *a*, cfu of P23 per plant; *b*, cfu of P23 per root; *c*, cfu of Ps6 per plant; *d*, cfu of Ps6 per frond. Closed and open bars indicate the average number of cells adhered to a frond and a root, respectively. Adhered cells from ten fronds or ten roots were dispersed using a homogenizer in a tube and spread on LB agar plate after appropriate dilution.

Fig. 4. Duckweed growth-promoting activity of P23 and Ps6 by single inoculation or co-inoculation. The number of fronds was counted after 10 days of culture. Mean ± SD are shown (n = 3, three independent flasks).

595 **Fig. 5. a.** Change in the number of P23 and Ps6 cells adhering and colonizing on *L. minor* either
596 by single inoculation or co-inoculation. Adhered cells from ten fronds or ten roots were dispersed
597 using a homogenizer in a tube and spread on LB agar plate after appropriate dilution. P23 and
598 Ps6 colonies were easily distinguished by the shape and size. **b.** Scanning electron microscopic
599 images of the root surfaces of axenic *L. minor* after 4 h of co-inoculation with P23 and Ps6. Rod
600 cells are Ps6, and coccoid cells are P23. The specimen was fixed in glutaraldehyde followed by
601 treatment with OsO_4. After dehydration by ethanol, critical point drying was performed through
602 CO_2 followed by Au coating. Samples were observed under S-2400 (Hitachi).

Table 1

Strains	Phosphate	Siderophore	IAA (mg/g biomass)
De1	−	−	+++ (35.0)
Ae2	+	+	++ (16.8)
Ps3	−	−	+ (7.8)
Sp4	+	−	− (0)
Ps6	+/−	−	+ (2.9)
Ps7	++	++	+/− (0.5)
Ps8	−	−	+ (6.6)
Ps10	−	−	+ (4.9)
P23	+++	+	+/− (1.1)

Fig. 1

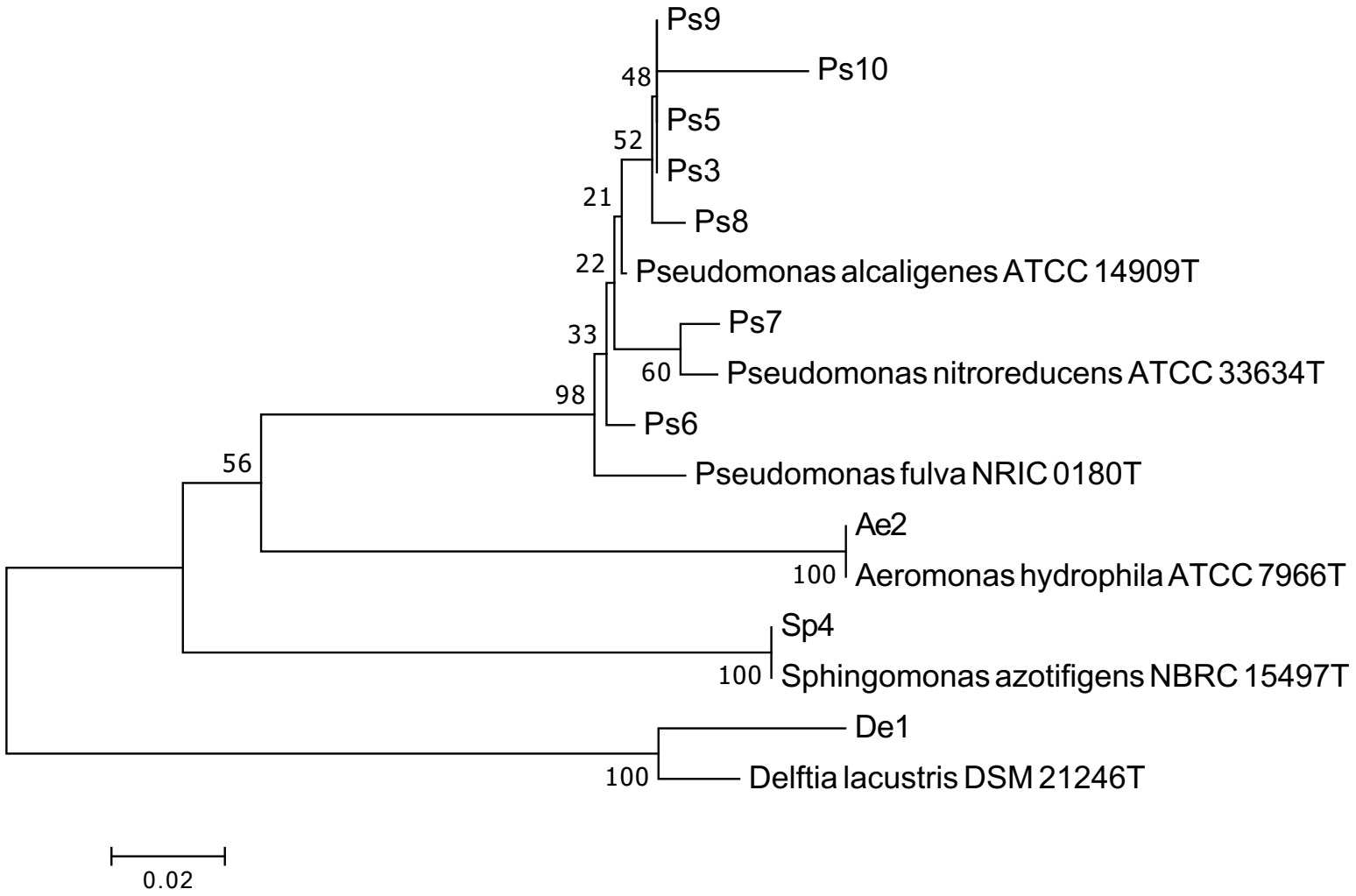
Ps9
48
Ps10
Ps5
52
Ps3
21
Ps8
22
Pseudomonas alcaligenes ATCC 14909T
Ps7
33
60
Pseudomonas nitroreducens ATCC 33634T
98
Ps6
56
Pseudomonas fulva NRIC 0180T
Ae2
100
Aeromonas hydrophila ATCC 7966T
Sp4
100
Sphingomonas azotifigens NBRC 15497T
De1
100
Delftia lacustris DSM 21246T
0.02

Fig. 2. a.

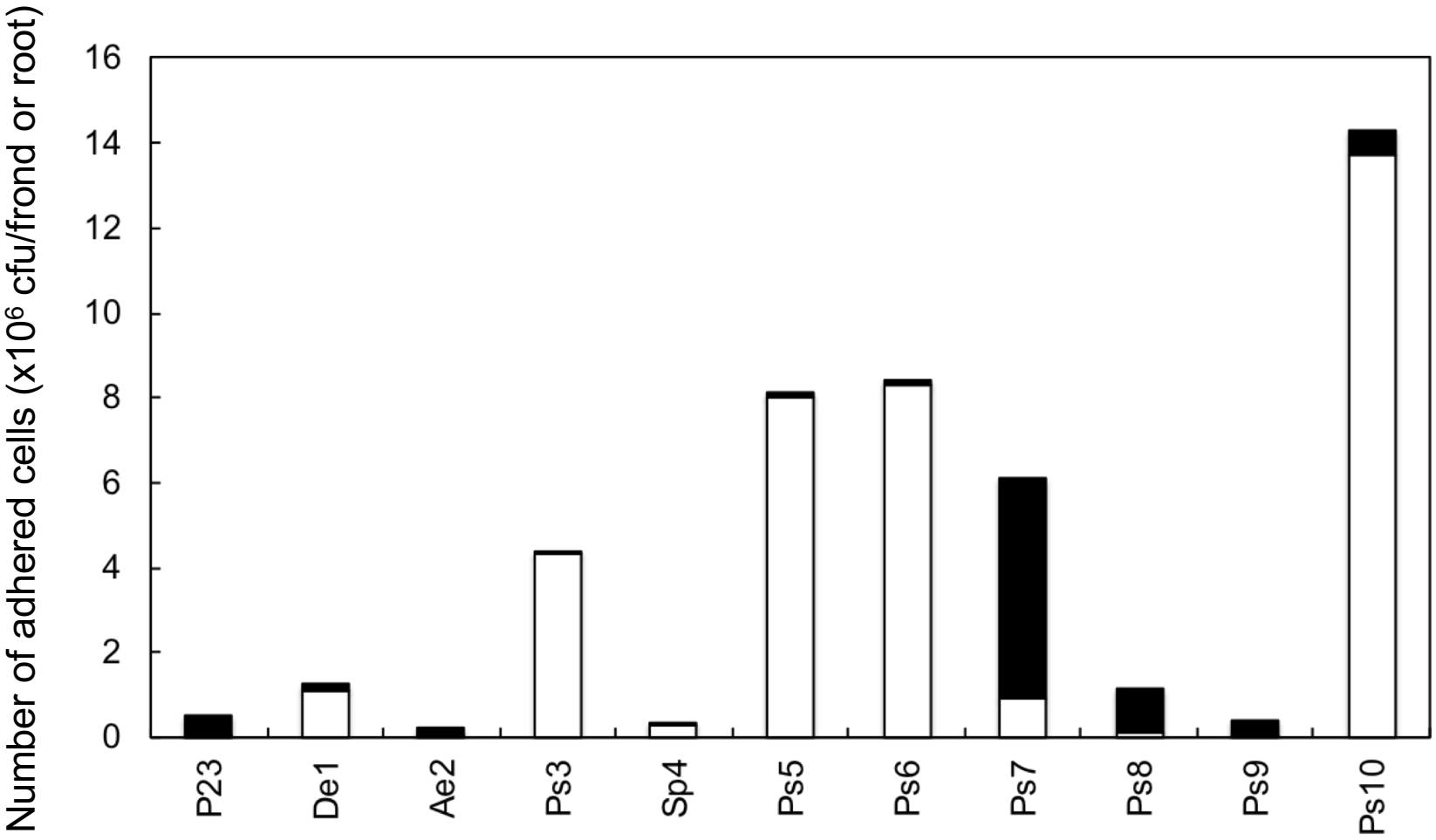
Number of adhered cells (x10^6 cfu/frond or root)
16
14
12
10
8
6
4
2
0
P23
De1
Ae2
Ps3
Sp4
Ps5
Ps6
Ps7
Ps8
Ps9
Ps10

Fig. 2. b.

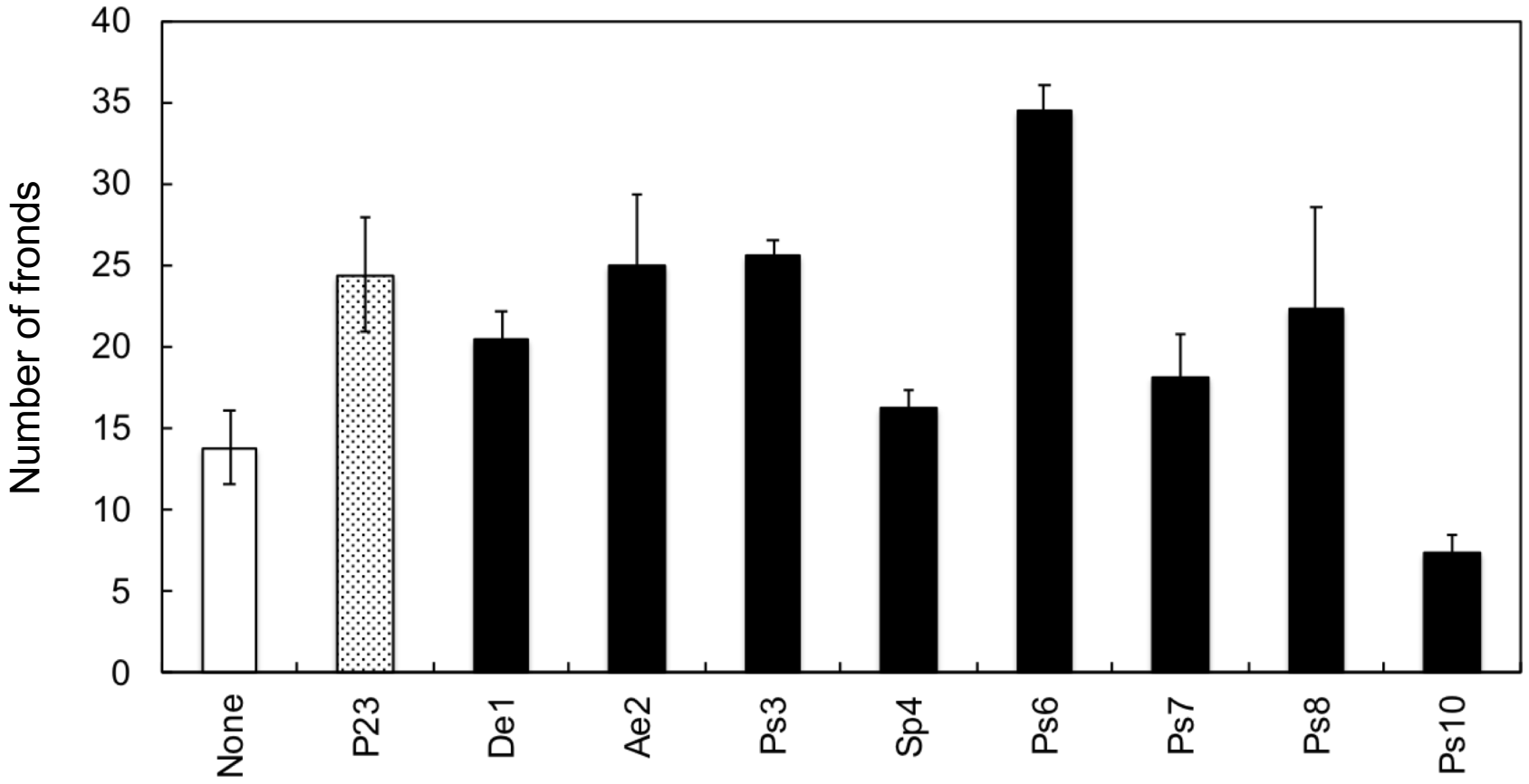
Number of fronds
40
35
30
25
20
15
10
5
0
None
P23
De1
Ae2
Ps3
Sp4
Ps6
Ps7
Ps8
Ps10

Fig. 3. a.

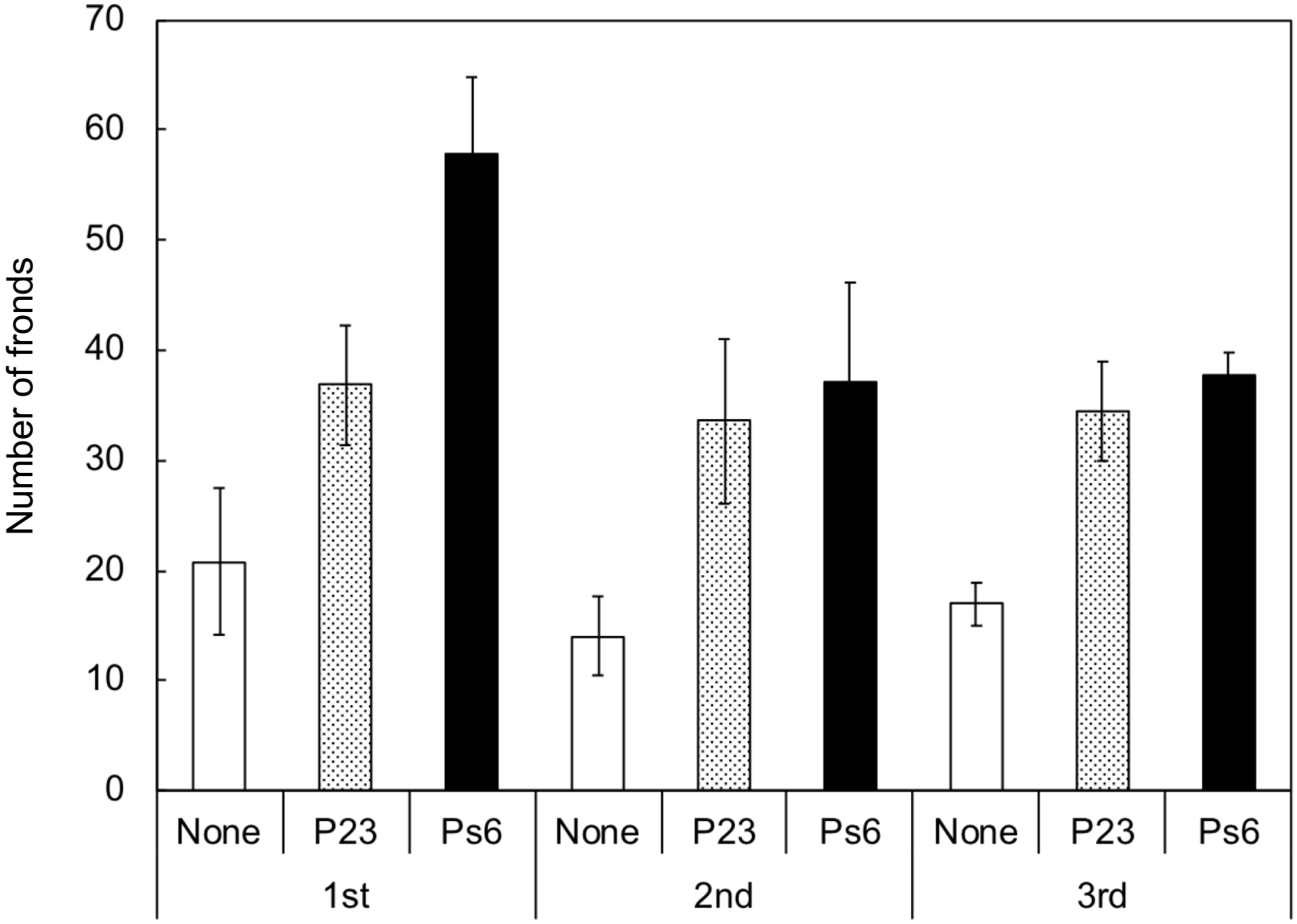
Number of fronds
70
60
50
40
30
20
10
0
None
P23
Ps6
None
P23
Ps6
None
P23
Ps6
1st
2nd
3rd

Fig. 3. b.

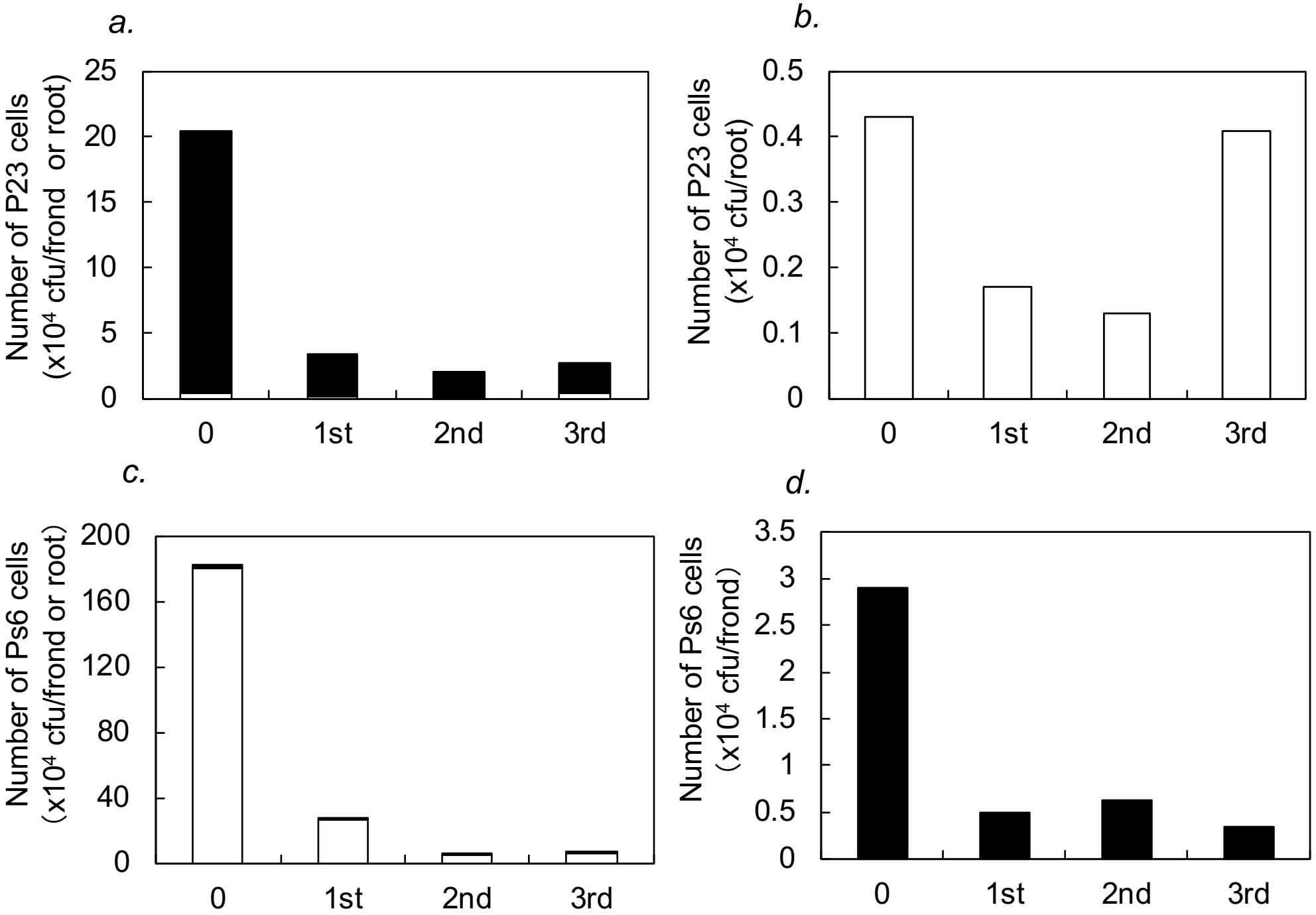
a.
Number of P23 cells
(x10⁴ cfu/frond or root)
25
20
15
10
5
0
0
1st
2nd
3rd
b.
Number of P23 cells
(x10⁴ cfu/root)
0.5
0.4
0.3
0.2
0.1
0
0
1st
2nd
3rd
c.
Number of Ps6 cells
(x10⁴ cfu/frond or root)
200
160
120
80
40
0
0
1st
2nd
3rd
d.
Number of Ps6 cells
(x10⁴ cfu/frond)
3.5
3
2.5
2
1.5
1
0.5
0
0
1st
2nd
3rd

Fig. 4.

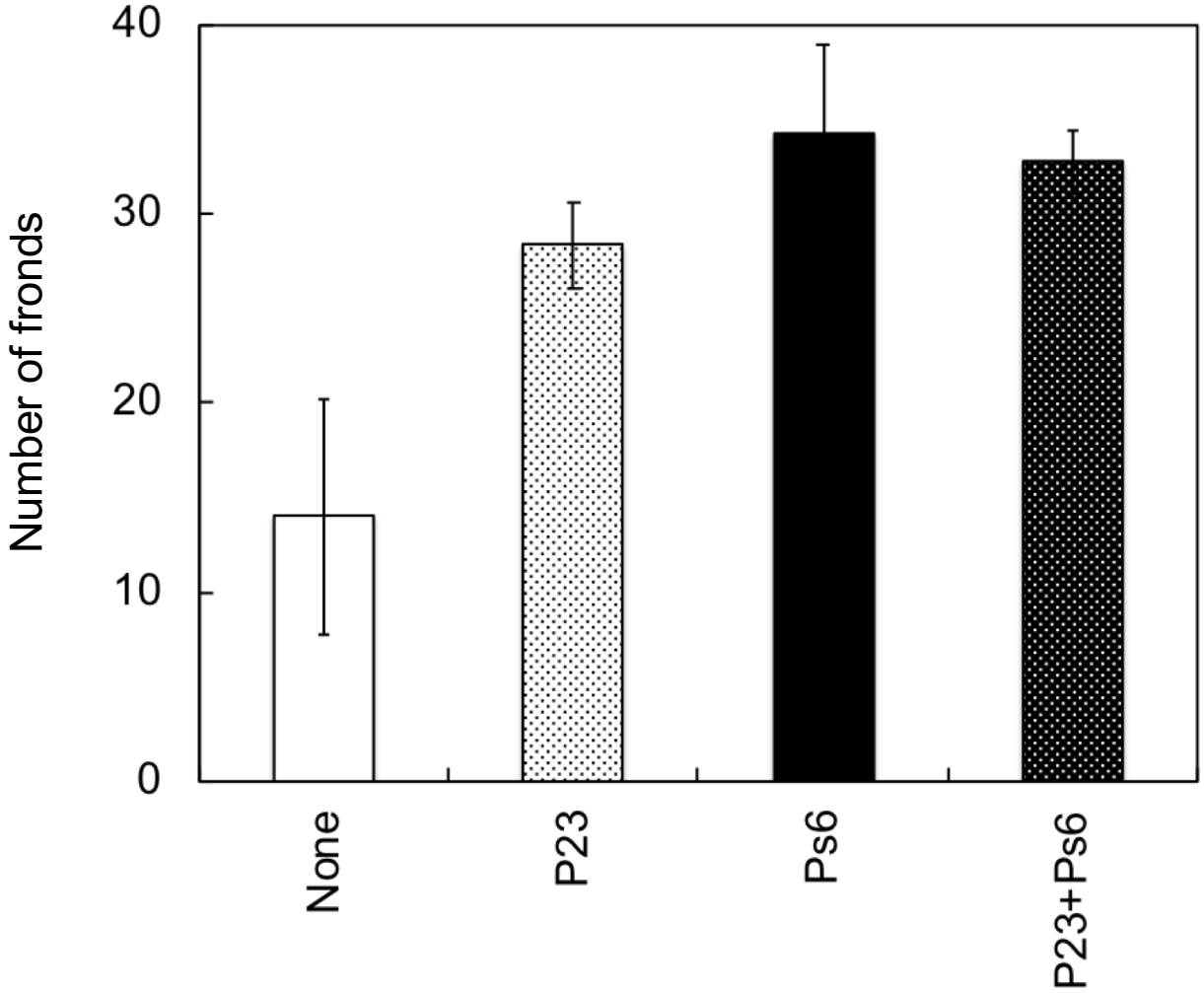

Fig. 5. a

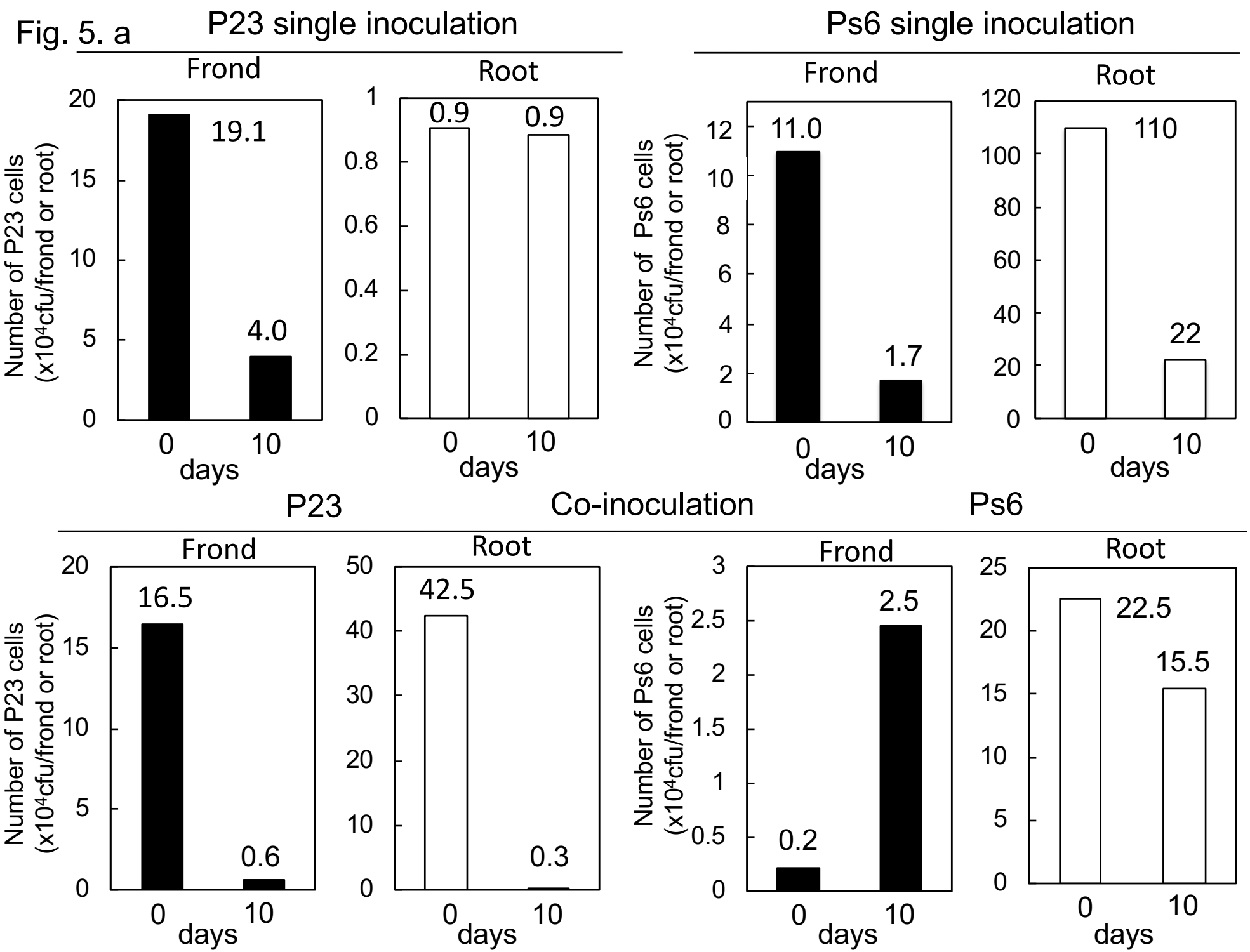
P23 single inoculation
Ps6 single inoculation
Frond
Root
Number of P23 cells (x10⁴cfu/frond or root)
Number of Ps6 cells (x10⁴cfu/frond or root)
19.1
4.0
0.9
0.9
11.0
1.7
110
22
0
10
days
P23
Co-inoculation
Ps6
16.5
0.6
42.5
0.3
0.2
2.5
22.5
15.5

Fig. 5. b.