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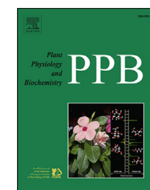

Research article

Differential oxidative and antioxidative response of duckweed *Lemna minor* toward plant growth promoting/inhibiting bacteria

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ABSTRACT

Bacteria colonizing the plant rhizosphere are believed to positively or negatively affect the host plant productivity. This feature has inspired researchers to engineer such interactions to enhance crop production. However, it remains to be elucidated whether rhizobacteria influences plant oxidative stress *vis-a-vis* other environmental stressors, and whether such influence is associated with their growth promoting/inhibiting ability. In this study, two plant growth-promoting bacteria (PGPB) and two plant growth-inhibiting bacteria (PGIB) were separately inoculated into axenic duckweed (*Lemna minor*) culture under laboratory conditions for 4 and 8 days in order to investigate their effects on plant oxidative stress and antioxidant activities. As previously characterized, the inoculation of PGPB and PGIB strains accelerated and reduced the growth of *L. minor*, respectively. After 4 and 8 days of cultivation, compared to the PGPB strains, the PGIB strains induced larger amounts of $O_2^{\bullet-}$, H_2O_2, and malondialdehyde (MDA) in duckweed, although all bacterial strains consistently increased $O_2^{\bullet-}$ content by two times more than that in the aseptic control plants. Activities of five antioxidant enzymes were also elevated by the inoculation of PGIB, confirming the severe oxidative stress condition in plants. These results suggest that the surface attached bacteria affect differently on host oxidative stress and its response, which degree correlates negatively to their effects on plant growth.

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1. Introduction

Plants experience a variety of environmental factors, and not all are ideal for plant growth (Boyer, 1982). Most of the factors, such as low temperature, salinity, ultraviolet radiation, and pathogen inhibit the growth of plants, commonly by inducing oxidative stress through the accumulation of reactive oxygen species (ROS). Although ROS are byproducts of normal cellular activities in the mitochondria, chloroplast, and peroxisome, they are capable of causing damage to plant cellular lipids, proteins, and DNA through radical reactions when present in excess. On the other hand, plants have developed well-tuned antioxidant systems to defend themselves from ROS, and these have been extensively reviewed (Mittler, 2002; You and Chan, 2015). However, it is also reported that the generation of ROS often exceeds the plants' antioxidant capacity and causes significant loss of the plant biomass and yield even under normal environmental conditions (Apel and Hirt, 2004). Up-regulation of antioxidants itself may inhibit growth through cross-talk between developmental and stress-response networks (Cabello et al., 2014). Therefore, maintaining ROS and antioxidants at low levels is essential to achieve enhanced productivity.

Recently, it became known that bacterial communities present in the plant rhizosphere are one of the factors impacting plant productivity akin to the environmental factors (Anderson and Habiger, 2012). Extensive research in this field has demonstrated the general occurrence of plant growth-promoting bacteria (PGPB) and plant growth-inhibiting bacteria (PGIB) or deleterious rhizobacteria (DRB) poses beneficial and deleterious effects on the host growth (Probanza et al., 1996; Suslow and Schroth, 1982). Our original research using duckweed *Lemna minor* as a model plant showed that both promotive and inhibitory effects by the co-existing bacterial community can be caused by complex and interactive influences of PGPB and PGIB existing in the root and frond zone of duckweed (Ishizawa et al., 2017). Therefore, to

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Abbreviations

ANOVA	analysis of variance
APX	ascorbate peroxidase
CAT	catalase
CFU	colony forming unit
DTNB	5,5′-dithiobis-2-nitrobenzoic acid
DRB	deleterious rhizobacteria
DW	dry weight
FW	fresh weight
GPX	guaiacol peroxidase
GR	glutathione reductase
GSSG	glutathione disulfide
MAMP	microbe associated molecular pattern
MDA	malondialdehyde
NBT	nitroblue tetrazolium
PGIB	plant growth-inhibiting bacteria
PGPB	plant growth-promoting bacteria
RGR	relative growth rate
ROS	reactive oxygen species
SD	standard deviation
SOD	superoxide dismutase
TCA	trichloroacetic acid

establish new approaches to improve crop productivity utilizing bacteria, it is critical that interactions between plants and bacteria, especially for PGPB and PGIB, are well understood.

It is likely that oxidative stress plays a role in determining the beneficial and harmful effects of rhizobacteria on the plant, considering that oxidative stress caused by environmental factors also affects plant growth (Apel and Hirt, 2004). Therefore, understanding plant–bacterial interactions from the viewpoint of oxidative stress in plants may offer clues to elucidate the mechanisms leading to promotive/inhibitory effects on plant growth involving rhizobacteria. Such knowledge is helpful in developing techniques/strategies to properly regulate the rhizobacterial community to improve plant growth. However, studies on the relationship between plant oxidative stress and coexisting bacteria have been scarce. The aim of this study is to examine how PGPB and PGIB affect oxidative stress levels of the host plant and its response. Toward this, sterile *L. minor* was co-cultivated with previously isolated PGPB and PGIB strains under laboratory conditions, and the changes in plant ROS and other stress associated indicators in duckweed were monitored.

2. Materials and methods

2.1. Plant and bacterial strains

Duckweed (*Lemna minor*, RDSC clone 5512) plants collected from a small pond in the botanical garden of Hokkaido University (Sapporo, Japan) were used in the experiments. The plants were sterilized by washing with 0.5% sodium hypochlorite for 7 min and followed by washing twice with sterilized water. The sterilized plants were successively cultured in flasks containing modified Hoagland medium (36.1 mg/L KNO_3, 293 mg L^{-1} K_2SO_4, 3.87 mg L^{-1} NaH_2PO_4, 103 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 147 mg L^{-1} $CaCl_2 \cdot H_2O$, 3.33 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 0.95 mg L^{-1} H_3BO_3, 0.39 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.03 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.08 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.254 mg L^{-1} $H_2MoO_4 \cdot 4H_2O$, and 5 mg L^{-1} EDTA·2Na; pH 7.0) and incubated at 28 °C, with a light intensity of 80 μmol $m^{-2}s^{-1}$ and a photoperiod of 16 h/8 h day/night cycle.

Four bacterial strains (*Aquitalea magnusonii* H3, *Acinetobacter septicus* M3, *Asticcacaulis exentricus* M6, *Pseudomonas otitidis* M12) isolated from the same duckweed strain were used for the study. The strains were previously characterized in terms of their effects on duckweed growth when co-cultivated with sterile *L. minor*: strains H3 and M12 were promotive (PGPB), while M3 and M6 were inhibitory (PGIB) (Ishizawa et al., 2017). These PGPB or PGIB strains were cultivated by inoculating a loop of bacterial colony into 20 mL of liquid LB medium and shaking the tube at 120 rpm at 28 °C until the culture reached the late exponential phase. Cells were then harvested by centrifugation (10,000 × *g*, 4 °C, 10 min), washed twice with sterilized Hoagland medium before using them in our experiments.

2.2. Experimental design

Sterile L. *minor* plants were co-cultivated with each one of the four bacterial strains under similar light and nutrient conditions as mentioned above. Attachment of bacterial strains to *L. minor* was enabled by growing it along with a suspension of bacterial cells (optical density at 600 nm = 0.1) added to the sterilized Hoagland medium and maintained for 24 h prior to the experiment. Then, 10 fronds of *L. minor* with the attached bacteria were transferred to 60 mL of fresh bacteria-free medium and co-cultivated. During co-cultivation, the medium was replenished at 48 h intervals. After 4 and 8 days of cultivation, the plants were harvested and subjected to the analyses of ROS and other stress associated indicators.

2.3. Plant growth evaluation

During the growth period, the number of *L. minor* fronds was periodically counted and recorded for evaluating the effect of PGPB/PGIB strains on plant growth. Relative growth rate (RGR, d^{-1}) was calculated as $(\ln FN_t - \ln 10)/t$, where FN_t is the frond number of *L. minor* on day t (4 or 8). In addition, fresh weight (FW) and dry weight (DW, 80 °C for 24 h) of plants were measured at the end of 8-d growth period.

2.4. Estimation of the amount of bacteria attached on duckweed

The amount of bacterial cells that attached to plants was estimated as the number of colony forming units (CFU) per gram fresh weight of the plants. At the end of each 4 and 8 day growth periods, 20 mg of plants were washed twice with 20 mL of sterile Hoagland medium and homogenized in 5 mg L^{-1} tripolyphosphate (TPP) using a BioMasher II (Nippi, Tokyo, Japan). The homogenates were spread onto solid 1:10 diluted LB medium containing 1.5% agar. Agar plates were incubated at 28 °C for 3 days and the number of bacterial colonies were counted.

2.5. Determination of chlorophyll content

Total plant chlorophyll content was determined spectrophotometrically (UV-1850, Shimazu, Kyoto, Japan). Pigment extraction was performed by soaking 30 mg of plants in 3 mL of methanol for 90 min in the dark. Chlorophyll content per milligram fresh weight was calculated using absorbance at 650 nm (A_{650}) and 665 nm (A_{665}) and applying the formula (Grimme and Boardman, 1972).

$$Chl\ a + b = 4.0 \times A_{665} + 25.5 \times A_{650}$$

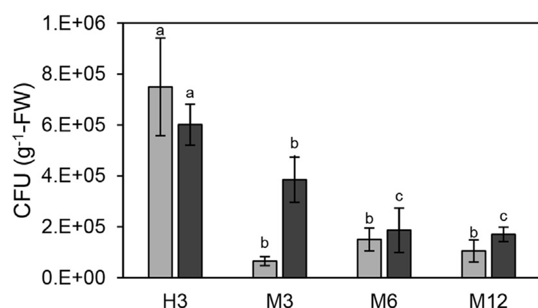

Fig. 1. Amount of bacterial strains that colonized on plant bodies after 4 days (grey bars) and 8 days (black bars) cultivations. Error bars show the standard deviations (n = 3). Significant differences between treatments are designated by different letters (ANOVA, $p < 0.05$).

2.6. Estimation of ROS

Plant superoxide anion ($O_2^{\bullet-}$) level was estimated by measuring the reduction of nitroblue tetrazolium (NBT) according to the method suggested by Doke (1983). Thirty milligrams of fresh plant samples were immersed in 1.5 mL of a mixture containing 10 mM sodium phosphate buffer (pH 7.8), 0.05% NBT and 10 mM NaN_3 in a test tube, followed by incubation at 23 °C for 60 min. Following this, 1.0 mL of the reaction mixture was incubated at 85 °C for 15 min. This reaction was stopped by cooling the solution with ice and absorbance at 580 nm (A_{580}) was measured to assay the reduction of NBT. The level of $O_2^{\bullet-}$ was expressed as the increase in A_{580} per hour per gram fresh weight.

Hydrogen peroxide (H_2O_2) content was measured according to Velikova et al. (2000). Thirty milligrams of plant material were homogenized in 1.5 mL test tube with 1.0 mL of ice-cold 0.1% trichloroacetic acid (TCA) using a high-power homogenizer (ASG50. AS ONE, Aichi, Japan) at 6000 rpm for 30 s. Homogenates were then centrifuged (10,000 × g, 4 °C, 15 min), and 0.5 mL of supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1.0 mL of 1 M KI. The mixture was incubated in the dark at 23 °C for 60 min, and the absorbance at 390 nm was measured. The content of H_2O_2 was calculated using a standard curve plotted with known concentrations of H_2O_2.

2.7. Determination of malondialdehyde (MDA) content

The level of lipid peroxidation was estimated indirectly by measuring the content of MDA, a by-product of lipid peroxidation (Heath and Packer, 1968). Thirty milligrams of plant material were homogenized in 1.5 mL test tube containing 0.5% 2-thiobarbituric acid in 1 mL of 20% TCA using a high-power homogenizer at 6000 rpm for 30 s. Homogenates were then transferred to centrifuge tubes and incubated at 95 °C for 15 min. The reaction was stopped by placing the tubes on ice, and followed by centrifugation (10,000 × g, 2 °C, 10 min). The absorbance of the supernatant was measured at 532 nm. Absorbance at 600 nm was also measured to account for non-specific turbidity or background. The content of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.8. Antioxidant enzyme assays

Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the change in absorbance at 240 nm which indicates decomposition of H_2O_2 (e = 40 $mM^{-1}cm^{-1}$) according to the method of Aebi (1984). The reaction mixture consisted of 900 μL of 50 mM potassium phosphate buffer (pH 7.0), 50 μL of 300 mM H_2O_2, and 50 μL of enzyme extract. The reaction was started by adding H_2O_2.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed by measuring the change in absorbance at 290 nm that accompanied the consumption of ascorbic acid (e = 2.8 $mM^{-1}cm^{-1}$) as described in Nakano and Asada (1981). The reaction mixture contained 800 μL of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM L-ascorbic acid, 50 μL of 100 mM H_2O_2, and 150 μL of enzyme extract.

For guaiacol peroxidase (GPX, EC 1.11.1.7) activity determination, increase in absorbance at 470 nm indicating the formation of tetraguaiacol (e = 26.6 $mM^{-1}cm^{-1}$) was measured as previously described (Horvat et al., 2007). The reaction mixture consisted of 900 μL potassium phosphate buffer (pH = 6.5), 15 mM guaiacol, 50 μL of 300 mM H_2O_2, and 50 μL of enzyme extract.

Glutathione reductase (GR, EC 1.8.1.7) activity was determined using the method described by Smith et al. (1988) by measuring the increase in absorbance at 412 nm that corresponds to the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) to 2-nitro-5-thiobenzoic acid (TNB, e = 13.6 $mM^{-1}cm^{-1}$). The reaction mixture contained 500 μL of 200 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 250 μL of 3 mM DTNB in 10 mM potassium phosphate buffer (pH 7.5), 100 μL of ultra-pure water, 50 μL of 2 mM NADPH, 50 μL of 20 mM glutathione disulfide (GSSG), and 50 μL of enzyme extract. The reaction was initiated by adding GSSG to the mixture.

Superoxide dismutase (SOD, EC 1.15.1.1) was assayed based on the inhibition of NBT's photochemical reduction by the decomposition of $O_2^{\bullet-}$ (Beauchamp and Fridovich, 1971). The reaction mixture consisted of 3 mL of 50 mM sodium phosphate buffer (pH 7.8), 13 mM L-methionine, 75 μM NBT, 0.1 mM EDTA, 300 μL of 24 μM riboflavin, and 0–200 μL of enzyme extract. The total volume of the mixture was adjusted to 3.5 mL by adding 50 mM sodium phosphate buffer (pH 7.8). The mixture was illuminated at a light intensity of 70 μmol $m^{-2}s^{-1}$ for 10 min, and the absorbance at 560 nm was measured immediately to evaluate the photochemical reduction of NBT. One unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition of the NBT photochemical reduction.

The activity of all the above antioxidant enzymes was expressed as unit mg^{-1} protein. For protein extraction, 50 mg of fresh plants in 1.5 mL test tube were homogenized with 1 mL of ice-cold 50 mM

Table 1
Effects of bacterial strains on the growth of *L. minor*, where the initial frond number = 10. Mean ± SD are shown.

	Frond number		RGR		FW (mg)	DW (mg)
	4 d	8 d	4 d	8 d	8 d	8 d
H3	45.8 ± 2.5 a	190.0 ± 4.9 a	0.380 ± 0.014 a	0.368 ± 0.003 a	236.0 ± 1.6 a	12.62 ± 0.20 a
M12	42.3 ± 2.2 b	177.8 ± 9.4 b	0.360 ± 0.013 b	0.360 ± 0.007 b	220.7 ± 11.9 b	12.26 ± 0.32 ab
M3	40.7 ± 1.2 c	158.7 ± 4.8 d	0.351 ± 0.008 c	0.345 ± 0.004 d	198.3 ± 2.9 c	11.33 ± 0.21 c
M6	39.3 ± 1.4 d	152.3 ± 5.9 d	0.342 ± 0.009 d	0.340 ± 0.005 d	194.3 ± 6.2 c	10.98 ± 0.33 c
Control	41.7 ± 1.7 bc	169.5 ± 3.9 c	0.357 ± 0.010 bc	0.354 ± 0.003 c	215.3 ± 3.3 b	11.94 ± 0.22 b

Significant differences between treatments and control are designated by different letters (ANOVA, $p < 0.05$).

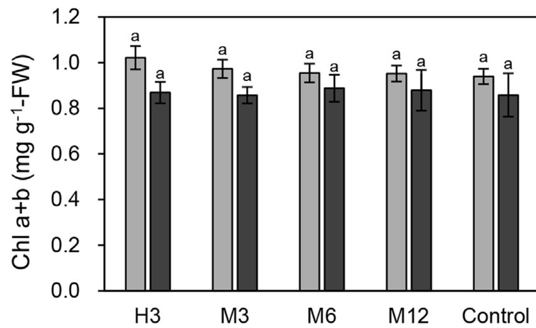

Fig. 2. Chlorophyll content of *L. minor* cultivated with and without inoculation of bacterial strain after 4 days (grey bars) and 8 days (black bars) of growth. Error bars show the standard deviations (n = 3). Values share the same letter indicate no significant difference (ANOVA, $p < 0.05$).

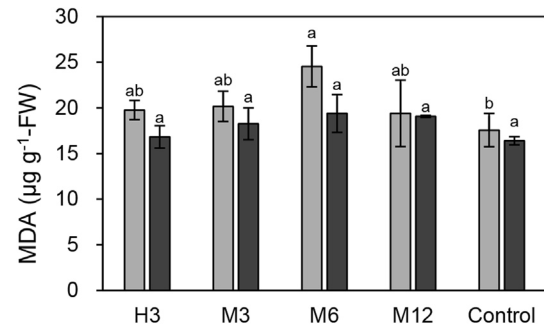

Fig. 4. Effect of bacterial strains on the plant malondialdehyde (MDA) content after 4 days (grey bars) and 8 days (black bars) of growth. Error bars show the standard deviations (n = 3). Significant differences between treatments are designated by different letters (ANOVA, $p < 0.05$).

potassium phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpolypyrrolidone. The homogenate was then centrifuged (20,000 × *g*, 2 °C, 20 min) and the supernatant was used for the enzyme assay. Soluble protein content of the supernatant was determined using the method of Bradford (1976) with bovine serum albumin as a standard.

2.9. Statistical analysis

All assays were performed with 3 biological replicates. Data was analyzed by one-way analysis of variance (ANOVA), and where it was significant, Duncan's multiple-range test was performed to separate the means. Significance at $p < 0.05$ was applied to all analyses. Statistical analyses were performed in R software v3.2.3 (http://www.r-project.org).

3. Results

3.1. Effects of bacterial inoculation on the growth and chlorophyll content of duckweed

In this study, two PGPB strains (H3, M12) and two PGIB strains (M3, M6) were individually co-cultivated with sterile *L. minor*. The amounts of bacteria colonized on the plants are shown in Fig. 1. Strain H3 had the highest CFU both after 4 and 8 days of cultivations. Although there was no carbon source to support bacterial growth in the medium, the CFU counts per plant fresh weight were maintained throughout the cultivation period, suggesting that these strains have stably attached to their host and established. Furthermore, as to strain M3, a notably higher number of CFU was observed after 8 days than at 4 days.

Table 1 summarizes the growth indices of *L. minor*. In the experiment, RGR of control plants (0.354 for 8-day cultivation) was at the same level as reported by Ziegler et al. (2014) which used the optimized culture condition for duckweeds. Similar to the previous study (Ishizawa et al., 2017), PGPB strain H3 and M12 accelerated *L. minor* growth, while PGIB strains M3 and M6 significantly delayed growth compared to aseptic control, in terms of the frond number on day 4 and 8. One notable difference is that the growth-promoting effect of strain M12 was smaller than reported previously. We believe this might be due to the higher growth rate of control plants owing to the difference in nutrient conditions.

Plant chlorophyll content did not show any significant differences among the plants co-cultivated with the four bacterial strains (Fig. 2), indicating the little effects of PGPB and PGIB on the pigment synthesis and/or destruction of *L. minor*. It should be noted that plant had lower levels of chlorophyll content after day 8 than day 4 for all the tests, suggesting some change in duckweed morphology or nutritional status have occurred in the later part of cultivation period, possibly due to the high plant density and/or formation of ethylene (Faber and Kandeler, 1990).

3.2. Plant ROS levels

We observed significant changes in the superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) levels of *L. minor*, depending on the bacterial strains used for co-cultivation. These free radicals serve as the major forms of ROS in plants. As shown in Fig. 3a, all bacterial strains significantly elevated $O_2^{\bullet-}$ content of the plants compared to the aseptic control. We have confirmed that simple introduction of bacterial cells (10^5~10^6 cfu) to the test reagent of $O_2^{\bullet-}$ did not affect the result of the assay (data not shown). Among the bacterial strains, PGIB strain M6, which had the strongest growth-inhibitory effects in this experiment, caused the largest increase in the $O_2^{\bullet-}$ content of *L. minor*. Another PGIB strain M3 also tended to induce

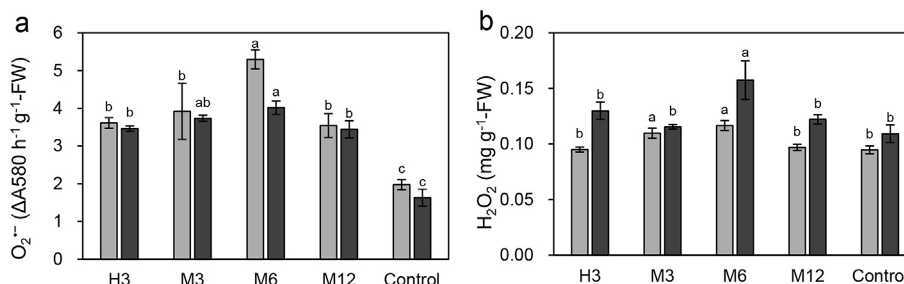

Fig. 3. Effect of bacterial strains on the plant superoxide anion level (a), hydrogen peroxide content (b) after 4 days (grey bars) and 8 days (black bars) of cultivations. Error bars show the standard deviations (n = 3). Significant differences between treatments are designated by different letters (ANOVA, $p < 0.05$).

more than PGPB strains, though the trend is not statistically significant.

As for H_2O_2 content, significantly higher value compared to the control were recorded for the plants cultivated with PGIB strain M6 at days 4 and 8 as well as strain M3 at day 4 (Fig. 3b). Although not significant, plants co-cultivated with PGPB strains H3 and M12 also showed higher H_2O_2 content than control plants, especially in the later part of growth period (day 8).

3.3. Lipid peroxidation

The level of duckweed lipid peroxidation was determined by estimating the amount of MDA accumulation in plant tissues (Fig. 4) as an indicator of cellular damage caused by ROS. Though the difference could not be confirmed due to large variation in results for both on day 4 and 8, PGPB strains increased MDA content in the range of 2.6–16.3%, while PGIB strains increased MDA in the range of 11.4–39.7% compared to the control plants. Thus, the general trend of the changes in MDA content during the cultivation period was similar to that of $O_2^{\bullet-}$ and H_2O_2 content, showing that the degree of lipid peroxidation is more or less correlated to ROS production. Specifically, it is worth noting that the significant increase of MDA content observed for the plants co-cultivated with M6 on day 4 coincided with the highest accumulation of $O_2^{\bullet-}$ (Fig. 3a).

3.4. Antioxidant enzyme activities

The effects of bacterial strains on the activities of five important antioxidative enzymes of the plants were assayed. Although we could not exclude bacterial-originated enzymes in these assays, their effects on the results are considered to be limited because the amount of bacterial protein extracted in the assays would be extremely smaller than plant proteins. Similar to the ROS and lipid peroxidation, the higher values were recorded for the plants cultivated with PGIB strains M3 and M6 in all enzyme studies except CAT (Fig. 5). Since plants are known to up-regulate antioxidant enzymes responding to adverse environments or ROS itself (Babu et al., 2003), the result would reflect the plant activation of antioxidant enzymes by perceiving the higher ROS content. In addition, we observed that activities of each enzyme were quite different between the plants collected after 4 and 8 days. Because the values were expressed based on the amount of total extracted protein, it suggests different protein allocation in plants depending on the growth stage.

4. Discussion

Despite a number of publications on growth promoting and growth inhibiting effects of plant rhizobacteria, their effects, particularly on plant oxidative stress response, are poorly understood. This study highlights the effects of bacterial strains on plant oxidative stress and antioxidant activities using duckweed *L. minor*, which is a useful model plant for investigating plant-microbe interactions (Appenroth et al., 2016).

As previously characterized (Ishizawa et al., 2017), the bacterial strains used in this study either promoted growth or inhibited the growth of duckweed (Table 1), while the influence on chlorophyll content was limited (Fig. 2). Interestingly, both PGPB and PGIB

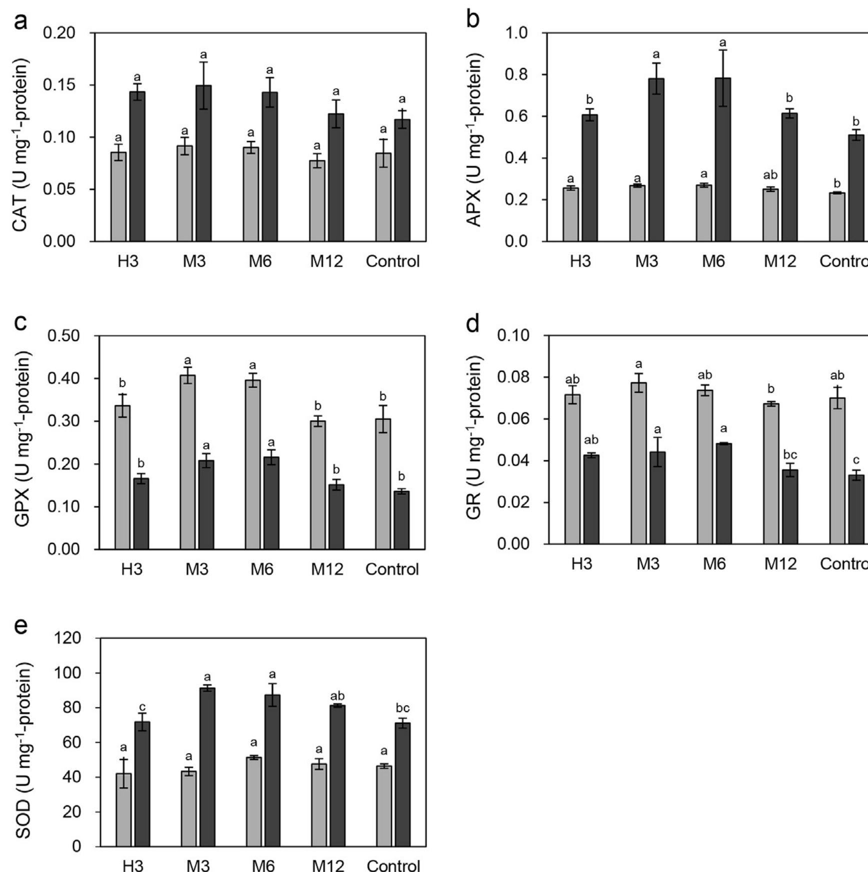

Fig. 5. Effect of bacterial strains on the plant enzymatic antioxidant activities after 4 days (grey bars) and 8 days (black bars) of cultivations. The activities of catalase (a), ascorbate peroxidase (b), guaiacol peroxidase (c), glutathione reductase (d), and superoxide dismutase (e) are shown. Error bars show the standard deviations (n = 3). Significant differences between treatments were designated by different letters (ANOVA, $p < 0.05$).

Table 2
Change of oxidative stress associated indicators induced by PGIB strains in comparison with copper (Panda, 2008). Mean percent change from control (non-treated) plants are shown.

Stress factor	Exposure term	O_2^-	H_2O_2	MDA	CAT	APX	GPX	GR	SOD	Reference
PGIB (M3)	4 d	98.3	15.9	14.8	8.3	15.0	33.4	10.3	−6.7	This study
	8 d	119.2	5.7	11.4	27.8	52.8	52.8	33.6	28.4	This study
PGIB (M6)	4 d	167.8	23.3	39.7	6.5	15.9	29.7	5.3	10.8	This study
	8 d	126.8	44.3	18.3	22.3	53.3	58.5	45.5	22.8	This study
20 μM Cu^{2+}	2 d	0.0	15.3	64.5	–	–	9.8	32.4	7.7	Panda (2008)
50 μM Cu^{2+}	2 d	13.0	24.7	89.4	–	–	0.0	50.9	6.3	Panda (2008)
100 μM Cu^{2+}	2 d	31.3	40.0	95.8	–	–	−9.8	82.4	−13.0	Panda (2008)

caused significantly different oxidative stress levels in *L. minor*, as seen by measuring the ROS content, MDA content, and antioxidant enzymatic activities (Figs. 3–5). In our experiments, the numbers of living bacterial cells colonized on the plants, which might be a kind of bacterial dose to the plant, were estimated as CFU counts. The CFU counts showed a large variation up to about ten times between treatments (Fig. 1). However, their effect on the plant oxidative stress levels could not be correlated to the CFU counts. Therefore, it is likely that the observed changes in plant oxidative stress levels depend on the nature and type of bacterial strains introduced rather than the amount of bacterial cells attached.

Among four bacterial strains used in this study, two PGPB strains, *Aquitalea magnusonii* H3 and *Pseudomonas otitidis* M12, showed relatively moderate influence on plant oxidative stress. However, these strains led to very high levels of $O_2^{\bullet-}$ content in inoculated plants relative to control plants, as well as PGIB strains (Fig. 3a). It is likely that bacteria generally stimulated the plant $O_2^{\bullet-}$ generation, though the degree was different among bacterial strains. Plants possess the ability to generate $O_2^{\bullet-}$ as an immune reaction when they recognize microorganisms through perception of microbe-associated molecular patterns (MAMPs) such as presence of chitin or flagellins (Smith et al., 2015). Other studies have shown that plants generate $O_2^{\bullet-}$ from the activity of NADPH oxidase during the establishment of plant–bacterial symbiosis as signal molecules (Nanda et al., 2010). This suggests that the observed phenomena were a result of spontaneous generation of $O_2^{\bullet-}$ by plants in response to bacterial colonization, which did not occur in the aseptic control plants.

On the other hand, there are some recent studies of PGPB associated with terrestrial plants that act to alleviate plant oxidative stress under harsh stress conditions through the regulation of antioxidant activities (Habib et al., 2016; Islam et al., 2014). In our study, we used favorable growth conditions for *L. minor*. As a result, the role of PGPB strains in alleviating plant oxidative stress compared to the aseptic control was not detected, suggesting the presence of growth-promoting mechanisms of PGPB other than through stress alleviation. However, in a few studies, other duckweed PGPB strains were reported to enhance the growth of duckweed even in the presence of toxic phenol (Yamaga et al., 2010) and chromium (Tang et al., 2015). Considering all these studies, the possibility that duckweed PGPB strains play a role in alleviating plant stress when plants are exposed to stressful environments cannot be ruled out. Therefore, to gain more insights on beneficial plant–microbe interactions additional studies are required.

Compared to the PGPB strains, two PGIB strains, *Acinetobacter ursingii* M3 and *Asticcacaulis excentricus* M6, caused considerably higher oxidative stress levels in *L. minor* (Figs. 3–5). Although there are several studies about the mechanism of plant growth promotion by PGPB such as nutrient supply and modulation of plant hormone (Glick, 2012), very little is known about how PGIB negatively affect plant growth and productivity. Thus far, hydrogen cyanide production (Astrom, 1991) and ammonia production (Weise et al., 2013) were proposed as possible mechanisms of existing PGIB strains. In this study, we show that PGIB strains induce higher levels of oxidative stress in the host plant than by the other bacterial strains, which explain at least some of the growth-inhibitory mechanisms employed by PGIB. The knowledge that PGIB induce a considerably high levels of plant oxidative stress may provide clues toward establishing strategies for crops to defend PGIB. For example, engineering plants for elevated abiotic stress tolerance or introducing outcompeting rhizobacterial strains with less influence on plant stress might be effective for managing the adverse effects of PGIB.

Duckweeds have been used in plant stress research and for chemical toxicity tests for a long time (Ziegler et al., 2016), and their responses toward environmental stress factors have been extensively studied. Thus far, a variety of heavy metals (Parlak and Yilmaz, 2012; Tkalec et al., 2008; Duman et al., 2010), salinity (Cheng, 2011), ammonium (Wang et al., 2016), and chemical compounds (Obermeier et al., 2015) have been shown to induce a dose-dependent response to oxidative stress, while the degree and type of activated antioxidants seemed to vary and depend highly on the culture conditions and/or duckweed strains used. In this study, similar responses of duckweed to the above-mentioned abiotic stressors are being reported for the first time for plant-associated bacteria, particularly in suppressing the growth of the host plant.

Table 2 summarizes the oxidative and antioxidative responses induced by PGIB strains, in comparison to Panda (2008) who investigated the response of *L. minor* fronds to copper (Cu^{2+}) exposure. Copper is one of the most typical and well-studied stress factors for duckweed, which induces severe oxidative stress in the plant at higher concentrations (Panda, 2008; Babu et al., 2003; Razinger et al., 2007). As shown in Table 2, increase in $O_2^{\bullet-}$ content and GPX caused by PGIB were much higher than that caused by copper, although the responses to H_2O_2 content was comparable to moderate concentrations of Cu^{2+}. Upregulation of antioxidant enzymes induced by PGIB were also comparable to that by copper, except for GPX. Even though we observed a considerable increase in MDA content for plants co-cultivated with PGIB, the level of lipid peroxidation did not seem to be as high as that caused by Cu^{2+}. Considering these information, we propose that the effects on the plant oxidative stress by PGIB strains are generally equivalent to the moderate dose of environmental contaminants such as copper, which should be certainly a focus of future research.

In summary, this is the first study to highlight the different effects of PGPB and PGIB strains on the oxidative stress and antioxidant enzyme activities of *L. minor*. Two PGIB strains were found to increase plant oxidative stress and antioxidant enzyme activities similar to abiotic stress factors reported previously. On the other hand, PGPB strains were less stressful, though they also caused certain levels of oxidative stress on the plant when compared to the control plant. Although the detailed mechanisms are still unknown, oxidative stress and its response may play important roles in growth inhibition caused by PGIB. In addition, a series of oxidative stress assays performed in this study can serve as useful tools to evaluate beneficial/deleterious effects of bacteria on duckweed productivity and possibly extend to studies on other plant species.

Contributions

Hidehiro Ishizawa designed and performed the experiments, interpreted the results, and drafted the manuscript. Masashi Kuroda designed the experiments, interpreted the results, drafted and revised the manuscript. Masaaki Morikawa interpreted the results, critically revised the manuscript, and supervised the project. Michihiko Ike designed the experiments, interpreted the results, drafted and revised the manuscript, and supervised the project.

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