Plant Physiology and Biochemistry 118 (2017) 667–673

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

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

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ARTICLE INFO

Article history:
Received 7 June 2017
Received in revised form
7 August 2017
Accepted 8 August 2017
Available online 9 August 2017

Keywords:
Duckweed
Plant growth-promoting bacteria
Plant growth-inhibiting bacteria
Oxidative stress
Reactive oxygen species
Plant-microbe interactions

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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http://dx.doi.org/10.1016/j.plaphy.2017.08.006
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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).

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

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