

Effect of nitrogen-deficiency on midday photoinhibition in flag leaves of different rice (*Oryza sativa* L.) cultivars

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Abstract

Effects of nitrogen (N)-deficiency on midday photoinhibition in flag leaves were compared between two contrastive Japanese rice cultivars, a traditional *japonica* cultivar with low yield, cv. Shirobeniya (SRB), and a *japonica-indica* intermediate type with high yield, cv. Akenohoshi (AKN). Both cultivars were grown under high-N and low-N conditions. At midday, low-N supply resulted in more intensive reductions in net photosynthetic rate, stomatal conductance, maximal quantum yield of photosystem II (PSII) and quantum yield of PSII electron transport in SRB than in AKN, indicating that SRB was more strongly photoinhibited than AKN under low-N condition. At midday, the low-N plants of two cultivars showed higher superoxide dismutase (SOD) activities than the high-N plants. However, ascorbate peroxidase (APX) activity was maintained in AKN but significantly decreased in SRB under low-N condition (N-deficiency). In contrast, hydrogen peroxide (H₂O₂) content in SRB significantly increased under low-N condition, indicating that the susceptibility to midday photoinhibition in the low-N plants of SRB is related to the increased H₂O₂ accumulation. It is suggested that the midday depression in photosynthesis may be a result of oxidative stress occurring in the low-N plants in which antioxidant capacity is not enough to cope with the generation of H₂O₂. Therefore, H₂O₂-scavenging capacity could be an important factor in determining the cultivar difference of midday photoinhibition in flag leaves of rice under low-N condition.

Additional key words: antioxidative system; chlorophyll fluorescence; cultivar difference; flag leaves; hydrogen peroxide; photoinhibition; photoprotection; rice.

Introduction

Photosynthesis cannot proceed in the absence of photons, but not all of those absorbed by antenna systems can be utilized for photosynthesis during exposure to full sunlight. The excessive photon energies produce reactive oxygen species (ROS), which can cause photooxidative destruction of the thylakoid membrane and stroma proteins in chloroplasts and finally inhibit photosynthesis (photoinhibition) (Foyer and Noctor 1999). Detoxification of ROS and avoidance of its formation are performed by integrated enzymatic and non-enzymatic antioxidative systems that are concentrated in chloroplasts, namely the water-water cycle (Asada 1999).

Nitrogen (N)-deficiency can severely affect photo-

synthetic rate at saturating irradiance and ambient CO₂ levels (Makino *et al.* 1983). It seems that decreased CO₂ assimilation capacity induced by N-deficiency potentially leads to an over-reduction of photosynthetic electron transport chain and therefore photoinhibition (Skillman and Osmond 1998, Bungard *et al.* 2000). Antioxidative systems are activated in N-deficient leaves. Both increase in superoxide dismutase (SOD, EC 1.15.1.1) activity and decreases in ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2) activities occur in response to enhanced formation rate of superoxide radicals in N-deficient coffee plants (Ramalho *et al.* 1998).

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Abbreviations: AKN – cv. Akenohoshi; APX – ascorbate peroxidase; C_a – atmospheric CO₂ concentration; C_i – intercellular CO₂ concentration; Chl – chlorophyll; F₀ – initial Chl fluorescence of a dark-adapted leaf; F_m – the maximal Chl fluorescence of a dark-adapted leaf; F_m' – the maximal Chl fluorescence detected in actinic light; F_s – steady Chl fluorescence in actinic light; F_v – variable Chl fluorescence; GR – glutathione reductase; g_s – stomatal conductance; N – nitrogen; NBT – nitroblue tetrazolium; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; PSII – photosystem II; Rubisco – ribulose-1, 5-bisphosphate carboxylase/oxygenase; ROS – reactive oxygen species; SOD – superoxide dismutase; SRB – cv. Shirobeniya; Φ_{PSII} – quantum yield of PSII electron transport.

The midday depression in photosynthesis is a common phenomenon for many species. Several studies showed that stomatal closure is at least partially responsible for the reduction of photosynthetic rate in sunflower (Quick *et al.* 1992) and rice (Hirasawa *et al.* 1989). However, it has been reported that the midday depression in photosynthesis in rice is coincided with photoinhibition of PSII (Horton and Murchie 2000, Wang *et al.* 2005). Photoinhibition causes a decrease of approximately 10 % in a daily carbon assimilation of a plant canopy (Long *et al.* 1994). The tolerance to photoinhibition of rice cultivars may significantly affect the rice grain yields under low-N input condition. Several studies on rice suggested the existence of difference among rice cultivars in both the susceptibility to photoinhibition and the activities of antioxidant enzymes such

Materials and methods

Plants and growth condition: Two Japanese rice cultivars, SRB and AKN, were used. The water-soaked seeds were sown in nursery boxes in a glasshouse at the beginning of June, 2007. At three weeks after sowing, the seedlings were transplanted into 8 dm³ pots filled with sandy loam soil, and divided into the high-N and the low-N groups which were fertilized with 1.6 g N and 0.4 g N in form of ammonium sulfate, respectively. In both groups, 1.6 g P and 1.6 g K were also applied in form of calcium superphosphate and potassium chloride, respectively. Plants were grown outdoors, and their growth was periodically surveyed. At two weeks after heading, four flag leaves from four plants were selected and used for measurements. The measurements of photosynthetic gas exchange and chlorophyll (Chl) fluorescence were made at midday (13:00) outdoors. After the measurements, Chl content at the midpoint of each leaf blade was evaluated by use of the SPAD meter (SPAD-502, Konica Minolta Sensing Co., Osaka, Japan). The SPAD values were means of three measurements. Then, the leaf blades were excised, frozen in N₂, and stored at -80 °C for assays of hydrogen peroxide (H₂O₂) content and enzymes activities. Thereafter, the plants were sampled and dried at 80 °C for 3 d in an oven to weigh their dry mass. Leaf area per plant was measured with an automatic area meter (AAM-8, Hayashi-denko, Tokyo, Japan). The measurements were repeated three times (days) using different plants, and we obtained similar results. A representative of results was shown in each figure.

Gas exchange and Chl fluorescence: At midday on a clear day at the beginning of August, 2007, photosynthetic gas exchange and Chl fluorescence were simultaneously measured by using a system that combined a portable photosynthetic system (LCpro, ADC, Herts, UK) and a portable fluorometer (PAM-2000, Waltz, Effeltrich, Germany). The chamber of the system was modified as follows: the fiberoptic of the PAM-2000

was attached onto the side of the chamber at a 60° angle without interfering with photosynthetic photon flux density (PPFD) distribution at the leaf surface, yet it allows for delivery of saturation pulse and measuring beam and the detection of measured signals. Incident PPFD beside the leaf chamber was measured with a photon sensor. The atmospheric CO₂ concentration (C_a) entering the chamber was 387 ± 3 cm³ m⁻³. Air temperature in the chamber was adjusted manually to the air temperature outside the chamber. Relative humidity in the chamber was also adjusted to the ambient humidity. Measurements were made under the following conditions: PPFD, 1883 ± 33 μmol m⁻² s⁻¹, leaf temperature, 35.3 ± 0.03 °C; water vapor pressure, 2.06 ± 0.05 kPa. Net photosynthetic rate (P_N), stomatal conductance (g_s), and intercellular CO₂ concentration (C_i) were recorded with a portable photosynthetic system. Using leaves that were dark-adapted for 30 min, the initial fluorescence (F₀) in non-photosynthetic conditions was determined with low intensity of a measuring beam; thereafter, the maximal fluorescence (F_m) was measured by applying a 0.8-s saturation pulse onto the leaf in order to reduce all the PSII centres. Then, the leaf was continuously exposed to natural irradiance. After the steady-state fluorescence (F_s) was recorded, a 0.8-s saturation pulse was applied to determine the maximal fluorescence in the light-adapted state (F_m'). Based on the data obtained, the following parameters were calculated: the maximal quantum yield of PSII, F_v/F_m = (F_m - F₀)/F_m (van Kooten and Snel 1990); and the quantum yield of PSII electron transport, Φ_{PSII} = (F_m' - F_s)/F_m' (Genty *et al.* 1989).

as SOD (Jiao and Ji 2001, Jiao *et al.* 2003). However, it is unknown whether there is a difference among cultivars in terms of the susceptibility to midday photoinhibition of leaves under low-N input condition.

In our previous study, we investigated the effects of N-supply restriction on PSII functions and CO₂ assimilation in flag leaves by using two contrasting Japanese rice cultivars, Shirobeniya (SRB, a traditional *japonica* with low yield) and Akenohoshi (AKN, a *japonica-indica* intermediate type with high yield), and found that under low-N conditions, the photosynthetic stability in flag leaves of AKN was superior than that of SRB (Kumagai *et al.* 2007). In this study, we attempted to identify the physiological factor responsible for the cultivar difference in midday photoinhibition of rice flag leaves under low-N condition.

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Enzyme extraction and assays: All extractions were carried out at 0–4 °C. For determination of SOD activity, leaf segments were frozen in liquid N₂ and homogenized with 50 mM HEPES buffer (pH 7.6) containing 0.1 mM Na₂EDTA. Homogenates were centrifuged at 13000 × g for 25 min at 4 °C. SOD activity was spectrophoto-

metrically assayed by monitoring the photochemical inhibition of nitroblue tetrazolium (NBT) reduction in 3 cm³ reaction mixtures at 25 °C according to the procedure of Yamane *et al.* (2004). For the determination of APX activity, leaf segments were frozen in liquid N₂ and homogenized with 25 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1 % (w/v) Triton X-100, 5 mM sodium ascorbate and 1 % (w/v) polyvinylpyrrolidone. Homogenates were centrifuged at 12000 × g for 10 min at 4 °C. APX activity was spectrophotometrically determined at 25 °C according to the procedure of Yamane *et al.* (2004).

Results and discussion

When the two cultivars, SRB and AKN, were grown under different N conditions, both dry mass and leaf area were decreased under low-N condition (Table 1). The degrees of the decrease were higher in SRB than in AKN. These data were consistent with our previous results (Kumagai *et al.* 2007). The decrease in Chl content is a phenomenon typical of plants grown under N-deficiency. Thus, the SPAD value was measured as an indicator of N-deficiency. In the flag leaves of the two cultivars, the SPAD values decreased from approximately 46 under high-N to below 30 under low-N. These data indicated that the two cultivars grown under low-N condition suffered from N-deficiency.

In the two cultivars, P_N was lower in the plants grown under low-N condition than in those under high-N condition (Fig. 1A). Under each N condition, P_N of SRB was significantly lower than that of AKN. The g_s value of AKN did not change between two N levels, while that of SRB decreased significantly under low-N condition as compared with high-N condition (Fig. 1B). C_i/C_a of the two cultivars tended to increase slightly under low-N condition (Fig. 1C). Some studies showed that decrease in P_N in N-deficient plants was accompanied by an increase in C_i , indicating that lower photosynthetic capacity was due to reduced metabolic activity of mesophyll cells rather than due to stomatal limitation (Ciompi *et al.* 1996, Huang *et al.* 2004). We also found that the decrease in P_N at midday of the low-N plants was accompanied by a slight increase in C_i/C_a . Therefore, we consider that the reduction of P_N under low-N condition at midday could be attributed to the decreased photo-

H₂O₂ content: Leaf segments were homogenized in cold 80 % (v/v) acetone in the ratio of 0.1 g tissue to 2 cm³ acetone. Homogenates were centrifuged at 12000 × g for 10 min at 4 °C. The H₂O₂ content was measured spectrophotometrically by monitoring the absorbance at 410 nm of Ti-H₂O₂ complex following the procedure of Patterson *et al.* (1984).

Statistic analysis: Data were statistically analyzed using one-way ANOVA with Tukey's test (*Sigmastat 3.1 for Windows, Systat Software, Inc., Richmond, USA*). Significant difference was analyzed based on $p < 0.05$.

synthetic activity of mesophyll cells, rather than the closure of stomata.

Since CO₂ assimilation acts as a major sink for the reducing equivalents (ATP and NADPH) generated by the primary photochemical reactions, it seems that light absorbed by the rice plants exceeded the light utilization capacity in chloroplasts of the flag leaves. Namely, the low-N leaves were exposed to excessive light for a significant part of the day, which may potentially result in photoinhibition. There was no significant difference in F_v/F_m measured in the morning between the two cultivars and between the two N conditions, and the values were approximately 0.800 (data not shown). At midday, there was no significant difference in F_v/F_m between the two cultivars grown under high-N condition. However, F_v/F_m of SRB was significantly reduced under low-N condition, although that of AKN was not reduced under the condition (Fig. 1D). F_v/F_m is used as an indicator for the degree of photoinhibition in PSII (van Kooten and Snel 1990). Therefore, the data indicate that the low-N plants of SRB are seriously photoinhibited during exposure to midday high irradiance. Furthermore, the decrease in Φ_{PSII} under low-N condition was clearer in SRB than in AKN (Fig. 1E). Since Φ_{PSII} represents the fraction of energy utilized for electron transport in thylakoid membranes of chloroplasts (Genty *et al.* 1989), this depression of Φ_{PSII} in SRB may reflect the limitation of CO₂ assimilation and photorespiration. In our previous study, we observed that SRB had a lower content of Rubisco in flag leaves than AKN over a wide range of N application (Kumagai *et al.* 2007). During the exposure to strong

Table 1. Dry mass [g per plant], leaf area [m² per plant], and SPAD of leaves of two cultivars (SRB = Shirobeniya, AKN = Akenohoshi) grown under high-N and low-N conditions. Values are given as the mean of SD ($n = 4$). Values followed by the same letters in the column had no significant difference as determined by the Tukey's test at 5 % level.

		Dry mass	Leaf area	SPAD
SRB	High-N	80.3 ± 2.4a	0.613 ± 0.04a	45.7 ± 2.6a
	Low-N	45.1 ± 0.8c	0.195 ± 0.04c	28.6 ± 1.4b
AKN	High-N	82.7 ± 5.8a	0.636 ± 0.04a	46.1 ± 0.2a
	Low-N	54.9 ± 3.4b	0.275 ± 0.02b	29.9 ± 0.4b

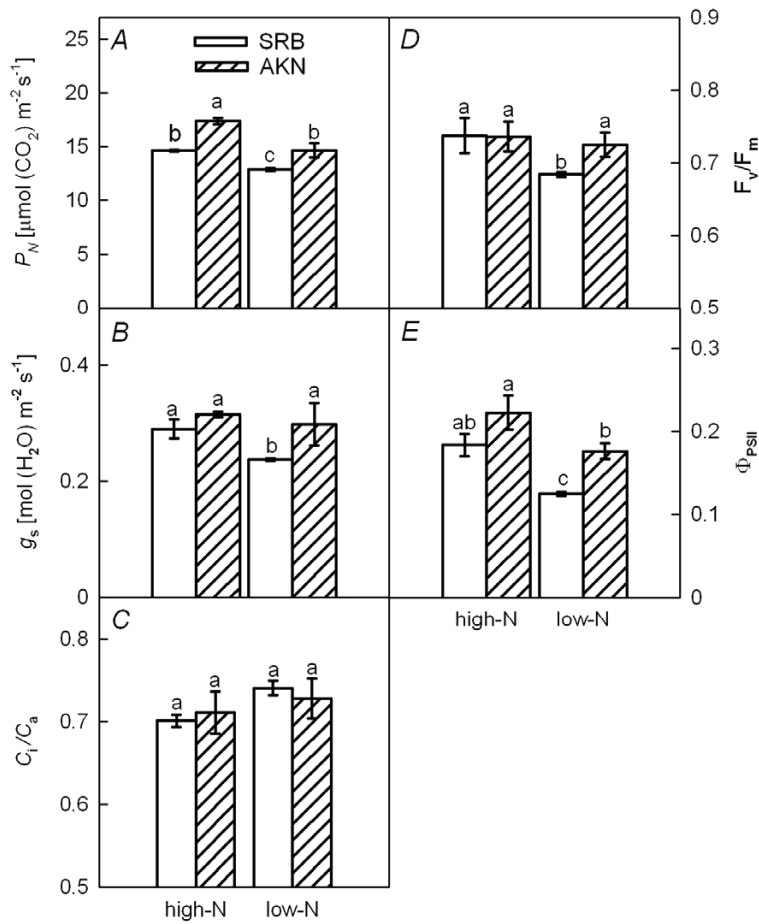


Fig. 1. Net photosynthetic rate (P_N , A), stomatal conductance (g_s , B), ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a , C), maximal quantum yield of PSII (F_v/F_m , D) and quantum yield of PSII electron transport (Φ_{PSII} , E) measured at midday (13:00) in flag leaves of two cultivars (SRB = Shirobeniya, AKN = Akenohoshi) grown under high-N and low-N conditions. Values are given as the mean \pm SD ($n = 4$). Bars followed by the same letters had no significant difference as determined by the Tukey's test at 5 % level.

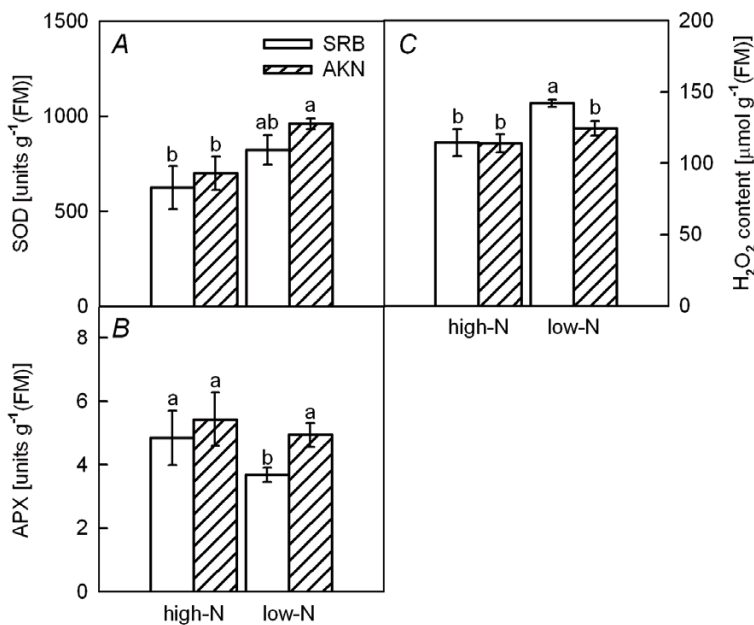


Fig. 2 Superoxide dismutase (SOD) activity (A), ascorbate peroxidase (APX) activity (B) and hydrogen peroxide (H_2O_2) content (C) in flag leaves taken at midday (13:00) of two cultivars grown under high-N and low-N conditions. Values are given as the mean \pm SD ($n = 4$). Bars followed by the same letters had no significant difference as determined by the Tukey's test at 5 % level.

midday sunlight, therefore, the flag leaves of the low-N plants of SRB may easily accumulate excessive energy, rendering them more susceptible to photoinhibition when the Rubisco content is reduced and both CO_2 assimilation

and photorespiration are limited due to N-deficiency.

Limitation of the CO_2 assimilation restrains the consumption of ATP and NADPH, resulting in a generation of ROS, especially under strong sunlight. Asada and

Badger (1984) reported that interruption of the photosynthetic CO₂ assimilation accelerates the production of H₂O₂ in spinach leaves. In general, H₂O₂ is rapidly scavenged through the water-water cycle in chloroplasts. At midday, the low-N plants showed higher SOD activities than the high-N plants (Fig. 2A). Interestingly, APX activity in AKN was almost the same between the two N conditions, while that in SRB was decreased significantly under low-N condition (Fig. 2B). The H₂O₂ level is thought to be regulated by SOD and APX, since these enzymes are involved in the generation and breakdown of H₂O₂, respectively (Asada 1999). In comparison with AKN, H₂O₂ content in SRB increased significantly under low-N condition (Fig. 2C). Furthermore, we found that there are significant correlations between the H₂O₂ content and APX activity ($r = -0.666$, $p < 0.001$), and between H₂O₂ content and the ratio of SOD to APX activity ($r = 0.829$, $p < 0.001$) in the two cultivars. These results indicate that the higher H₂O₂ levels in the low-N plants of SRB than in those of AKN would result from the lower APX activity and/or the higher SOD/APX ratio. Previous studies revealed that increased accumulation of H₂O₂ in stress-sensitive plants

as compared with stress-tolerant plants was associated with higher SOD/APX ratios under various stress conditions, such as salt stress (Mittova *et al.* 2003) and chilling stress (Zhou *et al.* 2006). H₂O₂ has effects on the fragmentation of large subunit of Rubisco (Ishida *et al.* 1998) and inhibits the synthesis of PSII proteins, in particular, that of the D1 protein (Takahashi and Murata 2008). Zhou *et al.* (2007) showed that a high negative correlation was observed between H₂O₂ content and Rubisco activity in rice plants grown under severe drought stress. The extent of photoinhibition depends not only on the rate of D1 protein degradation but also on the rate of D1 protein synthesis (Kyle *et al.* 1984). As a result, the reductions of F_v/F_m and Φ_{PSII} at midday in the low-N plants of SRB would be related to the increased H₂O₂ accumulation. Thus, it is suggested that the depression in photosynthesis may be a result of oxidative stress occurring in the low-N plants of SRB in which antioxidant capacity is not enough to cope with the generation of H₂O₂. Therefore, H₂O₂-scavenging capacity could be an important factor in determining the cultivar difference of midday photoinhibition in flag leaves of rice plants under the low-N condition.

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