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Effects of nitrogen deficiency on gas exchange, chlorophyll fluorescence, and antioxidant enzymes in leaves of rice plants

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Abstract

Gas exchange, chlorophyll (Chl) fluorescence, and contents of photosynthetic pigments, soluble proteins (ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBPCO), and antioxidant enzymes were characterized in the fully expanded 6th leaves in rice seedlings grown on either complete (CK) or on nitrogen-deficient nutrient (N-deficiency) solutions during a 20-chase period. Compared with the control plants, the lower photosynthetic capacity at saturation irradiance (P_{max}) was accompanied by an increase in intercellular CO_2 concentration (C_i), indicating that in N-deficient plants the decline in P_{max} was not due to stomatal limitation but due to the reduced carboxylation efficiency. The fluorescence parameters Φ_{PS2}, F_v'/F_m', electron transport rate (ETR), and q_P showed the same tendency as P_{max} in N-deficient plants. Correspondingly, a higher q_N paralleled the rise of the ratio of carotenoid (Car) to Chl contents. However, F_v/F_m was still diminished, suggesting that photoinhibition did occur in the photosystem 2 (PS2) reaction centres. In addition, the activities of antioxidant enzymes on a fresh mass basis were gradually lowered, leading to the aggravation of membrane lipid peroxidation with the proceeding N-deficiency. The accumulation of malonyldialdehyde resulted in the lessening of Chl and soluble protein content. Analyses of regression showed PS2 excitation pressure ($1 - q_P$) was linearly correlated with the content of Chl and inversely with soluble protein (particularly RuBPCO) content. There was a lag phase in the increase of PS2 excitation pressure compared to the decrease of RuBPCO content. Therefore, the increased excitation pressure under N-deficiency is probably the result of saturation of the electron transport chain due to the limitation of the use of reductants by the Calvin cycle. Rice plants responded to N-deficiency and high irradiance by decreasing light-harvesting capacity and by increasing thermal dissipation of absorbed energy.

Additional key words: catalase; leaf senescence; malonyldialdehyde; *Oryza sativa*; peroxidase; photosynthesis; photosystem 2; ribulose-1,5-bisphosphate carboxylase/oxygenase; stomatal conductance; transpiration rate.

Introduction

An increasing number of reports have focused on the photoinhibition and photoprotective mechanisms in plants subjected to nitrogen stress and high irradiance. In terms of photosystem 2 (PS2) photochemistry, some studies have demonstrated N-deficiency has no effect on the quantum yield of PS2 (Khamis *et al.* 1990, Henley *et al.* 1991, Ciompi *et al.* 1996, Lu and Zhang 2000). However, others have shown that N-deficiency reduces the maximal efficiency of PS2 photochemistry (F_v/F_m) and the quantum yield of PS2 (Φ_{PS2}) in spinach, indicating that N-deficiency causes damage to PS2 (Verhoeven *et al.* 1997). The similar or contradictory results may lie

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Abbreviations: AQY– apparent quantum yield; C_i – intercellular CO_2 concentration; Car – carotenoid; CAT – catalase; Chl – chlorophyll; CI – compensation irradiance; $D = 1 - F_v'/F_m'$, fraction of photon energy absorbed in PS2 antennae that is dissipated *via* thermal energy in the antennae; E – transpiration rate; $Ex = F_v'/F_m' (1 - q_P)$, fraction of energy neither dissipated in PS2 antennae system nor utilized for photosynthetic electron transport; ETC – photosynthetic electron transport chain; ETR – non-cyclic electron transport rate; F_m – maximal Chl fluorescence yield; F_0 – minimum fluorescence yield; F_v – variable Chl fluorescence; F_v/F_m – maximal photochemical efficiency; F_v'/F_m' – excitation energy capture efficiency of PS2 reaction centres; g_s – stomatal conductance to water vapour; MDA – malonyldialdehyde; P – fraction of photon energy absorbed in PS2 antennae that is utilized for photosynthetic electron transport, that is equal to Φ_{PS2}; P_{max} – photosynthetic capacity at saturation irradiance; P_N – net photosynthetic rate; POD – peroxidase; PPFD – photosynthetic photon flux density; q_N – non-photochemical quenching; q_P – photochemical quenching; SOD – superoxide dismutase; Φ_{PS2} – effective PS2 quantum yield.
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in the measurement under different treatment conditions. Recent research has revealed that F_v/F_m is decreased at midday in N-deficient wheat cultivars (Dong *et al.* 2002). The activity of xanthophyll cycle is enhanced in response to N limitation to dissipate excess energy under high irradiance (Lu *et al.* 2001, Cheng 2003). The characteristics of Chl fluorescence and photooxidation have been scrutinized during the senescence of flag leaf of different cultivars of a super high-yielding hybrid rice (Jiao *et al.* 2003).

Although more evidence has confirmed that N-deficiency induces early leaf ageing in plants (Crafts-Brandner *et al.* 1996, 1998), there is little knowledge about the initiation of leaf senescence. Huner *et al*. (1996) proposed that photosynthetic apparatus acts as not only a transducer of photon energy but also as a primary sensor of environmental change (irradiance, radiation quality, temperature, water availability, nutrient status, *etc*.) through modulation of the chloroplast redox signal as a consequence of imbalances between energy supply and energy consumption. PS2 excitation pressure is an estimate of the redox state of Q_A, the first and stable quinone electron acceptor of the PS2 reaction centre (van Kooten and Snel 1990). It is defined as $1 - q_P \approx Q_A(\text{red.})/[Q_A(\text{red.}) + Q_A(\text{ox.})]$ (Dietz *et al.* 1985, Ögren 1991, Schreiber *et al.* 1994, Ono *et al.* 2001), where q_P is the photochemical quenching coefficient. Ou *et al.* (2003) proposed that the PS2 excitation pressure might mediate natural leaf senescence in the super high-yielding rice. Does PS2 excitation pressure elicit premature leaf ageing under N-deficiency?

This is why the objective of our studies was to identify how N-deficiency affects the photosynthetic apparatus and photochemistry during later leaf development in rice plants. Furthermore, it was discussed whether PS2 excitation pressure induces leaf senescence of rice plants under N-deficiency.

Materials and methods

Plants: Seeds of rice (O*ryza sativa* L. subsp. *indica* cv. Zhengnong 952) were germinated and planted according to the method of Jiang *et al*. (1987). When the rice plants grew up to leaf stage 4, two plants per unit were transferred into *ca.* 7 000 cm^3 pots containing IRRI complete nutrient solution (Jiang *et al*. 1987). The plants were grown in the conservatory, Hua-jia-chi Campus of the Zhejiang University. Its daily temperature was 28±2 °C and the atmospheric relative humidity ranged between 76 and 94 %. When the 7th leaf grew out, the control and N-deficient groups were immediately grown with complete or N-free nutrient solution. The pH was adjusted to 5.0–5.5 regularly during the culture period and nutrient solutions were renewed twice a week. All the measurements of photosynthetic parameters and Chl fluorescence were conducted in the 6th leaves of the main stem every 5 d. After the measurements, the leaves were excised, frozen in liquid N_2, and stored at –80 °C for biochemical assays.

Determination of photosynthetic pigments: Ten leaf discs (diameter 5 mm) were removed from the 6th leaves and extracted with 5 cm^3 of 80 % acetone in the dark for 48 h at 25 °C until they were blanched. The concentrations of Chl and carotenoids (Car) were determined according to Wellburn and Lichtenthaler (1984). There were three replications for each determination.

Quantification of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) content: Frozen leaf blade (0.1 g) was ground into powder with the mortar and pestle in liquid N_2 and 1 % insoluble polyvinylpolypyrrolidone (PVP), and then homogenized with 1.9 cm^3 of the cooled extraction buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM $MgCl_2$, 12.5 % (v/v) glycerol, 10 % soluble PVP-40, and 10 mM β-mercaptoethanol. The homogenate was centrifuged at 15 000×*g* for 20 min. The supernatant was used for quantification of RuBPCO content. The quantification was made with the single radial immuno-diffusion method illustrated by Wang *et al.* (2003). The gel was prepared with 1 % agar, which was heated up and dissolved in the buffer containing 50 mM Tris-HCl (pH 7.0), 0.9 % NaCl, and 15 mM NaN_3. When the sol cooled down to 55 °C, the rabbit antiserum for RuBPCO was added and the sol was immediately poured on the glass plate. After the gel, which was 12.50 cm long, 9.50 cm wide and 0.15 cm thick, was formed, pores 2-mm in diameter were punched in it. The distance between the adjacent pores was 1.1 cm wide. 2 mm^3 of the supernatant and a gradient of standard RuBPCO samples purified from rice leaf were spotted in the pores, respectively. The gel was incubated at 25 °C for 48 h. Then it was stirred gently in 0.9 % NaCl for 30 min, several times washed with distilled water, afterwards the gel was covered with three layers of filter paper, on which 30 layers of tissue paper were laid for another 30 min. The gel was dried with a hair dryer. After the gel was stained for 10 min with the solution containing 0.2 % Coomassie Brilliant Blue R-250, 10 % acetate, and 25 % isopropanol, it was de-stained by the solution consisting of 10 % acetate and 25 % isopropanol. The gel was re-dried. The diameters of the precipitate rings were measured and the content of RuBPCO was calculated based on the standard RuBPCO contents.

Gas exchange was measured with a portable photosynthesis system (*LICOR–6400*, USA). All the photosynthetic measurements were taken at a constant airflow rate of 500 μmol s^{-1}. The concentration of CO_2 was 385±5 cm^3 m^{-3} and the temperature was 28±2 °C. Stomatal conductance to water (g_s), intercellular CO_2 concentration (C_i), transpiration rate (E), and P_{max} were performed at

saturating incident photosynthetic photon flux density (PPFD) of 1 000 μmol m^{-2} s^{-1}. The PPFD response and $P–C_i$ curves with the airflows of 500 and 300 μmol s^{-1}, respectively, were performed by means of the auto-programs given by *LI-6400*. A gradient of PPFD was set as follows: 0, 10, 20, 50, 100, 200, 500, 1 000, 1 500, and 2 000 μmol m^{-2} s^{-1} and the series of reference CO_2 concentrations was 400, 300, 200, 100, 50, 400, 400, 600, 800, and 1 000 μmol mol^{-1}. On the 20th day, the $P–C_i$ curve was employed to analyze the carboxylation efficiency.

Chl fluorescence was measured with an integrating fluorescence fluorometer (*LI-6400–40* leaf chamber fluorometer, *Li–Cor*, USA). After dark-adaptation of samples for 1 h, the minimal fluorescence (F_0) was measured with weak modulated irradiation (<0.1 μmol m^{-2} s^{-1}). A 600-ms saturating flash (>7 000 μmol m^{-2} s^{-1}) was applied to determine the maximum Chl fluorescence yield (F_m) and F_v/F_m. Immediately, the leaf was continuously irradiated with red-blue actinic beams (1 400 μmol m^{-2} s^{-1}) and equilibrated for 30 min to record F_s. Following this, another saturation flash (>6 000 μmol m^{-2} s^{-1}) was applied and then F_m' was determined. After the flash, actinic irradiation was removed, far-red irradation was given, and F_0' was determined. Other fluorescent parameters were calculated as follows (Genty *et al.* 1989): $\Phi_{PS2} = (F_m' – F_s)/F_m'$; $q_P = (F_m' – F_s)/(F_m' – F_o')$; $F_v'/F_m' = (F_m' – F_0')/F_m'$; $q_N = (F_m – F_m')/(F_m – F_o)$. PS2-driven electron transport rate, $ETR = (F_m' – F_s)/F_m' f I\ a_{leaf}$ (Bilger and Björkman 1990), where I is incident PPFD, f is the fraction of absorbed quanta that is used by PS2 (0.5 for C_3 plants), and a_{leaf} is leaf absorbance. Excitation pressure was defined as $1 – q_P$ (Huner *et al.* 1996). The following three derived Chl fluorescence parameters were employed to analyze the allocation of fraction of excitation energy: $D = 1 – F_v'/F_m'$ is the fraction of photon energy absorbed in PS2 antennae and dissipated *via* thermal energy in the antenna; P is the fraction of photon energy absorbed in PS2 antennae utilized for photosynthetic electron transport and is equal to $\Phi_{PS2} = q_P F_v'/F_m'$; thus $Ex = F_v'/F_m' (1 – q_P)$ is the estimate of fraction of excess excitation energy neither dissipated in the PS2 antennae nor utilized for photochemistry (Demmig-Adams *et al.* 1996, Guan *et al.* 2000).

Assay of antioxidant enzymes and soluble protein content measurements: Frozen leaf blade of rice (0.60 g) was homogenized in 12 cm^3 of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The homogenate was centrifuged at 15 000×*g* for 20 min at 4 °C. A small fraction of supernatant was used to measure soluble protein content according to the method of Bradford (1976). All operations were conducted at ice bath. Activity of superoxide dismutase (SOD) was measured by the photochemical method with nitro-blue tetrazolium (NBT) according to Beauchamp and Fridovich (1981). 25 mm^3 of crude enzyme extract was added to 3 cm^3 reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 63 μM NBT, 1.3 μM riboflavin, and irradiated at 25 °C. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the rate of NBT reduction at 560 nm. Catalase (CAT) activity was assayed according to Jiang and Wang (1982). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and crude enzyme extract. CAT activity was measured according to the decomposition of H_2O_2 at 240 nm (E = 39.4 mM cm^{-1}). One unit of CAT was defined as decomposition of 1 μmol H_2O_2 per min. Peroxidase (POD) was measured according to Kochba *et al*. (1977). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 20 mm^3 of guaiacol, 19 mm^3 of 30 % H_2O_2, and 20 mm^3 of enzyme extract. One unit of peroxidase was defined as an increase of 1 per min in optical density. The activities of SOD, POD, and CAT were represented both on a fresh mass and soluble protein bases.

Membrane lipid peroxidation state in the leaves was estimated using malonyldialdehyde (MDA) as indicator. The procedure was slightly modified according to Tang (1999). Frozen leaf blade (0.2 g) was ground into powder with the mortar and pestle in liquid N_2 and extracted with 10 cm^3 of 10 % trichloroacetic acid (TCA). The homogenate was centrifuged at 4 000×*g* for 10 min. 2 cm^3 of supernatant was added to 2 cm^3 of 0.6 % TBA (the solution was solved in 10 % TCA). After keeping in boiling water bath for 20 min and immediate cooling in an ice bath, the mixture was centrifuged at 4 000×*g* for 10 min again. The absorbance of supernatant was measured at 450, 532, and 600 nm, respectively, with a spectrophotometer *UV-1201* (*Shimadzu*, Japan). The formula of MDA determination was represented as $C\ [\mu M] = 6.45 (A_{532} – A_{600}) – 0.56\ A_{450}$, where 450 nm was the absorption peak of soluble saccharides. MDA content was calculated per fresh mass.

Results

Effects of N-deficiency on contents of photosynthetic pigments and proteins: As compared to the control, total Chl content in N-deficient plants decreased by 34 % on the 10th d (Fig. 1*A*). There was a transient increase in Chl *a/b* in N-deficient plants on the 1st d and then the ratio decreased as the N-deficiency lasted (Fig. 1*B*). The Car content was decreased by 36 % in N-stressed plants on the 15th d (Fig. 1*C*). The speed of decline in total Chl was faster in N-stressed plants than in the control ones. Consequently, the ratio of Car/Chl began to rise significantly on the 5th d ($p<0.01$) (Fig. 1*D*). The trend of loss of Chl was more pronounced until the N-deficient leaves

perished. The soluble protein content, which was acutely reduced by N deficiency on the 5th d, exhibited a continuous decline, while it declined only slightly in control plants (Fig. 1*E*). The content of RuBPCO, the predominant protein of chloroplasts, in N-deficient plants was diminished by 70 % on the 15th d (Fig. 1*F*).

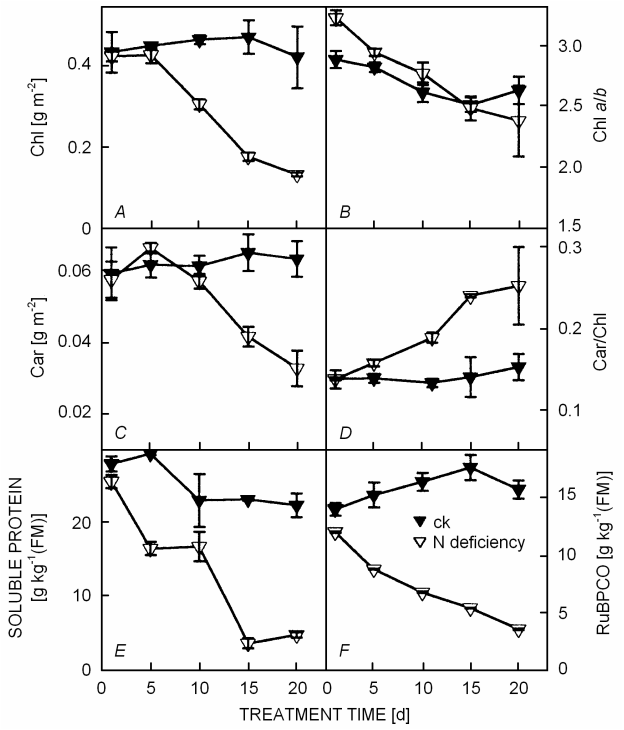

Fig. 1. Effects of N-deficiency on the contents of (*A*) total chlorophyll (Chl), (*E*) soluble protein, and (*F*) ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO), and the ratios of (*B*) Chl *a/b* and (*D*) Car/Chl in the 6th fully expanded leaves of rice seedlings during the 20-d chase period. Means ± SE of at least three replications, ck = control.

Effects of N-deficiency on photosynthetic characteristics: N-deficiency strongly reduced the photosynthetic performance in rice plants. P_{max}, g_s, and E in the N-deficient plants decreased by 32, 42, and 17 %, respectively, on the 5th d in comparison to the control (Fig. 2*A*,*C*,*D*). As N-deficiency progressed, the measured photosynthetic parameters in N-deficient plants declined more dramatically. On the 20th d, they were reduced by 77, 67, and 53 %, respectively. However, C_i in the N-deficient plants rose by 10 % on the average every 5 d (Fig. 2*B*). Analyses of PPFD-response curves demonstrated that the speed of decline of CI (compensation irradiance) was much faster than that in control (Fig. 2*F*). Likewise, there was a rapid decline in the AQY (apparent quantum yield) in N-deficient plants which was on the 20th day 28 % of that in the control (Fig. 2*E*), whereas it remained rather stable in control plants during the 20-chase period. The P–C_i curves illustrated that carboxylation efficiency in N-stressed plants was significantly decreased (Fig. 3*A*). Analyses of P–C_i curves showed that CO_2 compensation concentration in N-deficient plants was also reduced by 25 %. Besides, CI was significantly diminished on the 20th d deduced from the series of PPFD-response curves (Fig. 3*B*).

Effect of N-deficiency on Chl fluorescence parameters under steady state photosynthesis: Fig. 4*A*,*B*,*C*,*D* illustrates that F_0, F_m, F_v/F_m, and F_v'/F_m' were not affected on the 5th d, whereas Φ_{PS2} was reduced by 19 % in N-deficient plants as compared to that in control plants (Fig. 4*F*). Meanwhile, both q_P and ETR decreased significantly ($p<0.05$) (Fig. 4*E*,*H*). There was a correspondingly higher q_N in N-stressed plants than that in control plants (Fig. 4*G*). It corresponded with the finding that the ratio of Car/Chl increased markedly in N-deficient plants on the 5th d (Fig. 1*D*). Besides, PS2 excitation pressure ($1 - q_P$) also increased significantly ($p<0.01$) at the same time and remained rather stable (Fig. 4*I*). Not until the 15th d did F_m as well as F_v/F_m and F_v'/F_m' exhibit significant decrease in N-stressed plants in comparison to those in control (Fig. 4*B*,*C*,*D*). As nitrogen deficiency continued, F_v/F_m, F_v'/F_m', q_P, and ETR in N-deficient plants were lowered more rapidly than in the control (Fig. 4*C*,*D*,*E*,*H*). Not until on the 20th d did F_0 in N-stressed plants decline considerably compared to control plants (Fig. 4*A*).

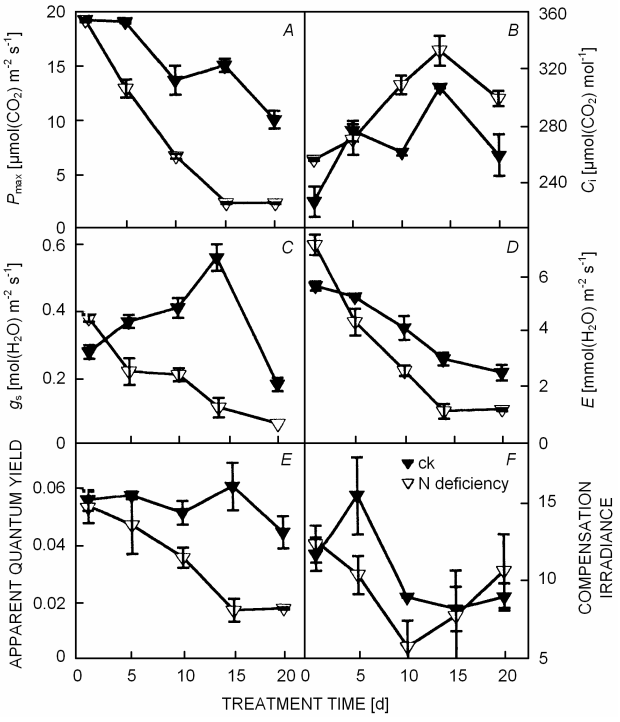

Fig. 2. Effects of N-deficiency on photosynthetic parameters (*A*: maximum photosynthetic rate, P_{max}; *B*: internal CO_2 concentration, C_i; *C*: stomatal conductance, g_s; *D*: transpiration rate, *E*; *E*: apparent quantum yield; *F*: compensation irradiance) in the 6th fully expanded leaves of rice seedlings during the 20-d chase period. Means ± SE of three replications, ck = control.

Effects of N-deficiency on antioxidant enzymes and lipid peroxidation: On a fresh mass basis, the activities of SOD declined in N-deficient plants (Fig. 5*A*), while the activities of POD and CAT initially increased (Fig. 5*C*,*E*). With N deficiency progress, the activities of the three key antioxidant enzymes concertedly fell (Fig. 5*A*,*C*,*E*). However, on a soluble protein basis, the activities of SOD as well as CAT and POD increased much more than in the control (Fig. 5*B*,*D*,*F*). The activities of three antioxidant enzymes reached the peaks on the 15th d and then fell quickly. The lowered capacity to scavenge the free radicals contributed to the over-accumulation of MDA in N-deficient plants (Fig. 5*G*).

Discussion

Crafts-Brandner *et al.* (1996) demonstrated that N-deficiency can trigger and accelerate leaf senescence at the time of full-expanded leaves. Crafts-Brandner *et al.* (1998) revealed that N-deficiency led to declines for proteins located in wheat leaves in the chloroplasts, peroxisomes, and cytosol. Degradation of photosynthetic pigments and proteins consisting of slow and rapid stages is the typical characteristic of leaf senescence (Deng *et al.* 2001). Our results showed that the decline in contents of photosynthetic pigments and soluble proteins (Fig. 1*A*,*C*,*E*) exhibited similar pattern as those reported. We also found that the decrease in P_{max} was accompanied by an increase in C_i, indicating that lower photosynthetic capacity was due to reduced carboxylation efficiency rather than to stomatal limitation. These findings correspond to the reports for sunflower by Ciompi *et al.* (1996). As regards the mechanisms of premature leaf ageing, RuBPCO is the first enzyme degraded in the Calvin cycle during senescence in leaves of wheat plants (Zhang *et al.* 1999). A significant reduction in RuBPCO content in plants on the 1st d of N-deficiency treatment (Fig. 1*F*) suggests that RuBPCO was very sensitive to the onset of leaf senescence. Judging from the P–C_i curve, the activity of RuBPC also might decrease in N-deficient plants (Fig. 3*A*), since there exists a good correlation between the initial slope and activity of RuBPC (Collatz 1977). Therefore, the lessening of RuBPCO content may be the key factor that causes the reduction in photosynthetic capacity and subsequent premature leaf ageing in N-deficient plants.

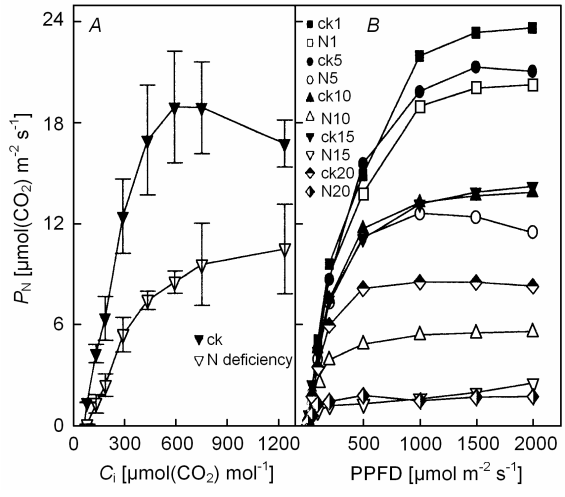

Fig. 3. *A*: Responses of photosynthetic rate to photosynthetic photon flux density (PPFD) under N-deficiency in the 6th expanded leaves of rice seedlings during the 20-chase period. *B*: Responses of P_N in the 6th leaves to C_i under N-deficiency on the 20th d in rice seedling plants. *Error bars* indicate SE (n = 3), ck = control.

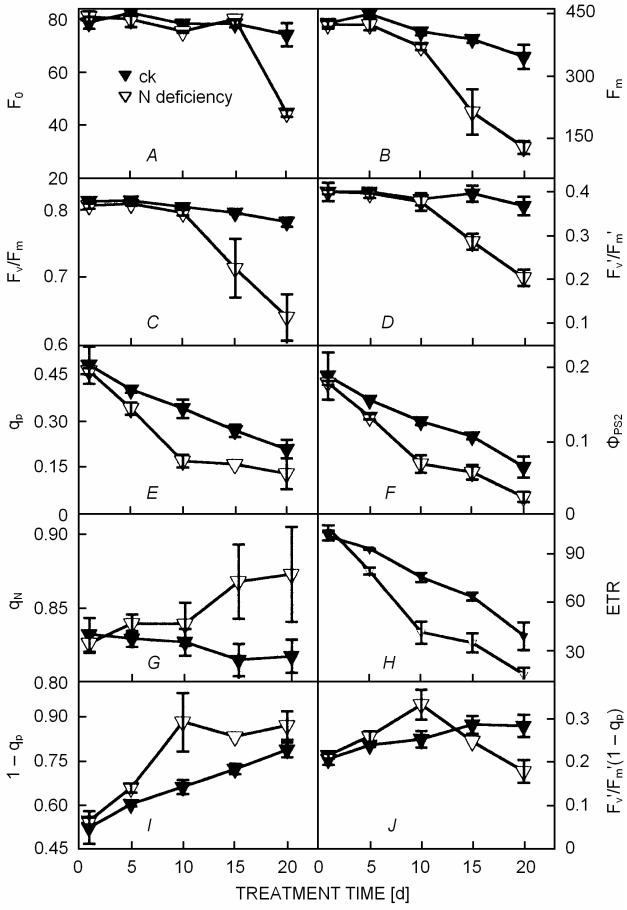

Fig. 4. Responses of chlorophyll fluorescence parameters [*A*: F_0; *B*: F_m; *C*: F_v/F_m; *D*: F_v'/F_m'; *E*: q_P; *F*: Φ_{PS2}; *G*: q_N; *H*: ETR; *I*: $1 - q_P$; *J*: F_v'/F_m' $(1 - q_P)$] to nitrogen deficiency in the 6th fully expanded leaves of rice seedlings during the 20-d chase period. Means ± SE of three replications, ck = control.

Chl fluorescence quenching analysis is frequently used to monitor the responses of photosynthetic apparatus to environmental stress (Krause and Weis 1991, Maxwell and Johnson 2000). With respect to the allocation of

excitation energy absorbed in PS2 antennae, there are three pathways for consumption of excitation energy. First, $D = 1 - F_v'/F_m'$, representing the fraction of excitation energy dissipated in PS2 antennae, was much larger in N-stressed plants than in the control (Fig. 4*D*). Accordingly, N-deficient plants had a higher q_N in N-stressed plants (Fig. 4*G*), which was parallel to the ratio of Car/Chl (Fig. 1*D*). Under high irradiance at midday, senescent leaves of wheat had a high q_N associating with a greater increase in the accumulation of zeaxanthin—one of Cars (Lu *et al.* 2001). A higher q_N was also observed in other plant species exposed to high irradiance and N-deficiency, *e.g.* in wheat (Lu and Zhang 2000, Dong *et al.* 2002), spinach (Verhoeven *et al.* 1997), and apple tree (Cheng 2003). Second, P or $\Phi_{PS2} = q_P F_v'/F_m'$, the fraction of excitation energy utilized for photosynthetic electron transport, was much lower in N-stressed plants than in the control (Fig. 4*F*), which is consistent with the impaired electron transport in N-stressed plants. Third, Ex = $F_v'/F_m' (1 - q_P)$, the fraction of excitation energy neither dissipated in PS2 antennae nor employed for photosynthetic electron transport, rose and reached the peak on the 10th d in N-stressed plants (Fig. 4*J*). The increase in Ex may lead to de-excitation of Chl in the least desirable path, potentially resulting in damaging singlet oxygen formation which could damage thylakoid membrane components (Barber and Andersson 1992). The damage to PS2 reaction centre in N-stressed plants can be detected from the decrease in F_v/F_m (Fig. 4*C*). The exceedingly lower q_P (Fig. 4*E*) in the N-deficient plants suggests that there is a greater fraction of reduction state of Q_A (Genty *et al.* 1989), which would result in a higher excitation pressure and a down-regulation of PS2 reaction centre when N-deficient plants are exposed to high irradiance. Similarly, the excitation pressure also culminated on the 10th d in N-deficiency plants, in accordance with the peak of Ex on the same day. Interestingly, not until 20th day did F_0 decline significantly in N-deficient plants as compared to that in the control (Fig. 4*A*). Studies have demonstrated that F_0 is one of the criterions for an estimate of the number of antenna pigments of the PS2 reaction centre. On the one hand, N-deficiency causes the loss of photosynthetic pigments (Fig. 1*A*,*C*), which in turn results in the fall of F_0. On the other hand, N-deficiency and high irradiance lead to damage to PS2 reaction centres, which can contribute to the rise of F_0 (Schnettger *et al.* 1994). Therefore, the delayed decline in F_0 may be the trade-off between the two interaction factors. So the decline in F_m plays a key role in contributing to the decrease in F_v/F_m in N-deficient plants. The decrease in F_v/F_m was reported as being accompanied by an enhanced production of superoxide (Jin and Tao 2000). Lin *et al.* (1999) reported photon energy conversion and metabolism of active oxygen were closely related to premature leaf ageing. Our present results show that during N-deficiency, the efficiency of excitation capture by open PS2 centres and the energy used for photochemical capacity (P_{max}) gradually declined in leaves of N-stressed plants; which led to the accumulation of Ex and probably subsequent overproduction of reactive oxygen species (ROS). However, the activity of a scavenger system, such as the activities of antioxidant enzymes (SOD, POD, CAT), per fresh mass decreased as the N-deficiency lasted, which in turn aggravated the peroxidation of membranes and downstream degradation of large biomolecules, such as RuBPCO and Chl. These results coincide with the report by Li and Mei (1989).

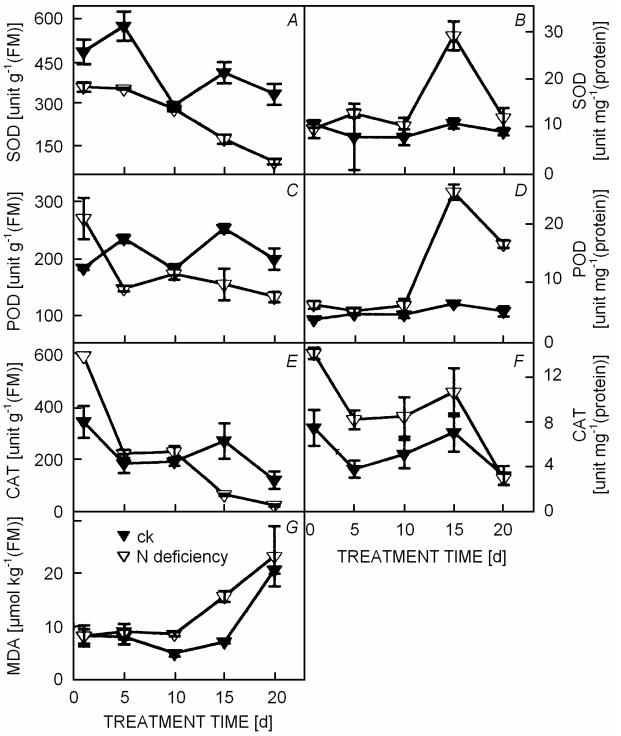

Fig. 5. Responses of antioxidant enzymes (*A, B*: superoxide dismutase, SOD; *C, D*: peroxidase, POD; *E, F*: catalase, CAT; *G*: malonyldialdehyde, MDA) presented both on the soluble protein and fresh mass bases and of membrane lipid peroxidation to N-deficiency in the 6th fully expanded leaves during the 20-d chase period. Means ± SE of three replications, ck = control.

Leaf senescence is a complex and highly regulated process, developmental phase in the life that results in the co-coordinated degradation of macromolecules and the subsequent mobilization of components to other parts of the plant (Buchanan-Wollaston 1997). The striking feature of leaf senescence is the breakdown of Chl and proteins. However, so far it is still an enigma what mediates the onset of leaf senescence. Excitation pressure reflects the redox state of PS2 and the balance between energy supply and consumption, which can be mediated by the redox state of electron transport components (Huner *et al.* 1996, 1998). It manifests that PS2 excitation pressure affects gene expression, such as *Rbc* gene family encoding RuBPCO in *Arabidopsis thaliana* (Robin and Horton 1994). Several excellent reviews show how the redox

state of plastid (chloroplast) controls both the plastid and nuclear gene expression (Rodermel 2001, Pfannschmidt 2003). Escoubas *et al.* (1995) reported that regulation of *cab* gene transcription by irradiation is signalled in a green alga by the redox state of plastoquinone pool, which can be represented as $1 - q_P$. Pfannschmidt *et al.* (1999) showed that the redox state of plastoquinone controls the rate of transcription of genes encoding reaction centre apoproteins of PS1 and PS2. Ou *et al.* (2003) proposed that PS2 excitation pressure mediated natural flag leaf senescence. Our results confirm a significant inverse linear correlation between the increased excitation pressure ($1 - q_P$) and the degradation of RuBPCO (r^{2**} = 0.9933, n = 5), which is consistent with the finding that both RuBPCO small and large units were down-regulated during natural flag leaf senescence (Ou *et al.* 2003). More importantly, our results revealed that both the soluble protein and RuBPCO contents were significantly reduced on the 5th d of N-deficiency or even earlier, and the declination was aggravated as N-stress lasted (Fig. 1*F*). However, the excitation pressure was not affected on the 1st d and slightly increased on the 5th d (Fig. 4*I*). So there was a lag phase in the increase of the PS2 excitation pressure compared to the decrease of RuBPCO content in chloroplast.

Our previous studies demonstrate a significant correlation between RuBPCO initial activity and net photosynthetic rate (P_N). RuBPCO content was also significantly correlated with total RuBPCO activity during leaf senescence in rice plants (Jiang and Xu 1995). In addition, N-deficiency greatly reduced not only the content of RuBPCO, but also P_{max}, RuBPCO carboxylation efficiency, and greatly P_N (Figs. 2*A* and 3*A*,*B*). Hence, the RuBPCO activity was impaired in N-deficiency plants, rendering the Calvin cycle blocked, which in turn contributed to over-accumulation of the assimilatory power in the chloroplasts. The over-production of ATP and NADPH can inversely render the saturated photosynthetic ETC and the over-reduced electron acceptor of Q_A, which leads to the increase in excitation pressure in PS2 centres. Therefore, the increased excitation pressure might be the result of a saturation of the electron transport chain due to the limitation of the use of reductants by the Calvin cycle under N-deficiency conditions. This proposal is contrary to the report that excitation pressure mediates the onset of leaf senescence of flag leaf senescence. Not until the 10th d the excitation pressure rose markedly in N-deficient plants, which is consistent with the sharp decline of Chl content on the same day. The excitation pressure led to the accumulation of excess excitation energy and subsequent overproduction of ROS, which may be partially responsible for the degradation of macromolecules, such as Chl and proteins. The coincidence indicates that the increased excitation pressure in turn accelerates the degradation of Chl. The excitation pressure is linearly correlated with the degradation of Chl (r^{2*} = 0.9056, n = 5) and soluble protein content (r^{2*} = 0.8815, n = 5). Subsequently, the increased excitation pressure may influence the down-stream events after Calvin cycle is inhibited during leaf senescence of rice plant.

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