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Oxidative Stress in Rice (*Oryza sativa* L.) Seedlings Induced by Flooding

Keun Pyo Lee and Jin Jung*

School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea

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Plant stress incurred by flooding was studied in terms of oxidative stress, using greened rice seedlings subjected to a complete submergence followed by re-exposure to air under illumination (30 W/m^2). It appeared that shoot tissues of the seedlings suffered oxygen deficiency during the flooding treatment, pertinent to the general concept. Interestingly enough, however, membrane peroxidation in shoots was enhanced by the submergence, as assessed by the content of 2-thiobarbituric acid-reactive substances (TBARS), and the re-aeration resulted in a rapid reduction of TBARS content. Such pattern of response was also seen in the change in the steady state level of H_2O_2. In contrast, superoxide dismutase and glutathione reductase that are involved in the detoxifying processes of superoxide in plant cells were significantly activated only during the re-aeration. These results allowed us to suggest the followings as a working hypothesis. Photorespiration-linked production of H_2O_2 may largely contribute to the increase in H_2O_2 level as well as TBARS production in shoots during the submergence. An abrupt re-supply of CO_2 by the re-aeration brings the photosynthetic apparatus back to full operation, suppressing photorespiration and probably causing a momentary, excess formation of superoxide and its dismutation product through side reaction, which gives rise to activating substrate-inducible antioxidative enzymes.

Key words: Oryza sativa, *flooding, lipid peroxidation, oxidative stress.*

Flooding is a common abiotic stressor in many vascular plants, imposing a hypoxic condition to them. One of the major biological consequences of flooding in plant cells is a decrease in the ATP production by respiration-linked phosphorylation due to the deficiency of oxygen available to mitochondria.[1,2] To compensate the reduced level of cellular energy production under limiting oxygen concentrations plant cells would mobilize substrate-level phosphorylation linked to glycolysis and hence put alcohol fermentation into full operation that regenerates NAD^+ required for continuing glycolysis. Since ATP production in glycolysis is far less efficient compared with oxidative phosphorylation, however, prolonged oxygen deficiency could lead to cellular dysfunctions.

Some plants have also been shown to suffer injury caused by a sudden exposure to air after a period of flooding, and this post-hypoxic injury has been implicated to be oxidative stress.[3] Flooding-induced oxidative stress has often been studied with etiolated seedlings germinated under water in the dark.[4,5] Metabolic feature of green tissues in the vegetative stage is different from that of etiolated tissues. The responses of photosynthetically active tissues to flooding as well as to the post-flooding re-aeration may therefore be distinguished from those of the photosynthetically inactive tissues.

Plant species differ considerably in their susceptibility to various environmental stresses. Paddy rice (*Oryza sativa* L.) is classified into drought-susceptible and flooding-tolerant species,[6,7] growing in soils that are usually covered with water. The objective of the present study was to determine whether complete submergence of rice plants causes oxidative damage to green tissues in light and whether the damage occurs during flooding, i.e. under hypoxic conditions, or during the re-aeration. Physiological implications of our results are to be discussed.

Materials and Methods

Plant materials and flooding treatment. Rice (*Oryza sativa* L., cv. Whaseong) seedlings were hydroponically cultivated in a growth chamber at relative humidity of 65% and at 261 under continuous illumination at 30 W/m^2 for 8 days. The growth medium (Hoagland solution) was changed once a day. On the 8th day of growth, rice seedlings were completely submerged in Hoagland solution and kept for up to 2 days. Then the seedlings were transferred to ambient air. The shoots were harvested at fixed time intervals during the submergence and the re-aeration, immediately frozen in liquid nitrogen, and stored at -70 until used for various biochemical assays.

Enzyme assays. Homogenates of rice shoots (1 g) in 10 m*l* of suitable buffer were centrifuged at 20,000 g for 10 min, and the supernatants were immediately used for the measurements of enzyme activities. Pyruvate decarboxylase (PDC) was assayed essentially according to Rivoal *et al.*[8] with minor modification. Alcohol dehydrogenase (ADH) activity was determined using native polyacrylamide gel electrophoresis as in Xie and Wu.[9] Superoxide dismutase (SOD) was assayed by measuring the rate of reduction of cytochrome c by O_2^-, which

*Corresponding author
Phone: 82-31-290-2406; Fax: 82-31-293-8608
E-mail: jinjung@snu.ac.kr

was supplied by the xanthine-xanthine oxidase system, as described by McCord and Fridovich.[10] Glutathione reductase (GR) was assayed by following GSSG-dependent oxidation of NADPH, as in Schaedle and Bassham.[11] Protein was quanitified by the Lowry method.[12]

Measurement of lipid peroxidation. Lipid peroxidation was estimated by measuring the content of 2-thiobarbituric acid-reactive substances (TBARS) in shoot homogenates prepared in 10% trichloroacetic acid containing 0.25% 2-thiobarbituric acid (TBA). The homogenates were heated at 95°C for 30 min, and the resulting pigment was spectrophotometrically quantified as in Heath and Packer.[13]

Hydrogen peroxide analysis. H_2O_2 in the shoot extracts was quantified following the procedures described by Warm and Laties[14] with a slight modification. Frozen rice shoots (1g) were ground in 10 m*l* of 5% TCA with 0.1 g of charcoal, and cheese cloth-filtered crude extracts were centrifuged at 20,000 g for 15 min. Supernatants were diluted with 0.2 M NH_4OH (pH 9.5), 5 μ*l* of which were transferred into test tubes and placed in the measuring cell of luminometer. Then chemiluminescence was initiated by injecting 100 μ*l* of 0.25 mM luminol and 100 μ*l* of 0.5 mM $K_3Fe(CN)_6$ into the samples. The emitted photons counted over 5 s were summed up.

Results and Discussion

Complete submergence has been shown to cause oxygen deficiency in etiolated plants that lack photosynthetic function.[1,6] It may be presumed that the severity of flooding-induced oxygen deficiency is relatively low in photosynthetically active cells under illumination because chloroplasts photogenerate O_2 through water-splitting reaction that can diffuse out of its generation site into various cellular compartments. However, assay results of enzymes involved in alcohol fermentation did not conform to such conjecture. As can be noted from Fig. 1, both ADH and PDC showed marked increase in their activities during flooding treatment of greened rice seedlings in light, the response of the former being far more drastic. This may be taken as an implication that even under intrinsically oxygen-evolving conditions the oxygen concentrations in photosynthetic cells are not high enough for mitochondria to properly function, promoting respiration-linked ATP synthesis, unless exogenous oxygen is supplied. Therefore the cells in green tissues in completely submerged rice plants are also thought to rely largely on glycolytic substrate-level phosphorylation for their energy needs during the submergence, activating alcohol fermentation as a physiological consequence.

Oxidative nature of a certain stress has frequently been studied by examining changes in activities of the antioxidant defence enzymes.[15,16] Of toxic oxygen species-processing enzymes in plants, in this investigation we focused on SOD that promotes detoxifying reaction of superoxide radical, one of the most common active oxygen formed in stressed organisms under aerobic conditions, and GR that takes part in the

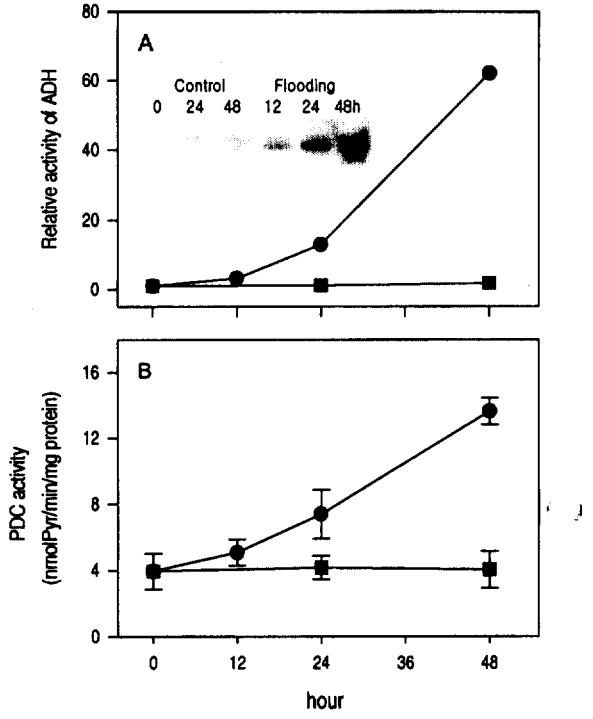

Fig. 1. Induction of ADH (A) and PDC (B) during flooding treatment of rice seedlings. ■, untreated control; ●, flooding treatment. Inset shows the native polyacrylamide gel electrophoresis of rice crude extract. Relative intensity of bands in scanned gel was calculated using image analysis program "Gel-Pro Analyzer" (Media Cybernetics Co.). The data were expressed as means ± SD (n = 4).

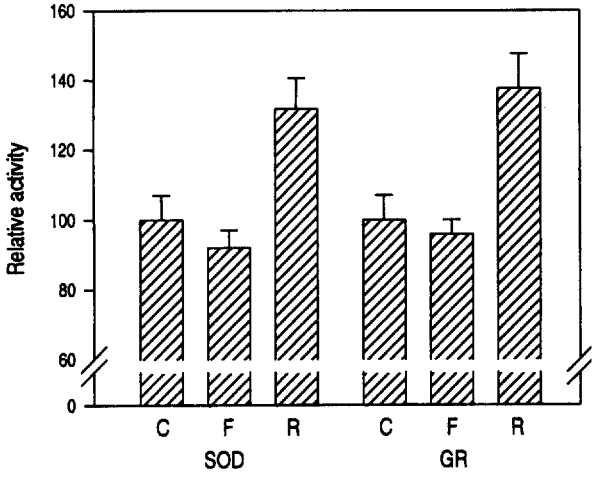

Fig. 2. Responses of antioxidative enzymes during flooding and re-aeration of rice seedlings. C, untreated control; F, flooding treatment; R, re-aeration after flooding treatment. The results were expressed as means ± SD (n = 4). The activities of enzymes in the shoot extracts of the untreated controls were: SOD, 44.5 ± 3.4 and GR, 0.072 ± 0.014 in units per mg protein.

ascorbate-glutathine cycle whose main function is to eliminate H_2O_2 in chloroplasts. It turned out that both enzymes responded only slightly, if any, to flooding treatment but did significantly to re-exposure to air after flooding, as shown in Fig. 2. The substantial activation of SOD and GR during the

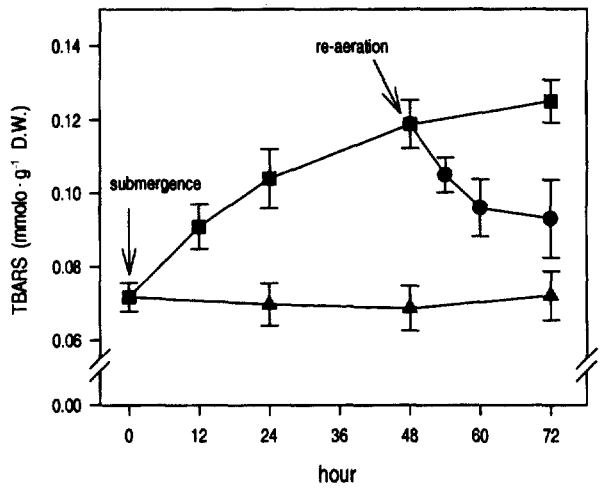

Fig. 3. Changes in the levels of TBARS in rice shoots during flooding and re-aeration. ▲, untreated control; ■, flooding; ●, re-aeration. The levels were expressed as means ± SD (n = 3).

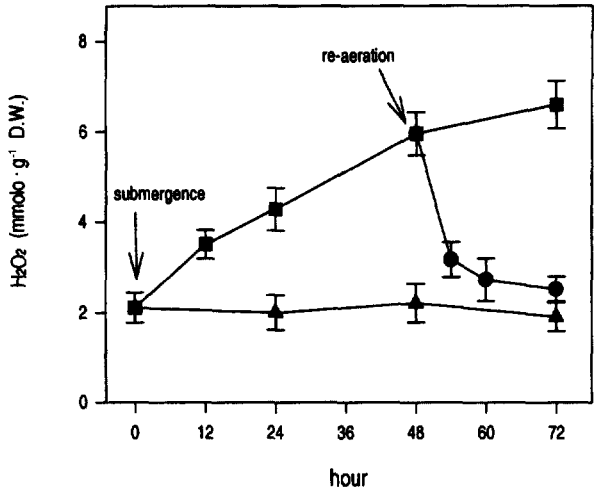

Fig. 4. Changes in the levels of H_2O_2 in rice shoots during flooding and re-aeration. ▲, untreated control; ■, flooding; ●, re-aeration. The levels were expressed as means ± SD (n = 3).

re-aeration period may suggest that oxygen reduction to superoxide and H_2O_2 proceeded somehow at an increased rate in plant cells when the cells that had been adjusted to a hypoxic condition were charged at a sudden with a high concentration of atmospheric oxygen.

It may be intuitive from the above results that reactive oxygen-mediated oxidative stress at cellular and molecular levels should occur during the re-aeration but not during the submergence. In order to ascertain this, we measured time-sequentially TBARS content of shoot tissues during the submergence and the re-aeration periods, for the TBARS level is generally taken as an index of lipid peroxidation of tissues, which is, in turn, regarded as a reliable parameter of oxidative stress in diverse organisms. Surprisingly, shoot tissues of rice plants suffered increased lipid peroxidation when they were placed under submergence, i.e. hypoxic conditions, whereas the re-aeration suppressed it to a significant extent, as shown in Fig. 3. Does this indicate that the formation of a certain activated oxygen species was enhanced by the submergence? To answer this question we monitored the steady state level of H_2O_2 over the whole period of treatment, because this long-lived toxic oxygen is capable of inducing lipid peroxidation and can be produced not only through normal cellular metabolism but also through dismutation of superoxide. As it turned out (Fig. 4), the pattern of change in H_2O_2 content of rice shoot tissues was similar to that in the TBARS content, suggesting possible association of the increased H_2O_2 production with the enhanced lipid peroxidation in completely submerged rice plants.

Then, the antioxidative emzymic response (Fig. 2) seems contradictory to the data related to oxidative chemical response during the treatment (Figs. 3 and 4). An explanation of this intriguing feature may be derived from the assumption that there are separate cellular compartments involved in the respective responses. Under submergence, the uptake by plant leaves of CO_2 and O_2 should be limited, and thus carbon fixation by chloroplasts and mitochondrial respiration would naturally be limited. Since the light phase reaction of photosynthesis can proceed to some extent, producing O_2 via water splitting, even under low CO_2 conditions, however, O_2 in chloroplasts could be fairly enough to initiate photorespiration, in which H_2O_2 is formed by glycolic acid oxidase activity in peroxisomes. This H_2O_2 production may be corresponding to what has been observed in rice seedlings during the submergence (Fig. 4). When the submerged shoot tissues are re-exposed to air, i.e. O_2 and CO_2 at physiologically functional levels, the electron transfer reactions of photosynthesis and mitochodrial respiration take place in leaf cells at abruptly increased rates, which inevitably gives rise to formation of superoxide as a side reaction and its dismutation product, H_2O_2. These activated oxygen species may then be efficiently eliminated *in situ* by the increased activities of antioxidative enzymes, such as SOD and GR, before they diffuse out and attack various cellular components including membrane lipid. It may be worth mentioning that SOD is a substrate-inducible enzyme[17] and also that GR activity of plant tissues becomes larger responding to oxidative stress as an antioxidant defense in photosynthetic cells.[16,18]

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