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Antioxidative Activities in Rice Leaves Exposed to Ozone

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ABSTRACT: Rice (*Oryza sativa* L.) plants were cultivated to examine changes in antioxidative defence mechanism induced by elevated ozone levels. Catalase activities in tolerant Jinpumbyeo and susceptible Chucheongbyeo under ozone fumigation were reduced at 5 hrs and 3 hrs after ozone fumigation, respectively. With the increased ozone supply, peroxidase activity in Jinpumbyeo was steadily enhanced whereas in Chucheongbyeo it was not changed. Four SOD-isozymes were detected by NBT staining of native-PAGE. Two isozymes of them were obviously induced by ozone supply, particularly in Jinpumbyeo. The continuous ozone fumigation increased remarkably putrescine levels in leaves whereas it did not affect the levels of spermidine and spermine. In this study, it was implied that ozone in cell inhibits strongly diamine oxidase and thus promotes ethylene biosynthesis which will cause the senescence in rice plants.

Keywords: rice, superoxide dismutase, catalase, peroxidase, polyamine

Ozone (O_3) is considered as a main phytotoxic air pollutant in industrialized countries causing more critical damage to crops and forests than the others known air pollutants. (Bowler et al., 1992). The phytotoxicity of O_3 is due to its high oxidative capacity through the induction of active oxygen species (AOS) on plant tissue, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen (O^-) (Malhotra & Khan, 1984). Plants have evolved protective scavenging systems in response to these AOS. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), as well as the enzymes of the ascorbate-glutathione cycle (Halliwell-Asada pathway) ascorbate peroxidase (APX), glutathione reductase (GR), monodehydro-ascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) provide endogenous defense against the accumulation of harmful AOS. However, they also have repeatedly been shown to be affected by O_3 (Lee *et al.*, 1984; Castillo & Greppin, 1988; Bender *et al.*, 1994; Brunschon-Harti *et al.*, 1995)

Polyamines (PAs), spermidine (Spd), spermine (Spm) and their diamine obligate precursor putrescine (Put), are small aliphatic amines that are ubiquitous in all plant cells. Evidences gathered in recent years supports their role as the regulators of cell proliferation and differentiation, which is similar to the results which have been proposed for animal and bacterial cells (Heby, 1981; Marton & Morris, 1987). PAs are basic molecules which are positively charged at physiological pH (Takeda *et al.*, 1983). They have been shown to bind strongly *in vitro* to negatively charged nucleic acids (Feurstein & Marton, 1989), acidic phospholipids (Tadolini *et al.*, 1985) and many types of proteins, including numerous enzymes in which the activities are directly modulated by polyamine binding (Carley *et al.*, 1983). These ionic interactions are important in regulating the structure and function of biological macromolecules, as well as their synthesis *in vivo* (Jacob & Stetler, 1989). Therefore, the main objective of this study was to examine antioxidants activities, such as SOD, POD, CAT and polyamines, affected by the elevated ozone levels in rice leaves.

MATERIALS AND METHODS

Plant growth and ozone treatment

Rice plants, cvs. Jinpumbyeo and Chucheongbyeo, were grown in an controlled environment chamber at day/night temperatures of 25/20°C, 70% relative humidity and 300~400 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR; 400-700 nm) in a 12 hrs-photoperiod. Rice plants were also grown under different nitrogen levels, i.e. standard nitrogen (11kg $10a^{-1}$, N1) and 2 fold nitrogen level (22 kg $10a^{-1}$, N2). P_2O_5 asd K_2O were applied with 7 kg $10a^{-1}$ and 8 kg $10a^{-1}$ as basal fertilizer, respectively. At panicle formation stage, two rice plants were exposed to ozone fumigation.

Ozone generated by ozone generator, which concentration was adjusted into 100±10 ppb was supplied for 0, 1, 3, 5, 8

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and 12 hrs in photo-periods. Each sample taken after ozone treatments was immediately harvested and frozen in liquid N_2 and stored at -70°C for further analysis.

Assays of antioxidative enzymes

Fresh leaf samples of both rice plants obtained at 0, 1, 3, 5, 8, and 12 hrs after ozone fumigation were used for enzyme analysis. For determination of antioxidant enzyme activities, leaves (1g) were homogenized in 100 mM sodium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, and 1% (w/v) PVP at 4°C. Soluble proteins were assayed by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Non-denaturing PAGE of the crude protein extracts was carried out on 10% polyacrylamide gels using a Bio-Rad electrophoresis system according to the manufactures instruction. Protein solutions (40 μg) were loaded onto the gel and separated for 3-4 hrs at 4°C, and 100 V. Immediately after electrophoresis, the activity of SOD isoenzymes was visualized using the nitroblue tetrazoliumchloride (NBT) staining procedure (Beauchamp & Fridovich, 1971). The gel was incubated in 100 μM NBT for 20 min at 25°C and then soaked for 30 min in the dark in 50 mM sodium phosphate buffer at pH 7.8 containing 3 mM riboflavin and 1 mM EDTA-Na. The gel was then illuminated to promote the photoreactive staining by SOD. The stained gels were finally photographed with a digital camera. In order to identify the nature of SOD enzymes with activity stain, gels were incubated for 30 min in 50 mM sodium phosphate buffer at pH 7.8 containing 2 mM KCN or 3 mM H_2O_2. Cu/Zn-SODs are inhibited by KCN and H_2O_2; Fe-SODs are inactivated by H_2O_2 but resistant to KCN and Mn-SODs are resistant to both inhibitors (Fridovich, 1986).

Catalase (E.C. 1.11.1.6) activity was determined by monitoring the decomposition of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm following the method of Aebi(1974). The reaction mixture contained 50mM potassium phosphate buffer (pH 7.0) and a proper amount of plant extract in a 3 mL. The reaction was initiated by adding 10 mM H_2O_2.

Peroxidase (E.C. 1.11.1.7) activity was determined by monitoring the formation of guaiacol dehydrogenation product (extinction coefficient 6.39 mM cm^{-1}) at 436 nm followed by the method of Pütter (1974). Three mL of reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 0.3 mM guaiacol and plant extract. The reaction was initiated by adding 0.1 mM H_2O_2.

Polyamine analysis

Fresh leaf tissues were ground with mortar and pestle in liquid nitrogen, and extracted with 5% cold perchrolic acid (PCA, 1 : 3, w/v). The homogenates were kept for 1 hr at 4°C, and then centrifuged at 10,000 g for 25 min. The supernatants were used for soluble polyamine determination. Benzoylation was performed with slightly modification of the method by Redmond & Tseng (1979). An aliquot of the supernatant (500 m*l*) and internal standard (1,3-diaminopropane, 100 m*l*) was mixed with 1 ml of 2N NaOH and 20 m*l* of benzoyl chloride. The mixture was incubated at room temperature for 20 min and the reaction was stopped by adding 2 ml of saturated NaCl. Benzoyl-polyamine was extracted into 2 ml of cold diethyl ether by vortexing for 30 sec. The diethyl ether phase was then evaporated to dryness, and the derivatized polyamine was redissolved in 64% MeOH(v/v). The benzoyl derivatives were separated on a 5 μm-2.0×50 mm (C_{18}) reverse phase column, using elution gradient from 40% to 60% acetonitrile in the mobile phase. Benzoyl-PAs was monitored by an UV detector (254 nm). LC-MS conditions were optimized to produce positive molecular ions via electrospray ionization. Other MS setting were capillary temperature (300°C), capillary voltage (16 eV), sheath gas flow (50 ml min^{-1}) and spray voltage (5 eV). Samples were analyzed with the mass spectrometer in full scan mode (50-800 amu). Analysis of LC/MS data was processed using with Xcalibur™ 1.3 software. The peaks were identified with reference to the retention times of PAs standard (Sigma, USA) prepared as described above. Quantitative determination was based on external standards.

RESULTS AND DISCUSSION

Antioxidative enzyme activity

The catalase activity in leaves of ozone-tolerant and ozone-susceptible rice cultivars was shown at Fig. 1. In ozone-tolerant Jinpumbyeo, the catalase activities in N1 and N2 were momently reduced at 1 hour and 3 hrs after ozone exposure, respectively. The activities were, however, rapidly recovered. Ozone supply for over 5 hrs caused catalase inactivation. Namely, the catalase activities in N1 and N2, at 8 hrs of ozone exposure, were severely inhibited to 41% and 54%, respectively. Regardless of the levels of nitrogen application, the catalase activities in leaves of ozone-susceptible Chuchenogbyeo were also similarly declined within 1 hr after ozone exposure. Thereafter, the activities were immediately elevated and inhibited at 3 hrs after ozone fumigation. No obvious difference in catalase activities appeared between nitrogen levels and cultivars. It is well-known that catalase eliminates H_2O_2 by breaking it down directly to water and oxygen (Winston, 1990). It was thought that prolonged fumigation reduced CAT activity for

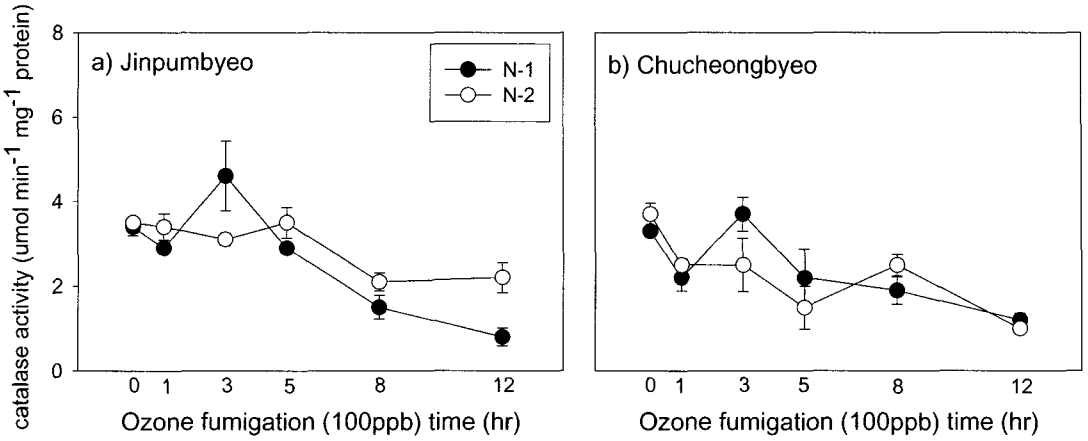

Fig. 1. Catalase activities in rice leaves with an increase (hr) of ozone fumigation time at panicle formation stage. The bars represent S.D. (n=3).

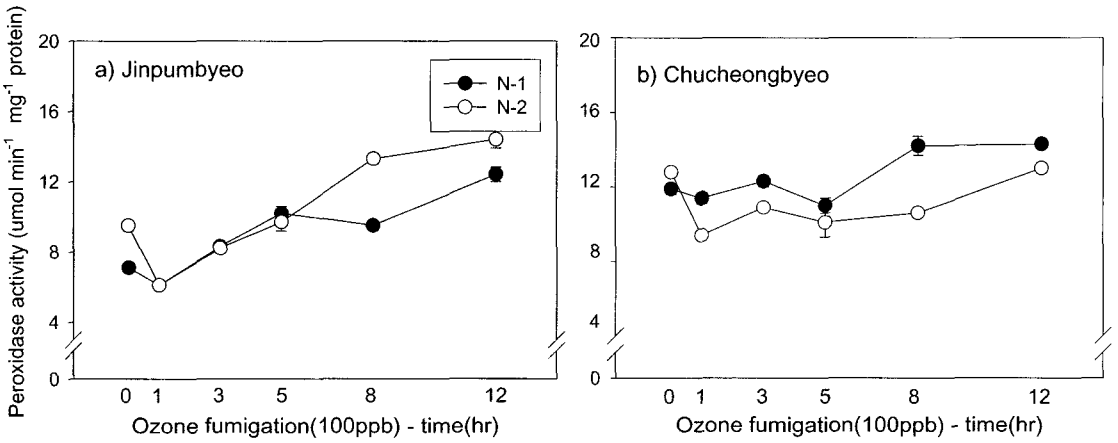

Fig. 2. Guaiacol-peroxidase activities in rice leaves with an increase of ozone fumigation time period at panicle formation stage. The bars represent S.D. (n=3).

both cultivars. The decreases in CAT activity would result in the accumulation of H_2O_2 which can react with O_2^- to produce hydroxyl radicals via the Herbert-Weiss reaction (Elstner, 1982; Bowler *et al.*, 1992). Declines in CAT activity in response to prolonged drought have been reported for other species (Dwivedi *et al.*, 1979). Thus, we would favor to conclude that CAT induction occurs commonly and is not related with susceptibility.

As shown in Fig. 2, peroxidase activities in both rice cultivars were significantly different and the activities were reduced in N1 (Jinpumbyeo) and N2 (Chucheongbyeo). In ozone-tolerant Jinpumbyeo, peroxidase activities in N1 and N2 were instantly reduced within 1 hr after ozone exposure, and then its activity was linearly increased in proportion to the increase of ozone fumigation. Peroxidase activities at 12 hrs after ozone fumigation in N1 and N2 were highly enhanced to 1.7- and 1.5-fold, respectively. Peroxidase activity in ozone-susceptible Chucheongbyeo seemed to be different compared to Jinpumbyeo, and the activity, also, was obviously affected by nitrogen levels. In N1 level, peroxidase activity was not changed up to 5 hrs after ozone fumigation and then strongly increased. In N2 level, the activity was, however, momently reduced by ozone exposure. Thereafter, the activity was slightly activated and then strongly elevated after 8 hrs after O_3 fumigation. It has been clearly demonstrated that PODs usually occur as multiple molecular forms (isozymes) and have a number of potential roles in plant growth, development, and differentiation (Gaspar *et al.*, 1991). PODs require H_2O_2 as an essential substrate and, therefore, POD metabolizes H_2O_2 to water. Anionic PODs are believed to utilize phenolic compounds such as coniferyl alcohol and H_2O_2 to initiate the chain reaction that leads to lignification (Otter & Polle, 1994). In this study, lignified spots near to stomata (data not shown) seemed to be a direct evidence of this result.

To compare the changes in SOD isozyme patterns in ozone-tolerant and ozone-susceptible cultivars, we analyzed SOD isozymes on native polyacrylamide gels. As shown in Fig. 3, SOD isozymes were identified as Cu/Zn-SOD whereas Fe-SOD and Mn-SOD were not observed in all treatments.

Although four Cu/Zn-SOD isozymes in the ozone-tolerant and ozone-susceptible cultivars during ozone exposure were detected, the Cu/Zn-SOD activity of each isozyme *per se* appeared to be differed according to cultivars and nitrogen levels.

SODs catalyze the dismutation reaction of superoxide anion (O_2^-) into H_2O_2 and O_2, and can be distinguished into three classes according to their metal co-factor: Cu/Zn-, Mn- or Fe-SOD (Bowler *et al*, 1994; Van Camp *et al.*, 1994). Despite the fact that these SODs can easily be differentiated on the basis of mRNA as well as activity levels with staining technique on gel, to date only a few studies have been conducted to study the expression of different SODs individually under abiotic stress (Perl-Treves & Galun, 1991; Willekens *et al.*, 1994). Drought stress has been shown to preferentially induce cytosolic Cu/Zn-SOD (Perl-Treves & Galun, 1991), and both UV-B and O_3 have been reported to have similar effects on chloroplastic and cytosolic Cu/Zn-

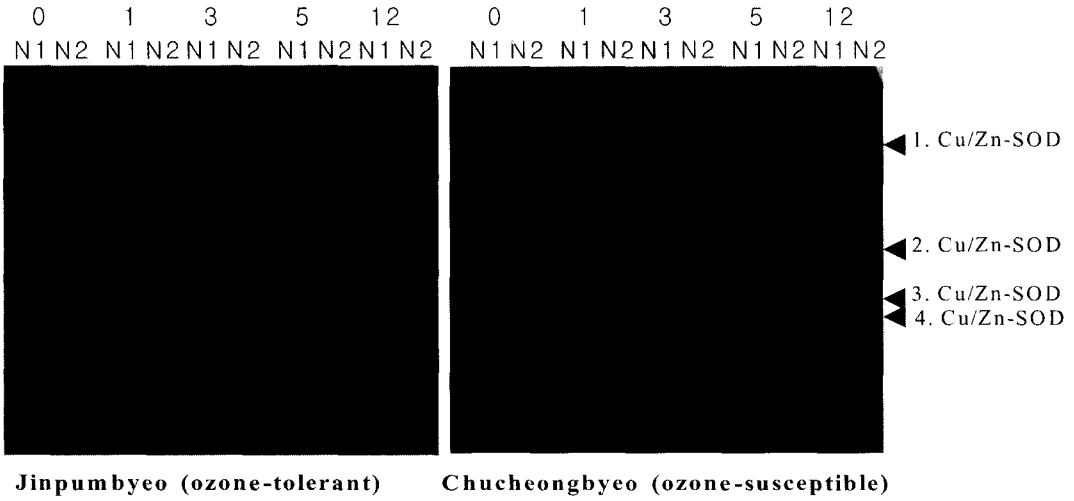

Fig. 3. Native gels stained for the activity of Cu/Zn-SOD from rice plants under ozone fumigation. Left (Jinpumbyeo), Right (Chucheongbyeo). Lane 1, N1-0h; Lane 2, N2-0h; Lane 3, N1-1h; Lane 4, N2-1h; Lane 5, N1-3h; Lane 6, N2-3h; Lane 7, N1-5h; Lane 8, N2-5h; Lane 9, N1-12h; Lane 10, N2-12h.

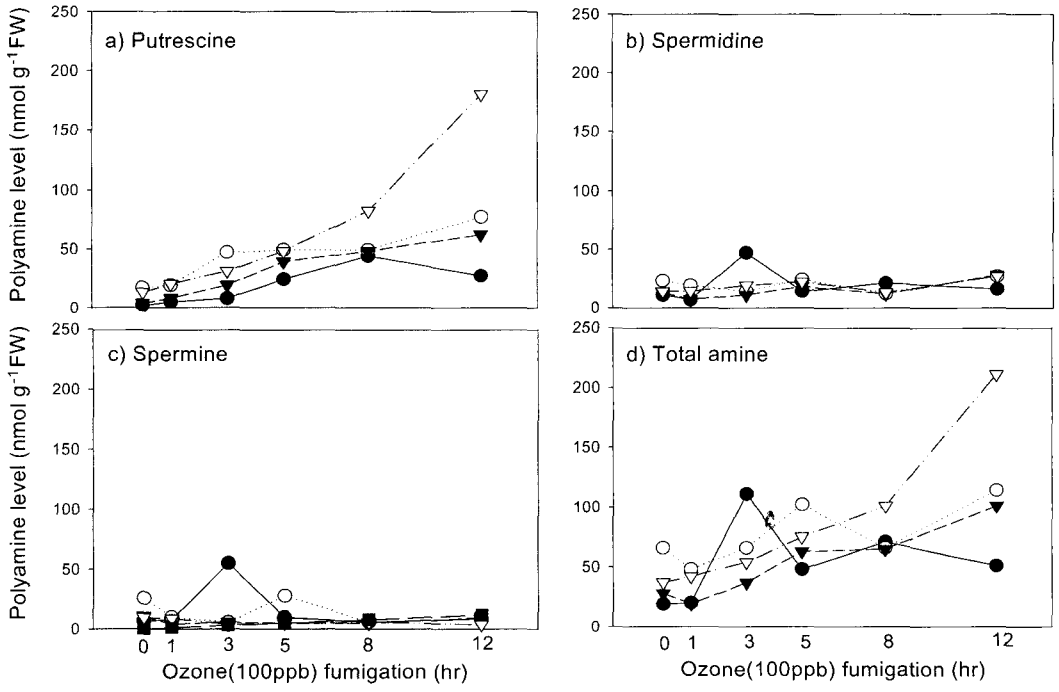

Fig. 4. Ozone effect on polyamine levels in two rice plants with different N level. Each symbol indicates Jinpumbyeo-N-1(●), Jinpumbyeo-N-2(○), Chucheongbyeo-N-1(▼) and Chucheongbyeo-N-2(▽), respectively.

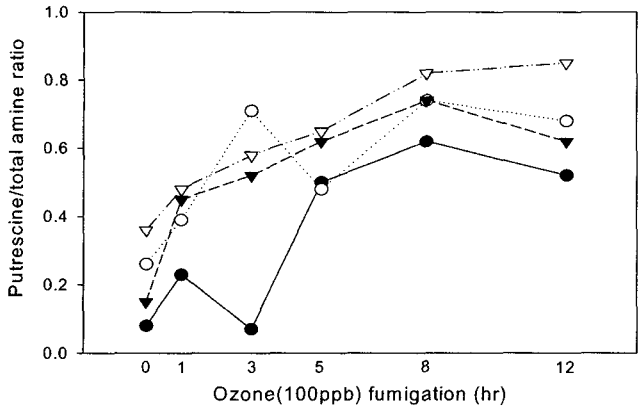

Fig. 5. Changes in the ratio of putrescine (diamine) to total amine with an increase of ozone fumigation period. Each symbol indicates Jinpumbyeo-N-1(●), Jinpumbyeo-N-2 (○), Chucheongbyeo-N-1(▼) and Chucheongbyeo-N-2(▽), respectively.

SOD (Willekens *et al.*, 1994). In our results, two isozymes in ozone-tolerant cultivar, i.e. No. 2 and No. 4, were evidently induced at least for 3 to 5 hrs after ozone exposure. Most strongly induced isozymes (No. 3) seemed to be always expressed in living plant cell. The biochemical characteristics of these isozymes will be elucidated in future works.

Polyamine biosynthesis

Continuous ozone fumigation increased the level of polyamines, especially putrescine content (Fig. 4). Moreover, it was clearly found that the increased nitrogen level affected polyamine biosynthesis. As soon as ozone was fumigated, putrescine was rapidly enhanced in both rice plants. In addition, the increased nitrogen level highly induced putrescine biosynthesis. Except 3 hrs ozone fumigation in Jinpumbyeo, contents of spemidine or spermine were not charged in rice plant. This result assumes that the increase of ozone blocked the biosynthesis pathway of spermidine or spermine and stimulated ethylene biosynthesis related to cell senescence.

The ratio of putrescine to total amine was estimated (Fig. 5). Due to the increase of putrescine contents, the ratio in each treatment was obviously elevated within 1 hr after ozone fumigation, and then slightly increased to the end of ozone supply except N1 and N2 in Jinpumbyeo. In this study, putrescine contents was considered as a physiological indicator to ozone stress. In conclusion, we would suggest that ozone has an inhibitory effect on diamine oxidase (Peter *et al.*, 1989) resulting in increase the putrescine whereas ozone stimulates the polyamine oxidase, finally resulting in the decrease of polyamine. On the other hand, the inhibition of spermine and spermidine biosynthesis induces ethylene production and thus leads to senescence.

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