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Response of Antioxidative Enzymes of Two Rice Cultivars to Ozone Exposure and Nutrient Supply

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ABSTRACT: Ozone (O_3)-induced changes in chlorophyll content and specific activities of antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were investigated in two rice cultivars (*Oryza sativa* L.) grown under variable nutrient treatments. For this study, two rice cultivars of Ilpumbyeo (IL) and Keumobyeo#1 (KM), which were known as resistant and susceptible to O_3, respectively, were exposed to O_3 at 0.15 ppm for 30 days and investigated with 10 days interval. The available nutrient regimes were varied by doubling the supply of nitrogen (N), phosphorus (P) and potassium (K) within a basic fertilizer status (N, P, K; 15, 12, 12 Kg/10a^{-1}). In both cultivars and at all nutrient status, chlorophyll content in O_3-treated plants decreased with prolonged treatment period, although higher N, P and K supply with O_3 treatment alleviated the decrease in chlorophyll content. The activities of almost all enzymes investigated for this study were decreased during initial stages of O_3 exposure except GPX which maintained higher activity throughout the exposure period than the non-treated plant. However, the antioxidant enzymes in O_3-treated plants showed almost the same or higher activities on 30 days after O_3 exposure. The most significant changes in activities were observed in GR of the O_3-treated leaves. With the prolonged treatment period, the activity of GR at 30 days was increased by 3-8 times compared to those in 10 days. Most of the investigated enzymes showed very similar tendency to O_3 treatment in all fertilizer status. There was no observed evidence for enhanced detoxification of O_3-derived activated oxygen species in plants grown under higher fertilizer status compared with that in plants grown under basic fertilizer status. The increase in the activities of SOD, APX and GR in rice leaves by relatively long-term treatment with O_3 at low concentration is considered to indicate that the plant became adapted to the O_3 stress and the protection system increased its capacity to scavenge toxic oxygen species. Our results in two rice cultivars indicated that there was little difference in the activities of antioxidant enzymes between IL and KM, which were known as resistant and susceptible cultivar to O_3.

Keywords **:** antioxidative enzymes, chlorophyll, nutrient supply, rice, ozone

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O_3, a chief constituent of photochemical air pollution, is a strong oxidant. In plants, decreased photosynthesis, foliar injury, reduction in shoot and root growth and in crop yield and premature senescence are observed effects (Michael *et al.*, 1993). There have been reported on the toxicity of active oxygen species associated with O_3, e.g., superoxide anion (O_2), hydrogen peroxide (H_2O_2), hydroxy radical (-OH) and singlet oxygen (O_2) (Alscher and Amthor, 1988; Runeckles and Chevone, 1991), which are formed directly by O_3 decomposition in aqueous sap in cells (Grimes *et al.*, 1983; Health, 1987), or indirectly by oxygen reduction in chloroplasts (Asada, 1980).

It has been established that plant tolerance of O_3 depends largely on its ability to detoxify O_3. The active oxygen is present in all plants at various degree as a result of normal aerobic metabolism. Allowed to accumulate, these active oxygen species can cause damage to cellular components, severely disrupting metabolic function (Elstner, 1987). Unless efficiently metabolized, O_3-derived activated oxygen species may alter plant metabolism by structurally modifying proteins and enhancing their susceptibility to proteolytic degradation (Pell and Dann, 1991).

Metabolism of O_3-derived activated oxygen species is largely dependent on the activities of certain antioxidant enzymes in plants such as superoxide dismutase (SOD), peroxidases (PODs), glutathione reductase (GR) and ascorbate peroxidase (APX) (Foyer *et al.*, 1994). SOD dismutates superoxide into H_2O_2 and O_2. Peroxidases, like ascrobate peroxidase (APX), subsequently reduce H_2O_2 into water using ascorbic acid as the electron donor. The resulting dehydroascorbic acid is cycled back to ascrobate using GSH (reduced glutathione) as the electron donor, and the GSSH (oxidized glutathione) formed is recycled back to GSH by glutathione reductase (GR). The evidence for an important

function of antioxidant enzymes in plant protection to O_3 is not convincing. Matters and Scandalios (1987) have shown that neither SOD nor catalase responded to O_3 in maize, and SOD was largely unaffected by O_3 in *Phaseolus vulgaris* (Chanway and Runeckles, 1984). Ascorbate peroxidase was proposed to be a key enzyme governing O_3 resistance in mung bean and pea (Mehlhorn, 1990).

In an O_3 enriched environment, a successful plant would be one that effectively removes the increased free radicals by O_3. When plants are exposed to O_3, their susceptibility is affected by a variety of environmental factors such as high CO_2, humidity, drought and temperature (Rantanen *et al.*, 1994; Rao *et al.*, 1995). The interaction effect of nutrient supply and O_3 stress on the activities of anti-oxidant enzymes has rarely been studied. Although numerous studies have demonstrated that antioxidant enzymes detoxify the O_3-derived activated oxygen species, there were many inconsistent results among enzymes and plant species and little information on antioxidant enzymes in rice plants O_3, in spite of the global importance of rice as a staple food crop. Therefore, the activities of antioxidant enzymes in two rice cultivars, the resistant and susceptible cultivars exposed to O_3 under different nutrient conditions were investigated.

MATERIALS AND METHODS

Plant materials and fertilizer levels

Two rice cultivars (Ilpumbyeo and Keumobyeo) belonging to the japonica group were used in this study. Sohn and Lee (1977) showed that Ilpumbyeo (IL) was resistant to O_3 and Keumobyeo (KM) susceptible. Seeds were pregerminated for 48 hours in tap water in the dark at 30°C and then 100 g of germinated seeds was sown in a seedling tray (10 × 60 × 2.6 cm). Ten days after sowing, seedlings were transplanted with three plants per pot into wagner pot (1/5000a). Each pot of soil was fertilized with four levels; basic status (N, P, K; 15, 12, 12 $10a^{-1}$), 2N (N, P, K; 30, 12, 12 $10a^{-1}$), 2P (N, P, K; 15, 24, 12 $10a^{-1}$), 2K (N, P, K; 15, 12, 24 $10a^{-1}$) status. Each pot was watered once or twice daily. All experiments were done in three replicates.

O_3 treatment

Exposure of plants to O_3 was started on the 20th day after transplanting. Mean O_3 concentration for daily 6 hr periods (10 a.m. to 4 p.m.) from June 1 until June 30 was 0.15 ppm, which was a half concentration of O_3 alarm in Korea. Each pot was randomly rotated every day within the chamber to increase the uniformity of O_3 exposure among plants. O_3 generator and monitoring system is shown in Table 1.

Table 1. O_3 generator and monitoring system.

Classification	Function
O_3 generator	
O_3 fumigated velocity	0~4 g hr^{-1}
Air volume	10 l min^{-1}
O_3 measuring instrument	
O_3 measuring range	0.00~9.99 μl l^{-1}
O_3 monitoring instrument	IN-2000 UV absorption analyzer
Chamber	
Mode	Open-top chamber
Dimension	200 × 100 × 150 cm

Chlorophyll and enzyme activity

To measure the interaction effect of O_3 and fertilizer supply on the activities of anti-oxidant enzymes in rice, whole leaf blades of third leaves from the bottom were cut three times with 10 days interval during 30 days O_3 treatment. Obtained leaves were placed in liquid nitrogen. Samples were stored at -80°C deep freezer for the later enzyme assays.

About 0.5 g of leaves were ground in liquid nitrogen and homogenized in 10 of 50 mM potassium phosphate buffer (pH 7.8) containing 0.5 mM ascorbic acid and 1 mM reduced form glutathione at 4°C. The homogenate was centrifuged at 15,000 g for 10 min. and the supernatant was used for the determination of the enzyme activities. A part of the supernatant solution was directly used to determine APX and DHAR activities and the remaining solution was dialyzed four times against about 50 volumes of 10 mML^{-1} of potassium phosphate buffer (pH 7.8) overnight and centrifuged at 18,000 g for 30 min. All the assays of the enzyme activities were performed by kinetics using spectrophotometer (UV-1200, Shimadzue Co., Japan) at 25°C and the final cuvette volume was 1 ml.

The SOD activity was assayed with the reduction of cytochrome c at A_{550} according to Schoner Krause (1990). The activity of APX was determined by the oxidation of ascorbate by H_2O_2 as a decrease in A_{290} (2, 8 mM cm^{-1}). The DHAR activity was assayed (Tanaka. *et al.*, 1982) by detecting the increase of A_{290} (6.2 mM cm^{-1}), as dehydroascorbate was reduced to ascorbate (Tanaka. *et al.*, 1982). GR activity was assayed by detecting the reduction of NADPH at A_{340} (26.6 mM cm^{-1}).

All the assays were carried out with three replications. The data were analyzed by analysis of least significant difference test ($P \leq 0.05$).

RESULT

At the time of the third leaf emergence (about 30 days

Table 2. Effect of O_3 and fertilizer levels on chlorophyll content in rice.

Treatment	Fertilizer (N-P-K kg $10a^{-1}$)	Days after O_3 treatment					
		10		20		30	
		IL†	KM†	IL	KM	IL	KM
O_3	15-12-12	100	93	86	76	78	66
	30-12-12	107	98	91	84	81	73
	15-24-12	106	106	94	82	82	72
	15-12-24	104	108	89	84	95	76
	Mean	104	101	90	81	84	71
Control	Mean	100	100	100	100	100	100
LSD(5%) :							
-between means of O_3 treatments		ns	ns	ns	ns	ns	2.4
-between means of fertilizers within O_3 treatment		2.2	ns	2.9	3.4	ns	3.6

†IL, Ilpumbyeo; KM, Keumobyeo#1
‡The unit represents percent against control.

after sowing), the seedlings were treated with 0.15 ppm O_3 for 30 days (6 hr per day). The samples were taken at 10 o'clock on the 10, 20 and 30th day for the chlorophyll content and enzyme activity.

The chlorophyll content in the leaves decreased with prolonged exposure period and was higher in IL of O_3 resistant cultivar compared to O_3 susceptible cultivar of KM (Table 2). On the 10th day after O_3 treatment, the difference in chlorophyll content between O_3-treated and non-treated plants was not observed. However on the 30th day, it was decreased by 22% and 34% for IL and KM, respectively, compared to the control (O_3 non-treated plants at basic nutrient status). There was little effect on the chlorophyll content by O_3 exposure among the variable nutrient supplies, although O_3-treated 2N, 2P and 2K status showed higher content than that of the O_3-treated basic nutrient status.

SOD activities in rice leaf tissue showed significant difference between O_3-treated and non-treated plants (Fig. 1). At 10 days, SOD activity in non-treated plants was higher than that of O_3-treated plants and also showed higher activity in 2K and 2P status than basic fertilizer status. However, this was no longer seen at 30 days. At 30 days after O_3 treatment, on the contrary, a slight increase in SOD activity was observed in O_3-treated plants and showed slightly higher activity than non-treated plants. This tendency was similar in two rice cultivars.

Increased SOD activity generates H_2O_2. Therefore, the activities of APX (Fig. 2) and GPX (Fig. 3) were determined. In both rice cultivars, major differences in the APX activity between O_3-treated and non-treated plants occurred at 10 days and was observed lower activity of APX 2 to 4 times in O_3-treated plants than non-treated plants. However, there was no difference between O_3-treated and non-treated plants in the activity of APX at 30 days. Contrary to the results of APX, the activity of GPX was higher in O_3-treated plants than non-treated plants. Activity also increased in O_3-treated plants with little change in non-treated plants with prolonged treatment period. At 30 days after O_3 exposure, GPX activity in O_3-treated plants was 2 to 3 times higher in O_3-treated plants compared to non-treated plants and there was no significant difference among the different nutrient treatments and between two rice cultivars.

The changes in the activities of DHAR are shown in Fig. 4. The activities of DHAR decreased up to the 20th day and then increased up to the 30th day after O_3 exposure. The activity change with treatment period was more significant in O_3-treated plants compared to non-treated plants. At 30 days, the activity of DHAR in O_3-treated plants was nearly 3 to 4 times higher compared to those on the 20th day.

No major changes higher throughout the 30 days treatment period were observed in the GR activity of plants grown under non-treatment with O_3, although, higher activity at the 10th day than O_3-treated plants was shown(Fig. 5). Prolonged O_3 exposure from 10 days to 20 days increased GR activity a little. However, the activity was significantly increased at 30 days by about 3-8 times compared to those at 10th days. The activity was higher in O_3-treated 2N or 2P status than O_3-treated basic fertilizer or 2K status at 30 days. The activities in antioxidant enzymes according to variable nutrient supplies was similar tendency between the two rice cultivars.

DISCUSSION

Decreases in photosynthetic pigments have been widely used as an indicator of O_3 stress (Darrall, 1989). O_3 is

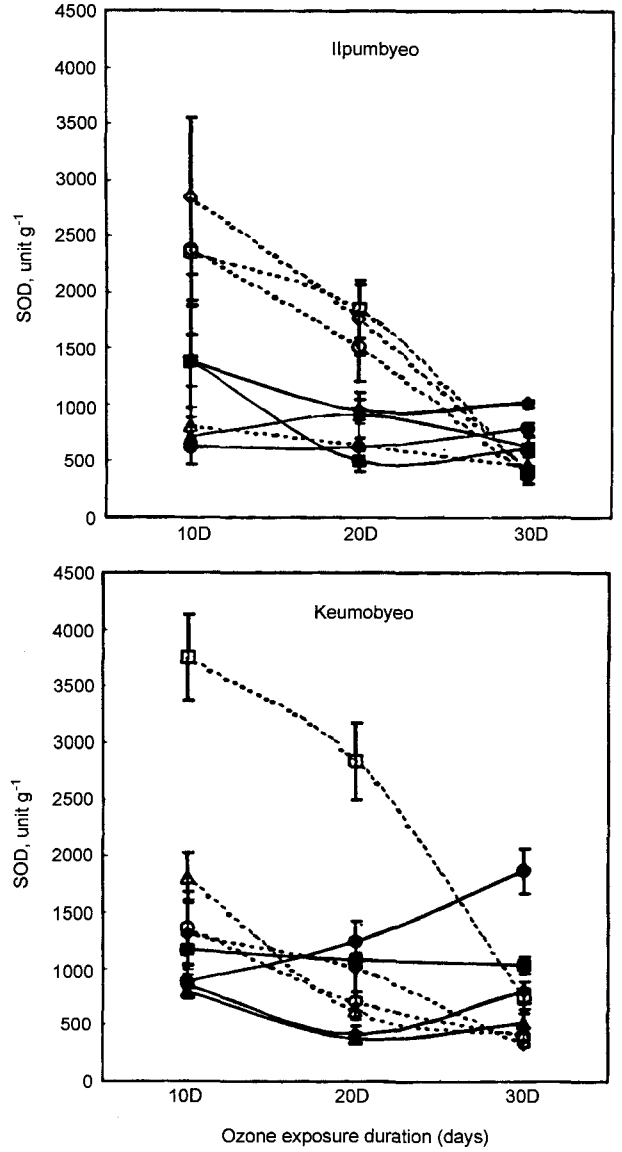

Fig. 1. Changes of superoxide dismutase (SOD) activity in rice leaves applied with different fertilizer levels during 30 days of O_3 (0.15 ml l^{-1}, 6 hr day^{-1}) treatment, Solid symbol; O_3 treatment, Open symbol; control. (●○); N-P-K= 15-12-12 kg $10a^{-1}$), ((▲△); N-P-K=30-12-12 kg $10a^{-1}$), ((◆◇); N-P-K=15-24-12 kg $10a^{-1}$), ((■□); N-P-K=15-12-24 kg $10a^{-1}$)

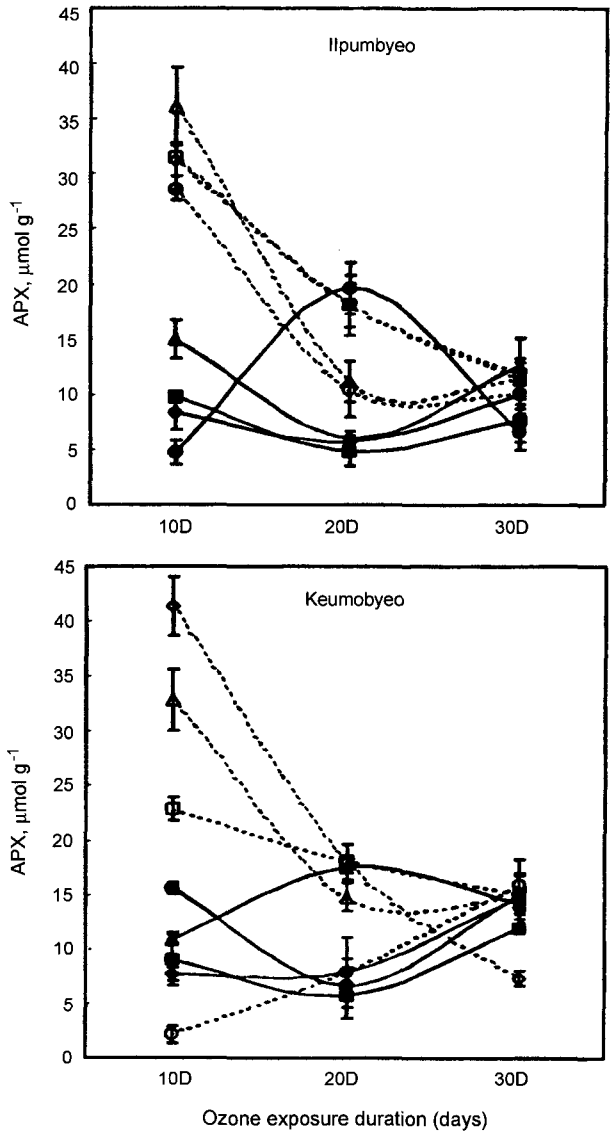

Fig. 2. Changes of ascorbate peroxidase (APX) activity in rice leaves applied with different fertilizer levels during 30 days of O_3 (0.15 ml l^{-1}, 6 hr day^{-1}) treatment. Solid symbol; O_3 treatment, Open symbol; control. ((●○); N-P-K=15-12-12 kg $10a^{-1}$), (▲△); N-P-K=30-12-12 kg 10^{-1}), ((◆◇); N-P-K=15-24-12 kg $10a^{-1}$), ((■□); N-P-K=15-12-24 kg $10a^{-1}$).

believed to induce degradation of chlorophyll or inhibit their biosynthesis (Darral, 1989; Health, 1994). Study results showed that the chlorophyll content in O_3-treated plants decreased with the prolonged treatment period (Table 2). O_3 exposure for 30 days decreased chlorophyll content in IL and KM by 22 and 34, respectively. This is in agreement with the results obtained by Mulholland *et al.* (1997) and Nouchi (1993).

It has been established that plant tolerance of O_3 depends largely on the ability to detoxify O_3. Plants are endowed with an array of enzymes and small molecules such as ascorbate and glutathione to cope with free radicals. SOD catalyzes the dismutation of O_3 to H_2O_2 and O2, whereas peroxidase metabolize H_2O_2 (Foyer *et al.*, 1994). In addition, plants are equipped with enzymes such as glutathione reductatse and ascorbate peroxidase that are capable of metabolizing H_2O_2 by utilizing ascorbate and glutathione (Creissen *et al.*, 1994; Foyer *et al.*, 1994). The available literature, in general, relates plant tolerance of O_3 with enhanced antioxygenic activities and/or gene expression of

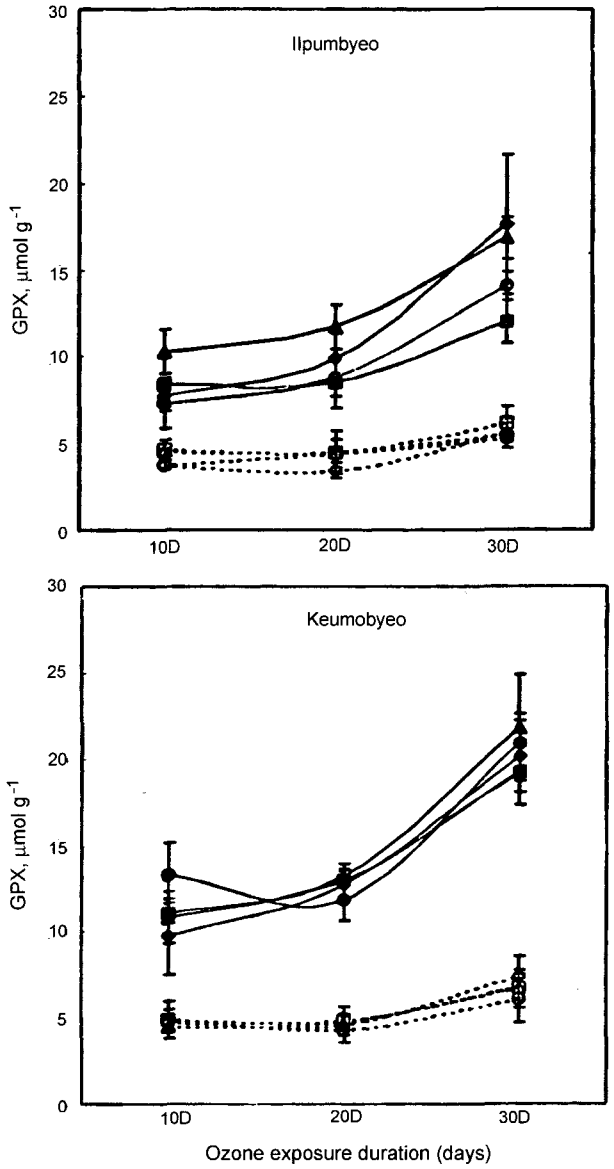

Fig. 3. Changes of guaiacol peroxidase (GPX) activity in rice leaves applied with different fertilizer levels during 30 days of O_3 treatment, Open symbol; control. ((●○); N-P-K=15-12-12 kg $10a^{-1}$), ((▲△); N-P-K=30-12-12 kg $10a^{-1}$), ((◆◇); N-P-K=15-24-12 kg $10a^{-1}$), ((■□); N-P-K=15-12-24 kg $10a^{-1}$).

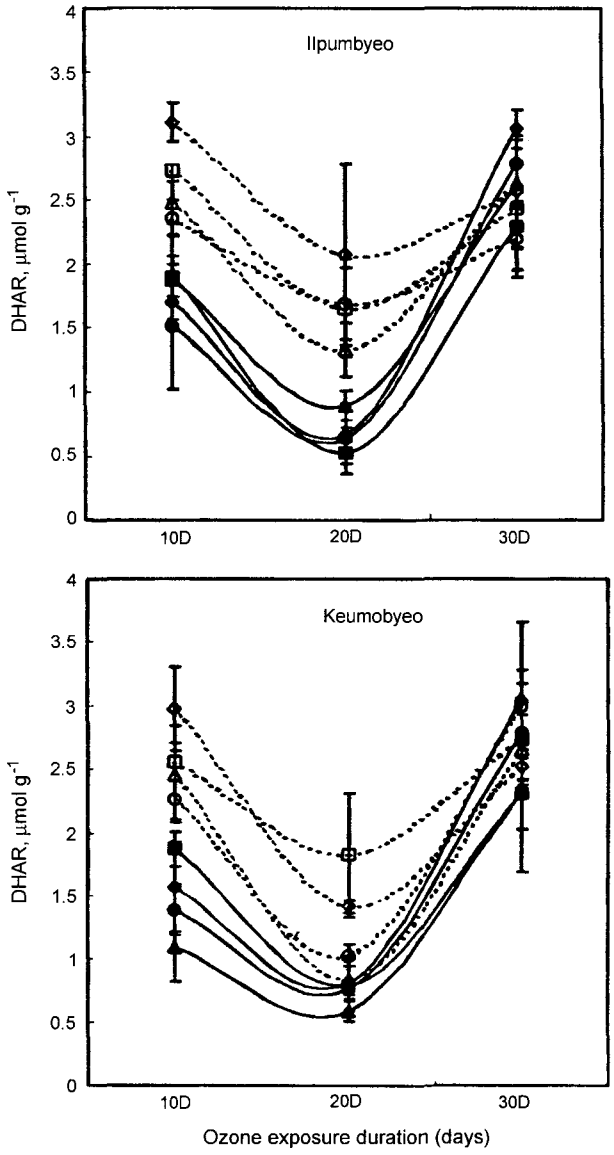

Fig. 4. Changes of dehydroascorbate reductase (DHAR) activity in rice leaves applied with different fertilizer levels during 30 days of O_3 (0.15 ml l^{-1}, 6 hr day^{-1}) treatment. Solid symbol; O_3 treatment, Open symbol; control. ((●○); N-P-K=15-12-12 kg $10a^{-1}$), ((▲△); N-P-K=30-12-12 kg $10a^{-1}$), ((◆◇); N-P-K=15-24-12 kg $10a^{-1}$), ((■□); N-P-K=15-12-24 kg $10a^{-1}$).

various antioxidant enzymes (Kangasjarvi *et al.*, 1994; Van Camp *et al.*, 1994; Willekens *et al.*, 1994).

In the present study, evidence for enhanced detoxification of O_3-derived activated oxygen species in plants grown under higher fertilizer status compared to that in plants grown under basic fertilizer status was not obversed. Most of the investigated enzymes showed very similar tendency to O_3 treatment in all fertilizer status, although O_3-treated 2N status showed higher activities in GPX and GR compared to those in O_3-treated basic fertilizer status. The activities of almost all enzymes investigated in this study were decreased during initial stages of O_3 exposure, except GPX (Fig. 3). In both cultivars and all nutrient status, GPX activities in the leaves exposed to 0.15 ppm O_3 continuously increased up to the 30th day and the activity was higher compared to non-treated plants throughout the treatment period. This is in agreement with the results obtained by Nouchi (1993). He also reported that the guaiacol peroxidase activity increased significantly when injury was occured by exposure to O_3 at

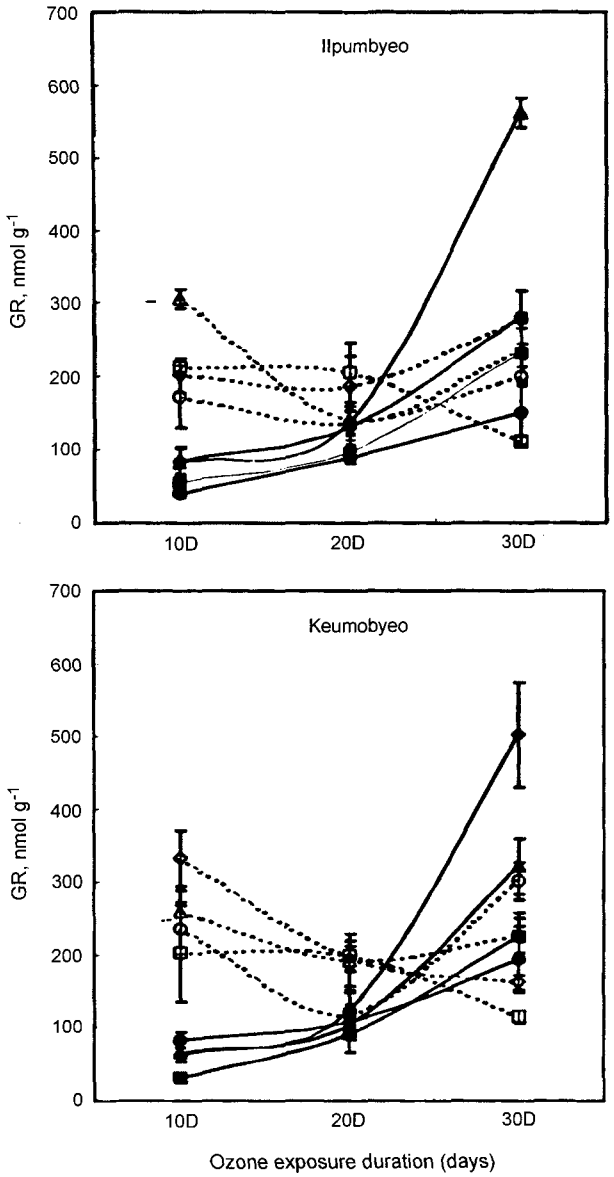

Fig. 5. Changes of glutathione reductase (GR) activity in rice leaves applied with different fertilizer levels during 30 days of O_3 (0.15 ml l^{-1}, 6hr day^{-1}) treatment. Solid symbol; O_3 treatment, Open symbol; control. ((●○); N-P-K=15-12-12 kg $10a^{-1}$), ((▲△); N-P-K=30-12-12 kg $10a^{-1}$), ((◆ ◇); N-P-K=15-24-12 kg $10a^{-1}$), ((■□); N-P-K=15-12-24 kg $10a^{-1}$)

a high concentration for 8 hours or at low and relatively low concentrations for five weeks. It is well known that activity of peroxidase increases under stress condition such as drought, low temperature and air pollution (Endress *et al.*, 1980; Varshney, 1985). The increase in the guaiacol peroxidase activity indicates the formation of large amounts of H_2O_2 in cells due to O_3 stress.

SO_2 fumigation of spinach leaves and chloroplasts caused the accumulation of H_2O_2 (Tanaka *et al.*, 1982) that could be reduced somewhat by cytochrome C and by SOD, catalase, ascorbate peroxidase and glutathione reductase activities, together with a reversible inhibition of Calvin cycle enzymes (Tanaka *et al.*, 1982). Decreased activities of SOD and APX due to 10 days exposure to O_3 indicate that accumulated H_2O_2 and O_3 stress in the initial period of O_3 treatment may be associated with the decreased activity in SOD and APX. Significant changes in activities were observed in GR of the O_3-treated plants with the prolonged treatment period. The activity of GR on the 30th day was increased 3 to 8 times as compared to those on the 10th day (Fig. 5).

SOD, which scavenges O_2, plays an important role in the protection system against O_3 injury (Lee and Bennett, 1982). However, since there are discrepancies in the changes of SOD activity associated with O_3 exposure in plants (Tanaka *et al.*, 1988; Mckersie *et al.*, 1982), it remains unclear whether the sensitivity or tolerance of plants to O_3 is correlated with SOD activities. In this study, plants exposed to lower levels of O_3 (0.15 ppm for 6 hours daily) for 30 days showed signs of injury. The symptoms include minute and localized chlorosis areas which result in decreased chlorophyll content of both rice cultivars (Table 2). At the onset of visible symptoms, the SOD level in the treated plants showed the increased values as compare to the non-treated plants although SOD in O_3-treated plants during the initial exposure showed very low values (Fig. 1). Chanway and Runeckles (1984) reported that the increase in injury symptoms may be attributed to the increasing SOD activity. Contrary to this report other workers have shown that in maize neither SOD nor catalase responded to O_3 or SO_2 treatment (Matters and Sandalios, 1987). Spinach leaves fumigated with O_3 exhibited the decreased SOD and catalase activities, but the increased APX activity (Sakaki *et al.*, 1983; Tanaka *et al.*, 1988).

The increase in the activities of SOD, APX and GR of rice leaves with relatively long-term treatment of O_3 at low concentration indicates that the plants became adapted to the O_3 stress and the protection system increased its capacity to scavenge toxic oxygen species. Little difference was observed in the activities of antioxidant enzymes between IL and KM rice cultivars, which were known to be resistant and susceptible to O_3, respectively (Sohn and Lee, 1997).

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