Physiological and Biochemical Responses to Ozone Toxicity in Five Species of genus *Quercus* Seedlings

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참나무속 5종의 오존 독성에 대한 생리생화학적 반응

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ABSTRACT

Physiological and biochemical changes of five species of genus *Quercus* exposed to ozone fumigation were investigated to assess their tolerance against ozone toxicity. At the end of 150 ppb ozone fumigation, chlorophyll contents, photosynthetic characteristics, malondialdehyde (MDA) and antioxidative enzyme activities were measured in the leaves of five *Quercus* species (*Quercus acutissima*, *Q. aliena*, *Q. palustris*, *Q. serrata*, and *Q. variabilis*). Chlorophyll and carotenoid contents, net photosynthesis and carboxylation efficiency decreased after ozone treatment, indicating that O₃-exposed plants underwent physiological inhibition. The reduction rate of total chlorophyll contents and carboxylation efficiency were respectively 15% and 34% for *Q. aliena* and 38% and 62% for *Q. variabilis*. The amount of MDA increased with the highest increase rate of 140% in *Q. acutissima* which also showed the highest increase rate (60%) of superoxide dismutase (SOD). Ascorbate peroxidase (APX) activity increased in *Q. variabilis*, *Q. serrata* and *Q. acutissima* by ozone treatment. Based on our results, ozone tolerance of the five *Quercus* species was ranked as *Q. aliena* > *Q. palustris* > *Q. serrata* > *Q. variabilis* > *Q. acutissima*. We concluded that chlorophyll contents, photosynthesis, MDA content and antioxidative enzymes were the important physiological markers for tolerance against ozone stress, which were closely related with one another.

Key words : Oak, Chlorophyll content, Photosynthesis, MDA, Antioxidative enzyme

I. INTRODUCTION

For more than 50 years, the phytotoxicity of ozone (O₃) has been demonstrated for forest tree species (Karnosky *et al.*, 2007). Studies concerning the dynamics of ozone (O₃) between atmosphere and plants have been carried out under the United Nations Economic Commission for Europe-Convention on Long Range Transboundary Air Pollution (UNECE CLRTAP) (Mills *et al.*, 2000). It is now clearly established that O₃ can

cause a range of effects including visible leaf injury and reduction of growth and yield, and can alter the sensitivity of plants to biotic and abiotic stresses (Fuhrer and Achermann, 1994). Despite the increasing environmental awareness and regulations designed to limit industrial and vehicular emissions, ozone levels potentially harmful to human health and vegetation have increased every year in Korea (Ministry of Environment, 2005).

Oak trees, *Quercus* species, which are one of the

most abundant tree species in Korean, are important economically and ecologically as the ornamental or roadside tree. They are known as a tolerance species to air pollutants, but the information on the biochemical and physiological responses and tolerant mechanisms for *Quercus* species under environmental stress was few.

It appears that distinct O₃ induced effects are largely confined to fast-growing tree species (Skärby *et al.*, 1998). Investigations on the effects of O₃ on the slow-grown species, such as *Quercus*, are scarce and did not consider O₃ to be a significant factor in the complex of damage to the oak (Thomas *et al.*, 2002, Samuelson *et al.*, 1996; Hanson *et al.*, 2005). On the other hand, Hanson *et al.* (1994) and Kelting *et al.* (1995) reported reductions in foliar assimilation to O₃ exposures in red oak (*Q. rubra*). No information has been provided to compare the sensitivity among *Quercus* species to O₃ that this study focuses on. However, difficulties in ranking the O₃ sensitivity of different species may arise since intra-specific variations have been reported in several plant species (Inclán *et al.*, 1999; Minnocci *et al.*, 1999; Ranieri *et al.*, 2001). Inclán *et al.* (1999) indicated the differential sensitivity to O₃ of five Mediterranean woody species, and Elvira *et al.* (2004) reported intra-specific variations in two population of *Q. coccifera* to O₃.

During the past three decades, occurrence of oak declines has been recorded in many European countries (Thomas *et al.*, 2002). In the Mediterranean region, mainly *Q. ilex* has been affected (Müller-Edzards *et al.*, 1997), and a decline has been also reported for deciduous oak species (*Q. cerris*, *Q. frainetto*, *Q. pubescens* and *Q. robur*) in Italy (Ragazzi *et al.*, 1998), and for *Q. suber* on the Iberian Peninsula (Sousa Santos and Moura Martins, 1993). On the contrary, studies of red oak, however, showed an enhanced sensitivity to O₃ in 30-year-old mature trees than two year-old seedlings due to higher stomatal conductances in the older trees (Samuelson *et al.*, 1996; Weinstein *et al.*, 1998; Wullschleger *et al.*, 1996).

The factors determining plant sensitivity or tolerance are not clearly understood but it is thought to be related to many underlying physiological, anatomical, biochemical and environmental factors (Alonso *et al.*, 2001; Pääkkönen *et al.*, 1998, Ribas *et al.*, 2005). Ozone effects on tree biomass are the result of several processes occurring at the cellular and physiological levels. Acceleration of leaf senescence has been widely

reported as one of the most characteristic processes derived from ozone exposure. These processes involve chlorophyll degradation and reductions in CO₂ assimilation (Elvira *et al.*, 1998; Zheng *et al.*, 2002). Physiological effects of ozone exposure include photosynthesis reduction and an increase of antioxidant systems. Photosynthesis reduction results in a decreased growth rate in either volume or biomass.

The biochemical markers have been used to select the best tolerant species, cultivar and variety to O₃ or other pollutants (Han *et al.*, 2006). Paoletti *et al.* (2003) also adopted antioxidant levels as biomarkers of ozone sensitivity that could be used for screening of great diversity of tree species' potential sensitivity to ozone. However, there was not an exact answer about the differences of biochemical and physiological responses among species, cultivar or varieties as well as among biochemical makers.

Therefore, this study was undertaken to investigate cellular responses to ozone as mechanism for oxidant impact on photosynthesis in seedlings of oak trees. The aims of the present study were to (1) determine the effects of ozone exposure on photosynthetic pigments, photosynthesis, antioxidant enzyme activities and lipid peroxidation, (2) evaluate the potential occurrence of interspecific variations in O₃ sensitivity in *Quercus*, and (3) evaluate a differential sensitivity to ozone exposure in *Quercus*.

II. MATERIALS AND METHODS

Seeds of five species of genus *Quercus* (*Q. acutissima*, *Q. aliena*, *Q. palustris*, *Q. serrata*, and *Q. variabilis*) were germinated in sand soil on March 12, 2007. One-year-old seedlings were transplanted into plastic pots (H 20 × W 15 cm) containing artificial soil which consisted of 1:1:1 sand: peat moss: vermiculite (volume basis). Five seedlings per treatment were transferred into O₃ chamber and were arranged in two blocks. O₃ treatment was divided into two chambers: control chamber was circulated with the clean air and the other one was fumigated with 150 ppb O₃. O₃ fumigation time was 8 hrs a day. O₃ concentration in chamber was registered 5 ± 1 ppb in control and 150 ± 10 ppb in treatment chamber during fumigation period. The fumigation system has been described in detail by Lee *et al.* (2003). The experiment was started on June 22, 2007 and it was conducted for four weeks.

2.1. Photosynthetic pigments

At the end of O₃ fumigation, the leaves of control and O₃-treated seedling of oak trees were excised and soaked in dimethyl sulfoxide (DMSO) in a glass vial. The vial was tightly capped and incubated at 70 °C for 2 hrs in the dark. The concentration of the extracted pigments (total chlorophyll, chlorophyll a, chlorophyll b and carotenoid) was calculated on the basis of their absorbance values at 664, 645 and 470 nm according to Lichtenthaler (1987).

2.2. Photosynthesis and biomass

Net photosynthesis of fully expanded leaves was measured with an infrared gas analyzer (Li-6400, Li-COR, USA). Environmental parameters were maintained stably for measuring (mean temperature: 20.0 ± 0.1 °C; relative humidity: 68.2 ± 3.2%; leaf-to-air vapour pressure deficit: 1.2 ± 0.2 kPa). All determinations were performed at 1100 µmol m⁻² s⁻¹ photon flux density (PFD). Net photosynthesis (A, µmol CO₂ m⁻² s⁻¹) was determined at light saturation level between 10 a.m. and 3 p.m.

To calculate carboxylation efficiency, *ACi*-curve was made (Farquhar *et al.*, 1980; Kim and Lee, 2001). The carboxylation efficiency was determined from the initial slope of a linear regression using the linear portion of the *ACi*-curve (0-150 ppb intercellular CO₂).

For biomass measurements, shoots and roots were carefully removed, and then thoroughly rinsed twice with distilled water. Shoot and root dry weights were recorded after drying the tissues at 70 °C.

2.3. Lipid peroxidation

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by thiobarbituric acid reaction as described by Heath and Parker (1968). The crude extract was mixed with the same volume of 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95 °C for 30 min. and then quickly cooled in an ice-bath. The mixture was centrifuged at 3000 × g for 10 min. and the absorbance of the supernatant was monitored at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm), MDA concentration was determined by its molar extinction coefficient (155 mM⁻¹ cm⁻¹) and the results were expressed as µmol MDA g⁻¹ FW.

2.4. Antioxidative enzyme activities

Fresh leaves (0.1 g) were homogenized under ice-

cold condition with 5 mL of 50 mM phosphate buffer (pH 7.0), 10 mM ascorbic acid (AsA) and 1.0% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 20,000 × g for 30 min. and the supernatant was collected for enzyme assays.

Superoxide dismutase (SOD) was assayed based on the inhibition of reduction of nitro-blue tetrazolium in the presence of xanthine at 530 nm according to the method of Beauchamp and Fridovich (1971). Ascorbate peroxidase (APX) activity was determined by the method of Nakano and Asada (1981). The assay was carried out in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM EDTA, 0.1 mM H₂O₂, and 0.1 mL enzyme extract. The change in A₂₉₀ was recorded for 1 min. after the addition of H₂O₂. Activity of glutathione reductase (GR) was assayed as described in Carlberg and Mannervik (1985). The assay was carried out in a reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 mM NADPH, 0.5 mM GSSH and 0.1 mL enzyme extract. The change in A₃₄₀ was recorded for 5 min. after the addition of enzyme extract. Catalase (CAT) activity was determined by following a two-step procedure (Fossati *et al.*, 1980). The rate of dismutation of H₂O₂ to water and molecular oxygen is proportional to the concentration of catalase. Therefore, the sample containing catalase was incubated in the presence of a known concentration of H₂O₂. After incubation for exactly one minute, the reaction was quenched with sodium azide. The amount of H₂O₂ remaining in the reaction mixture was then determined by the oxidative coupling reaction of 4-aminophenazone (4-aminoantipyrene) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) in the presence of H₂O₂ and catalyzed by horseradish peroxidase (HRP). The resulting quinoneimine dye was measured at 520 nm. All enzyme activities were measured using UV-120 (SHIMADZU, Japan).

2.6. Tolerance and injury index

Based on physiological and biochemical responses, ozone tolerance ability of five *Quercus* species was ranked. Injury index was determined by the effect of ozone on MDA, total chlorophyll content, carboxylation efficiency and biomass. Tolerance index was calculated using increase rate of SOD, APX, CAT and GR activities. Standard index on each parameter was calculated as (X-Xavg)/SD, where X is the average difference rate between control and ozone treatment for each species, Xavg is the average difference rate between

Table 1. Effects of ozone fumigation on photosynthetic pigments in the leaves of five species of genus *Quercus*

Species	Ozone	<i>Chl a</i>	<i>Chl b</i>	<i>Chl_{a+b}</i>	<i>Car</i>	<i>Chl a/b</i>	<i>Chl/Car</i>
		mg g ⁻¹					
<i>Q. acutissima</i>	Control	3.77 ± 0.02	1.81 ± 0.02	5.58 ± 0.03	0.86 ± 0.01	2.08 ± 0.02	6.50 ± 0.05
	150 ppb	2.49 ± 0.01	1.46 ± 0.02	3.95 ± 0.03	0.68 ± 0.10	1.70 ± 0.02	5.79 ± 0.07
	%*	-33.9	-19.3	-29.2	-20.9	-18.3	-10.9
<i>Q. aliena</i>	Control	2.96 ± 0.01	1.02 ± 0.01	3.98 ± 0.02	0.83 ± 0.01	2.90 ± 0.01	4.77 ± 0.02
	150 ppb	2.20 ± 0.01	1.20 ± 0.01	3.40 ± 0.01	0.68 ± 0.01	1.83 ± 0.01	5.02 ± 0.03
	%	-25.7	17.7	-14.9	-18.1	-36.9	5.2
<i>Q. palustris</i>	Control	2.70 ± 0.01	0.92 ± 0.01	3.62 ± 0.01	0.70 ± 0.01	2.93 ± 0.01	5.15 ± 0.02
	150 ppb	2.29 ± 0.02	0.67 ± 0.01	2.96 ± 0.02	0.60 ± 0.01	3.44 ± 0.02	4.89 ± 0.01
	%	-15.2	-27.2	-18.2	-14.3	17.4	-5.1
<i>Q. serrata</i>	Control	3.73 ± 0.06	1.72 ± 0.03	5.45 ± 0.05	0.83 ± 0.01	2.17 ± 0.06	6.53 ± 0.04
	150 ppb	3.10 ± 0.02	1.40 ± 0.01	4.50 ± 0.02	0.75 ± 0.01	2.21 ± 0.02	5.96 ± 0.05
	%	-16.9	-18.6	-17.4	-9.6	-1.8	-8.7
<i>Q. variabilis</i>	Control	3.40 ± 0.05	1.67 ± 0.01	5.10 ± 0.06	0.83 ± 0.06	2.05 ± 0.01	6.18 ± 0.03
	150 ppb	1.90 ± 0.02	1.25 ± 0.01	3.15 ± 0.03	0.60 ± 0.01	1.51 ± 0.01	5.28 ± 0.02
	%	-44.1	-25.2	-38.2	-27.7	-26.3	-14.6
Species (S)		***	***	***	***	***	***
Ozone (O ₃)		***	***	***	***	***	***
S × O ₃		***	***	***	***	***	***

All the values are means of five replicates ± SD; mixed effects linear model: *** $p < 0.001$.

*Reduction rate (%) was calculated as: % = [(control-treatment)/control] × 100.

control and ozone treatment for all five species and SD is the standard deviation of difference rate for five species. Standard index calculated by multiplied with weight value 12.5 to make even impact for all 8 parameters. The total sum of tolerance index that is calculated from 4 antioxidative enzymes and injury index including sum of MDA, chlorophyll content, carboxylation efficiency and biomass were aggregated to evaluate the tolerance against ozone stress.

2.6. Statistical analysis

To compare the effect on control and O₃ treatment, ANOVA was performed on experimental data (statistical significance, $p \leq 0.05$). Statistical analyses were performed using the statistical package SAS System for Windows, Version 8.01 (SAS Institute, USA).

III. RESULTS

3.1. Photosynthetic pigments

After 28 day of ozone exposure, the contents of photosynthetic pigments showed significant ($p < 0.05$) dif-

ferences among five species, between control and ozone treatment and species × ozone treatment interaction. Chlorophyll and carotenoid contents were decreased by ozone treatment, indicating that O₃-exposed plants underwent physiological inhibition (Table 1). Ozone treatment negatively affected on the photosynthetic pigments in the leaves of five *Quercus* species. The content of chlorophyll *a* in the seedlings of five species was 44.1% (*Q. variabilis*) to 15.1% (*Q. palustris*) lower in O₃-exposed plant when compared with the control, respectively. For the content of chlorophyll *b*, *Q. palustris* and *Q. serrata* showed reduction rate ranged from 27.2% to 18.6%, whereas *Q. aliena* showed an increase of 17.8% under ozone exposure. The reduction rate of total chlorophyll contents in five species ranged from 14.9% (*Q. aliena*) to 38.2% (*Q. variabilis*) in O₃-exposed plant comparing control, respectively. Carotenoid content showed highest reduction in *Q. variabilis* (27.7%) and lowest reduction in *Q. serrata* (9.5%) under ozone fumigation. The highest reduction rate on the ratio of chlorophyll *a* to chlorophyll *b* was observed in the leaves of *Q. aliena* (38.9%), whereas *Q. palustris*

increased by 17.4% under ozone exposure, respectively. For the ratio of total chlorophyll to carotenoid,

Fig. 1. Effects of ozone fumigation on net photosynthesis, carboxylation efficiency and biomass in the leaves of five species of genus *Quercus*. Qa: *Q. acutissima*, Qal: *Q. aliena*, Qp: *Q. palustris*, Qs: *Q. serrata*, and Qv: *Q. variabilis*. All the values are means of five replicates $\pm$ SD; mixed effects linear model. Net photosynthesis: species $p=0.0003^{***}$, ozone $p=0.0001^{***}$, species $\times$ ozone $p=0.2213$; carboxylation efficiency: species $p=0.0001^{***}$, ozone $p=0.0006^{***}$, species $\times$ ozone $p=0.7530$; dry weight: species $p<0.0001^{***}$, ozone $p<0.0001^{***}$, species $\times$ ozone $p=0.2626$. *, ** and *** significant at $p \leq 0.05$, 0.01 and 0.001.

Q. variabilis showed highest reduction (14.6%).

3.2. Net photosynthesis, carboxylation efficiency and biomass

The net photosynthesis showed significant ($p < 0.05$) differences among species and between control and ozone treatment at the end of ozone exposure (Fig. 1). However, there was no significant interaction between species and ozone treatment for net photosynthesis. The reduction rate of net photosynthesis comparing with control in the seedlings of five species varied from 23.8% (*Q. variabilis*) to 58.6% (*Q. acutissima*). Carboxylation efficiency differed significantly ($p < 0.05$) among species and between control and ozone treatment at the end of ozone exposure (Fig. 1). Species $\times$ ozone treatment interaction was not significant for carboxylation efficiency in the seedlings of five species. The reduction rate of carboxylation efficiency was the highest in the leaves of *Q. acutissima* (62.1%), and *Q. aliena* that showed lowest reduction rate was also decreased 33.6% for carboxylation efficiency in comparison with control. Substantial progression of photosynthetic-pigment destruction by ozone fumigation suggested the hinder of photosynthesis and decrees of photosynthesis resulted in reduction of biomass. Biomass showed the same result with net photosynthesis and carboxylation efficiency. There were significant ($p < 0.05$) differences among species and between control and ozone treatment while there was no significant difference with species $\times$ ozone treatment interaction. Among five species, *Q. acutissima* (30.9%) and *Q. ser-*

Fig. 2. Effect of ozone fumigation on MDA content in the leaves of five species of genus *Quercus*. Qa: *Q. acutissima*, Qal: *Q. aliena*, Qp: *Q. palustris*, Qs: *Q. serrata*, and Qv: *Q. variabilis*. All the values are means of five replicates $\pm$ SD; mixed effects linear model. Species $p=0.0001^{***}$, ozone $p=0.0001^{***}$; species $\times$ ozone $p=0.0001^{***}$ significant at $p \leq 0.001$.

Table 2. Effects of ozone fumigation on antioxidative enzyme activities in the leaves of five species of genus *Quercus*

Species	Ozone	SOD (unit g ⁻¹)	APX (μmol g ⁻¹)	GR (nmol g ⁻¹)	CAT (unit g ⁻¹)
<i>Q. acutissima</i>	Control	4343 ± 422	373 ± 35	111 ± 59	1807 ± 96
	150ppb	6939 ± 890	402 ± 66	499 ± 114	1988 ± 216
	%*	59.8	7.8	350.0	10.0
<i>Q. aliena</i>	Control	4768 ± 477	348 ± 26	246 ± 48	1925 ± 266
	150ppb	6288 ± 147	289 ± 27	399 ± 127	1945 ± 242
	%	31.9	-17.0	62.2	1.0
<i>Q. palustris</i>	Control	3212 ± 301	474 ± 19	84 ± 12	1896 ± 391
	150ppb	2346 ± 378	399 ± 29	94 ± 34	1794 ± 372
	%	-27.0	-15.8	11.9	-5.4
<i>Q. serrata</i>	Control	4059 ± 281	345 ± 44	673 ± 55	1938 ± 273
	150ppb	4015 ± 336	422 ± 57	668 ± 63	1906 ± 229
	%	1.1	22.3	-0.7	-1.7
<i>Q. variabilis</i>	Control	6698 ± 1836	272 ± 46	252 ± 43	1803 ± 277
	150ppb	6724 ± 755	343 ± 51	365 ± 44	1898 ± 255
	%	0.4	26.1	44.8	5.3
Species (S)		***	***	***	n.s.
Ozone (O ₃)		**	n.s.	***	n.s.
S × O ₃		***	**	***	n.s.

All the values are means of five replicates ± SD; mixed effects linear model: ** $p < 0.05$, *** $p < 0.001$, and n.s.: not significant.

*Increasing rate (%) was calculated as: % = [(control-treatment)/control] × 100

Q. palustris (33.3%) showed high reduction and *Q. variabilis* (14.0%) showed low reduction in biomass.

3.3. Lipid peroxidation and antioxidative enzyme activity

There was a significant difference in leaf MDA content among five species and between control and ozone treatment (Fig. 2). Especially, the amount of MDA was increased under ozone fumigation and showed the highest increase rate of 140% in *Q. acutissima* among five species, whereas it was reduced in *Q. aliena* and *Q. palustris* under ozone fumigation.

The responses of antioxidative enzymes varied each other under ozone fumigation (Table 2). For SOD, there was a significant ($p < 0.05$) difference among five species. There was a significant difference between control and ozone treatment, and species × ozone treatment interaction showed a significant difference for SOD activity. *Q. acutissima* showed the highest increase rate of SOD activity (59.8%), however, *Q. palustris* showed highest reduction of SOD activity (-27%). APX activity differed significantly ($p < 0.05$) between control and ozone treatment as well as among five species, and species × ozone treatment interaction was also significant for APX activity. APX activity increased in

Fig. 3. Correlation of tolerance and injury indices. Data for this figure were obtained from the same measurements as in Tables 1, 2 and Figs. 1, 2. *Quercus* Qa: *Q. acutissima*, Qal: *Q. aliena*, Qp: *Q. palustris*, Qs: *Q. serrata*, and Qv: *Q. variabilis*. The differences between the values of exposed ozone and unexposed ozone on photosynthetic pigment content, photosynthesis, biomass and MDA content were evaluated for injury index, and four antioxidative enzyme activities were used for tolerance index.

the leaves of *Q. acutissima* (7.8%), *Q. serrata* (22.3%) and *Q. variabilis* (26.1%) under ozone exposure, whereas

Q. aliena (-17.0%) and *Q. palustris* (-15.8%) decreased in comparison with control plants. GR activity differed significantly ($p < 0.05$) between control and ozone treatment as well as among five species, and species $\times$ ozone treatment interaction was also significant for GR activity. GR activity remarkably increased in the leaves of *Q. acutissima* (350%) and *Q. aliena* (62.2%) under ozone exposure. However, *Q. serrata* showed no significant difference in comparison with control plants. For CAT activity, the differences among species, treatments and species $\times$ ozone interaction were not significant.

3.4. Rank of tolerance ability using tolerance and injury index

Based on physiological and biochemical responses mentioned above, ozone tolerance ability of five *Quercus* species was ranked as *Q. aliena* > *Q. palustris* > *Q. serrata* > *Q. variabilis* > *Q. acutissima* (Fig. 3).

IV. DISCUSSION

In our research, the degree of ozone-injury was quantitatively estimated and compared among five oak species, based on four injury and four tolerance indices. A gradient in O_3 sensitivity was found for the species involved in this experiment. The results indicate that the differences between control and treatment in the sensitivity to ozone were distinct in aspects of low levels of chlorophyll and carotenoid content of O_3 -fumigated leaves (Table 1). Reduction in the chlorophyll content of leaves, following exposure of plants to ozone, has been reported in many species such as *Pinus ponderosa* (Anderson *et al.*, 2003), *Acer* species (Han *et al.*, 2007), Citrus (Iglesias *et al.*, 2006) and *Quercus ilex* (Ribas *et al.*, 2005). Knudson *et al.* (1977) and Sakaki *et al.* (1983) found higher reduction in chlorophyll *a* than chlorophyll *b* in O_3 -exposed plants of *Phaseolus vulgaris* L. and *Spinacia oleracea* L. and our results also support this founding (Table 1). Ozone exposure induces the activation of leaf senescence-related process that was linked to chlorophyll degradation, photosynthetic decline and lipid peroxidation (Ribas *et al.*, 2005; Bielenberg *et al.*, 2002).

The most apparent effect of oxidative stress induced by ozone described in numerous species, is leaf damage. In our experiments, visible injury symptoms (i.e., dark pigmented stipple in *Q. serrata*, brown pigmented stipple in *Q. palustris* and necrosis in *Q. acutissima*)

were observed in O_3 -treated plants. It has been reported that a main detrimental effect of ozone at the subcellular level is photosystem damage and reductions in photosynthesis (Anderson *et al.*, 2003), and collapse of mesophyll cells (Long and Naidu, 2002)

Adult holm oaks (*Q. ilex*) may be considered to be O_3 tolerant in terms of photosynthesis, as only 9% reduction was recorded in the O_3 -treated leaves compared to the controls (Paoletti *et al.*, 2007) and also, Felzer *et al.* (2007) reported higher sensitivity to ozone in 30-year-old mature trees than two year-old seedlings due to greater photosynthetic rates leading to higher stomatal conductances in the older trees. However, we observed severe reduction rate of net photosynthesis ranging from 23.8% to 58.6%, and carboxylation efficiency ranging from 33.6% to 62.1% by ozone treatment for all five species in spite of one-year-old seedlings. Photosynthesis reduction leads to growth and biomass reduction, and reduction rate ranged from 14% to 33.3% (Fig. 1A, 1B, and 1C). Vitale *et al.* (2008) showed severely limited photosynthesis in three-year-old seedling of *Q. ilex* by 250 ppb of O_3 fumigation like our results. Therefore, the reasons of differential results about photosynthesis and biomass to O_3 tolerance could be variance among species or difference of O_3 concentration.

We analyzed the level of lipid peroxidation and antioxidant activity, including SOD, APX, GR, CAT, because each of these traits have been previously associated with oxidative stress induced by O_3 (Puckette *et al.*, 2007; Iglesias *et al.*, 2006). MDA concentration, which estimates the state and integrity of membrane through the degree of lipid peroxidation, has been shown to correlate with the level of ozone exposure. In addition, O_3 -treated plants showed an increase in MDA content, indicating the state of membrane lipid peroxidation has a correlation with the degree of O_3 exposure to plants (Prince *et al.*, 1990; Yoshida *et al.*, 1994; Ranieri *et al.*, 1996; Iglesias *et al.*, 2006). In our results, there was the increase of MDA content after exposure of O_3 in *Q. acutissima*, *Q. serrata* and *Q. variabilis* species that showed severe reduction of chlorophyll content and net photosynthetic rate. However, the other two species, *Q. aliena* and *Q. palustris*, which showed no reduction rate in the ratio of chlorophyll *a* to *b*, did not increase in MDA content (Fig. 2). Therefore, the reduction of chlorophyll content and net photosynthesis had a tendency of the increase of MDA content in sensitive species.

Lipid peroxidation is the ultimate effect of O₃ attack to membranes, once the antioxidant defenses of membranes fail to cope with O₃ and oxygen radicals (Calatayud *et al.*, 2002; Iglesias *et al.*, 2006). Plant responds to O₃-induced oxidative stress by activation of a number of antioxidative stress-related defense mechanisms. Carotenoid is also protective agents against oxidative stress, and has been associated with photoprotective mechanisms and ascorbate pool that plays a crucial role to the defense of hydrogen peroxides reaction (Tauz *et al.*, 2002; Iglesias *et al.*, 2006). However, in our study, we did not find a concomitant decrease in both carotenoid content and antioxidative enzyme activities.

In the present study, there were significant differences in the activities of SOD and GR between treatments. On the contrary, APX and CAT had no significant differences between treatments. SOD and APX activities were higher in O₃-treated *Q. palustris* than those of control plants, whereas GR activities were not significantly increased (Table 2). Additionally, *Q. acutissima* that showed high reduction rate in photosynthetic pigments content, photosynthesis and highest increase rate of MDA content had highest increase rate in SOD and GR activities (see above). Although cellular responses to ozone are dependent on multiple factors, in general higher activities of scavenger antioxidant metabolite usually increase protection against stress (Sharma and Davis, 1997; Calatayud *et al.*, 2002; Iglesias *et al.*, 2006). In our experiment, sensitive species induced increase of APX activity, on the other hand, it was decreased in tolerance *Quercus* species (*Q. aliena* and *Q. palustris*). APX is a component of ascorbate-glutathione pathway and both chloroplast and cytosolic isozymes of APX. Elevated APX activities in O₃-treated plants contribute to tolerance against O₃ through the removal of H₂O₂ by APX. SOD represents another major class of antioxidant enzymes that play an important role in eliminating O₂ during oxidative stress. No difference in total SOD and GR mRNA amount and enzyme activity levels were observed in which plants were treated with 150 ppb O₃. However, different ecotype plants of *Arabidopsis thaliana* exposed to 200 ppb O₃ had higher 83% SOD activity and 98% GR activity than controls (Sharma and Davis, 1997). No detectable changes in CAT activity were observed in O₃-treated plants (Kubo *et al.*, 1995; Rao *et al.*, 1996). Sharma and Davis (1997) concluded that differences in these results were due to the dissimilar ecotypes used and the O₃ treatments. As indicated in the previous

studies, we may assume that APX, SOD and GR activities were highly connected with O₃ induced stress in sensitive species as much as their activities highly increased. However, the 150 ppb O₃ treatment for tolerance species did not alleviate the oxidative stress or those enzymes already used detoxification as defensive response. We also may not observe changes in CAT activities in O₃-treated plants. Our data indicate that the exposure of five *Quercus* species to high ozone levels (150 ppb) altered several physiological parameters, and antioxidant mechanisms were triggered especially in sensitive species.

O₃-induced physiological and biochemical responses can be used as a valuable criterion to rank the sensitivity of these species to the ozone pollutant (Fig. 3). Among five species, *Q. acutissima* was the most sensitive species that has detrimental effects on its highest reduction rate of chlorophyll, net photosynthesis and carboxylation efficiency, and highest increase rate of lipid peroxidation by ozone fumigation in our research. Therefore, in conclusion, the results of this experiment support three-tested hypotheses. Ozone exposure enhanced leaf senescence-related processes and induced reduction of chlorophyll content, photosynthetic decline and depletion of biomass accumulation. Our present study suggests that 1) physiological markers such as chlorophyll contents, photosynthesis, MDA content and antioxidative enzymes were considered as the very important indicators in order to evaluate the tolerance against ozone stress, and 2) parameters were closely related with each other and thus they cannot be used as a single marker. The results of this study highlight the species specificity of ozone responses.

적 요

오존에 노출된 참나무속 5종의 오존에 대한 내성 능력을 평가하기 위하여 생리생화학적 변화를 조사하였다. 150ppb 오존에 노출된 참나무속 5종(상수리나무, 갈참나무, 대왕참나무, 졸참나무, 굴참나무)의 잎에서 엽록소 함량, 광합성 특성, MDA 함량 및 항산화효소 활성이 측정되었다. 엽록소, 카로테노이드 함량, 순광합성 속도 및 탄소고정효율은 오존 처리 후에 감소하였다. 오존에 노출된 수목의 총 엽록소 함량과 탄소고정효율의 감소율은 갈참나무의 경우 15%와 34%였으며, 굴참나무의 경우 38.3%와 62.1%였다. MDA 함량은 오존 처리 하에서 증가하였으며, 상수리나무에서 140%까지 증가를 보였다. 상수리나무의 SOD 활성 증

가을(60%)은 가장 높았으며, APX 활성은 굴참나무, 졸참나무, 상수리나무에서 증가를 보였다. 생리생화학적 반응을 기초로 한 참나무속 5종의 내성 능력은 갈참나무, 대왕참나무, 졸참나무, 굴참나무, 상수리나무 순이었다. 결론적으로 엽록소 함량, 광합성 특성, MDA 함량, 항산화효소와 같은 생리학적 지표들은 오랜 스트레스에 대한 내성을 평가하기 위한 매우 중요한 지표들로 생각되며, 이러한 모수들은 서로 밀접한 관계를 가진다.

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