Environ Monit Assess (2007) 128:109–119
DOI 10.1007/s10661-006-9409-0

Ozone and/or Water Stresses Could have Influenced the *Betula ermanii* Cham. Forest Decline Observed at Oku-Nikko, Japan

Hideyuki Shimizu · Yan Wen Feng

Received: 6 February 2006 / Revised: 25 April 2006 / Accepted: 26 April 2006 / Published online: 14 December 2006

Abstract A serious forest decline of *Betula ermanii* Cham. has been observed at Mt. Mae-Shirane, Oku-Nikko, Japan, where high ozone (O_3) concentration and severe water deficiency have been measured. In order to consider the possibility whether O_3 and/or water stresses could have been the causes of the forest decline of *B. ermanii*, plant growth experiments were conducted in environment-controlled growth cabinets. Two-year-old seedlings of *B. ermanii* were exposed to either charcoal-filtered air (O_3 concentration < 5 ppb) or 50 ppb O_3 (daily average, ranging between 20–100 ppb) for 123 days at 20.0/12.5 ± 1.0°C (day/night) and 70/80 ± 7% relative humidity (day/night). Simultaneously, seedlings were treated with three watering regime: 1.0 < pF < 1.8 (no water stress), 1.8 < pF < 2.5 (mild water stress) or 2.5 < pF < 3.0 (severe water stress). O_3 exposure significantly reduced the dry weights of leaf, root and the whole plant, while water stress significantly reduced the dry weights of each organ and the whole plant. Significant reductions of net photosynthesis, transpiration and stomatal conductance were also observed under O_3 and/or water deficiency treatments, while contents of RuBP carboxylase/oxygenase (Rubisco), chlorophyll$_{a+b}$ and some essential nutrient elements (N, P, K, Mg and Ca) were not markedly changed. It was suggested that the decrease in net photosynthetic rate induced mainly by stomatal closure was the major cause of the growth reduction under O_3 and/or water stresses. No significant interactions between O_3 and water stresses were observed in terms of the depression of dry matter production, which suggested that simultaneous stress treatments of O_3 exposure and water deficiency could affect the tree growth of *B. ermanii* additively.

Keywords Additive effect · *Betula ermanii* Cham. · Forest decline · Growth reduction · Ozone (O_3) · pF · Photosynthesis · Stomata · Water stress

H. Shimizu (✉) · Y. W. Feng
National Institute for Environmental Studies,
16-2 Onogawa, Tsukuba,
305-8506 Ibaraki, Japan
e-mail: hshimizu@nies.go.jp

Present address:
Y. W. Feng
Central Research Institute of Electric Power Industry,
1646 Abiko, Abiko, 207-1194 Chiba, Japan

1 Introduction

Air pollution, particularly ozone (O_3) pollution, has been considered to be implicated in widespread damage to the forest trees in Eastern and Western Europe and throughout the United States (Ashmore, Bell, & Rutter, 1985; Johnson & Siccama, 1983; McLaughlin, 1985; Sandermann, Wellburn, & Heath, 1997). O_3 concentrations in the troposphere above Europe have increased and reached up to 70 ppb in summer in the forests at elevations of about 800–1,200 m (Feister & Warmbt, 1987). Similar trends of

O_3 concentration increase were recorded in some high altitude areas (Herman, Smidt, Loibl, & Bolhar-Nordenkampf, 2005; Oltmans et al., 1998).

At high mountains or northern regions, not only O_3 but also other environmental stresses, especially drought (water deficiency) might impact the forest trees (Dobbertin et al., 2005; Oksanen, 2005). There has been much interest in the relative magnitudes and interactive effects of these two stresses (Gorissen, Joosten, Smeulders, & Van Veen, 1994; Matyssek et al., 2006; Pääkkönen, Vahala, Pohjola, Holopainen, & Kärenlampi, 1998), while previous research results were inconsistent. Dixon, Thiec, and Garrec (1998) showed that a drought relieved the effects of O_3 on beech (*Fagus sylvatica* L.), but not on Norway spruce (*Picea abies* (L.) H. Karst.). Temple, Riechers, and Miller (1992) found that O_3 accentuated the decrease in photosynthate translocation under drought conditions in ponderosa pine (*Pinus ponderosa* Douglas ex Lawson & C. Lawson). Pearson and Mansfield (1994) found that in the following year of a drought, both O_3-treated and control beech trees (*F. sylvatica*) exhibited a reduction in shoot length. In some cases, stomatal closures under drought conditions might protect trees against O_3 uptake and damage (Beyers, Riechers, & Temple, 1992; Matyssek et al., 2006; Pääkkönen et al., 1998), while O_3 showed stomatal responses to be sluggish, which could enhance the negative effects of water stress (Paoletti, 2005).

In Japan, forest declines have been observed in several areas with several different tree species, such as Japanese fir (*Abies firma* Siebold & Zucc.) in Fukuoka Prefecture and Japanese fir (*A. firma*) and Siebold's beech (*Fagus crenata* Blume) at Mt. Tanzawa in Kanagawa Prefecture. Research evidences suggested that photochemical oxidants, especially O_3, acidic fog and water deficiency might be related to these declines (Nakane, Sakugawa, & Igawa, 2000; Suda, Sugi, Utsunomiya, Oishi, & Hamamura, 1992).

A serious damage to *Betula ermanii* Cham. and the subsequent forest decline have been reported at Mt. Mae-Shirane, a famous national park in Oku-Nikko region, Japan (Tanimoto, Liu, Satomichi, Ohkubo, & Nihei, 1996), where higher than 100 ppb O_3 concentrations have been recorded by continuous measurement with O_3 analyzers (Hatakeyama, 1999). As Mt. Mae-Shirane is located to the north-northwest (NNW) of the Tokyo metropolitan area, pollutants produced around Tokyo might be transported to Mt. Mae-Shirane with changing by photochemical reactions (Hatakeyama, 1999).

In order to clarify the damaged situation of *B. ermanii* and the environmental conditions around Mt. Mae-Shirane, field surveys were conducted during 2000 and 2001 (Feng, Ohta, & Shimizu, 2005; Feng, Shimizu, Ohta, & Izuta, 2002; Shimizu, Feng, Tobe, Kohno, & Izuta, 2002; Tamura et al., 2002). As a result, *B. ermanii* trees grown on the SE-facing slope declined apparently in stem volume, stand density and tree decline degree compared to those grown on the NW-facing slope (Feng et al., 2002, 2005). Higher O_3 concentrations were observed on the SE-facing slope than on the NW-facing slope during the survey period, and the continuous O_3 monitoring system at NIES' Oku-Nikko field station indicated that the maximum O_3 concentration could easily exceed 100 ppb in summer time (Feng et al., 2002, 2005; Fujinuma, 1991).

Although we could not detect any marked differences on soil acidification (pH decrease) nor on contents of essential elements (K, Ca and Mg) and heavy metals (Mn and Al) in soils between the SE- and the NW-facing slopes, the soil water content on the SE-facing slope was significantly lower than that on the NW-facing slope during the growing season from July to October (Feng et al., 2002; Tamura et al., 2002). These surveys suggested that O_3 and/or water stresses might have played important roles in the observed forest decline of *B. ermanii*.

In the present study, we investigated the effects of O_3 exposure and soil water stress treatments, singly or in combination, on plant growth and physiological activities such as photosynthesis and stomatal conductance of *B. ermanii* seedlings, and discussed the influence of O_3 and/or water stresses on the forest decline of *B. ermanii* observed at Oku-Nikko region, Japan.

2 Materials and Methods

2.1 Plant material and experimental design

On 4 April 2001, we transplanted 2-year-old seedlings of *B. ermanii* from a nursery, NIES Experimental Farm, into plastic pots (12 cm diameter, 20 cm height). Each seedling was grown in each pot, which contained a soil mixture of red clay (the loamy layer soil of the Kanto Region from NIES Experimental

Farm, 50%), vermiculite (25%) and Akadama (artificial horticultural soil, 25%) in an environment-controlled glasshouse under natural sunlight, with day/night temperatures of 20/15 ± 1°C (day/night) and a constant relative humidity of 70 ± 7%, until 14 June 2001. Plants were fertilized regularly (three times per week) with 100–200 ml of 0.1% Hyponex solution (Hyponex Co., Osaka, Japan) and watered daily as necessary.

These seedlings were exposed to one of two levels of O_3 and one of three soil watering regimes from 14 June till 15 October 2001 at 20.0/12.5 ± 1.0°C and 70/80 ± 7% relative humidity. Separate growth cabinets were used for the two levels of O_3 exposure: one received the charcoal-filtered air (O_3 concentration < 5 ppb), while the other received 50 ppb O_3 (daily average concentration). In the latter, O_3 concentration was gradually increased from 20 to 100 ppb between 08:00 and 13:00, maintained at 100 ppb between 13:00 and 17:00, gradually decreased from 100 to 20 ppb between 17:00 and 22:00, and maintained at 20 ppb between 22:00 and 08:00. The total exposure doses for the 123 days were 147.6 ppm h (SUM0) or 57.2 ppm h (AOT40). This exposure periodicity of O_3 concentration was based on the data of atmospheric O_3 concentrations reported for the Oku-Nikko region, where dieback of *B. ermanii* has been observed and relatively high concentrations of O_3 were recorded from May to October (Feng et al., 2002, 2005; Fujinuma, 1991; Hatakeyama, 1999).

Simultaneously, *B. ermanii* seedlings were treated with three levels of soil water stress, as conducted with three levels of water supplying regime; W0: almost no water stress condition (1.0 < pF < 1.8; 0.98 kPa < water potential < 6.19 kPa), W1: mild water stress condition (1.8 < pF < 2.5; 6.19 kPa < water potential < 30.96 kPa) or W2: severe water stress condition (2.5 < pF < 3.0; 30.96 kPa < water potential < 97.98 kPa). The pF is a soil water tension or suction and is defined as the logarithm of the negative of the water potential (matric potential) calculated as follows:

$$\mathrm{pF} = \log(-10.2\Phi)$$

where Φ is the soil water potential (kPa).

The pF values were measured continuously by using the pF porous ceramic cup (sensor) putting into the center of potted soil and pF meters (DIK-3060, Daiki Rika Kogyo Co., Tokyo, Japan). All seedlings were irrigated to maintain the water contents within a desired range of pF values, and the average soil water contents during the experiments were 38% (W0), 25% (W1) and 17% (W2), respectively.

We called these six treatments as follows: NW0 (< 5 ppb O_3, W0), NW1 (< 5 ppb O_3, W1), NW2 (< 5 ppb O_3, W2), OW0 (50 ppb O_3, W0), OW1 (50 ppb O_3, W1) and OW2 (50 ppb O_3, W2). For each treatment, eight seedlings of *B. ermanii* were grown for 123 days.

2.2 Growth analysis

We harvested eight seedlings of *B. ermanii* just before the treatments for initial value, and harvested the rest of seedlings in each treatment at the end of the experiment. Seedlings were divided into leaf, stem and roots. Leaf area was measured using an automatic leaf-area meter (LI-3100, LI-COR, Inc., Lincoln, NE, USA). Each plant organ was then dried at 80°C for 72 h and weighed. We calculated the relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), leaf weight ratio (LWR) and specific leaf area (SLA) from the mean values of dry weights and leaf area as follows (Evans, 1972):

$$\begin{aligned}\mathrm{RGR} &= 1/W\ dW/dt \\ &= (\ln W_2 - \ln W_1)/(t_2 - t_1)\end{aligned} \tag{1}$$

$$\begin{aligned}\mathrm{NAR} &= 1/A\ dW/dt \\ &= [(W_2 - W_1)(\ln A_2 - \ln A_1)]/ \\ &\quad [(t_2 - t_1)(A_2 - A_1)]\end{aligned} \tag{2}$$

$$\begin{aligned}\mathrm{LAR} &= A/W \\ &= [(A_2 - A_1)(\ln W_2 - \ln W_1)]/ \\ &\quad [(W_2 - W_1)(\ln A_2 - \ln A_1)]\end{aligned} \tag{3}$$

$$\begin{aligned}\mathrm{LWR} &= L/W \\ &= [(L_2 - L_1)(\ln W_2 - \ln W_1)]/ \\ &\quad [(W_2 - W_1)(\ln L_2 - \ln L_1)]\end{aligned} \tag{4}$$

$$\begin{aligned}\mathrm{SLA} &= A/L \\ &= [(A_2 - A_1)(\ln L_2 - \ln L_1)]/ \\ &\quad [(L_2 - L_1)(\ln A_2 - \ln A_1)]\end{aligned} \tag{5}$$

where W_i, A_i and L_i are the mean values of dry weight of the whole plant (g), the leaf area (cm^2) and the leaf dry weight (g) at time t_i (t_1: initial and t_2: final harvests), respectively.

Then, RGR is the product of NAR by LAR, and LAR is the product of LWR by SLA.

2.3 Gas exchange measurements

We measured CO_2 gas exchange rates of the fourth to seventh leaf from the apex of six randomly selected seedlings per treatment at about 16 weeks after initiation of O_3 exposure and/or water stress treatments with a Portable Photosynthesis System (CIRAS-1, PP Systems Co., Hitchin, UK) equipped with a 250 cm^3 cuvette. The system's software calculated the net photosynthetic rate, the transpiration rate and the stomatal conductance of the leaves. During the gas exchange measurements, atmospheric CO_2 concentration, air temperature and relative air humidity in the cuvette were regulated at 380 ± 10 ppm, 20 ± 0.5°C and 70 ± 5%, respectively. Photosynthetic photon flux density at the leaf surface was averaged at 580 ± 20 μmol m^{-2} s^{-1}.

2.4 Measurement of chlorophyll$_{a+b}$, RuBP carboxylase/oxygenase (Rubisco) and element contents

At the end of the experiment, chlorophyll contents were measured using the fourth to seventh leaf from the apex of the six seedlings per treatment, which were used for the gas exchange measurements. We extracted the chlorophyll from leaf discs using 5 ml of 80% acetone. The absorption of extracts was measured at 663 and 645 nm with a spectrophotometer (UV-1200, Hitachi Co., Tokyo, Japan), and the concentration of chlorophyll$_{a+b}$ was calculated using the formulae proposed by Arnon (1949).

We homogenized 0.1 g fresh leaves which had been used in the gas exchange measurements in 1 ml extraction buffer containing 100 mM HEPES (pH 7.5, 25°C), 5 mM EDTA, 0.7% polyethylene glycol 20,000 (w/v), 2% polyvinylpolypyrrolidone (PVPP, insoluble, w/v), 1 mM phenylmethylsulfonylfluoride (PMSF) and 0.2% β-mercaptoethanol (v/v). The leaf homogenates were then centrifuged at 9,000 × g for 10 min at room temperature. We diluted the supernatant fluid twice with the extraction buffer mentioned above, then treated the supernatant with the following sample buffer at 95°C for 5 min: 0.1 M Tris-HCl (pH 6.8), 12% β-mercaptoethanol (v/v), 20% glycerol (v/v), 4% sodium dodecylsulfate (SDS, w/v) and 0.01% bromophenol blue. The amounts of Rubisco were determined spectrophotometrically after formamide extraction of Coomassie brilliant blue R-250-stained subunit bands separated by SDS-PAGE (Shimizu et al., 2002).

We digested 1.2 g dried leaf in 9 ml HCl (36%) and 3 ml HNO_3 (61%), and determined the concentrations of potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P) by means of inductively coupled plasma mass spectrometry (ICP-750, Nippon Jarrell-Ash., Kyoto, Japan). The nitrogen (N) concentrations were analyzed using a CN analyzer (NC-90A, Shimadzu Co., Kyoto, Japan).

2.5 Data analysis

We performed the analysis of variance (ANOVA) of our results using SPSS software (SPSS Japan Inc, 2002. The significances of main and interactive effects on each parameter were calculated using a two-way ANOVA with O_3 and water stresses as independent factors. We considered the values to be significant at $P < 0.05$ or $P < 0.01$.

3 Results

3.1 Growth responses

During the experimental period of 123 days, we did not observe any visible symptoms caused by O_3 exposure and/or water deficiency on leaves of *B. ermanii* seedlings, whereas plant growth was remarkably affected by O_3 and water stresses, singly or in combination. The dry weights of all organs and the whole plant of *B. ermanii* seedlings at the final harvest are given in Figure 1 and the significances of main and interactive effects of O_3 and water stresses by ANOVA are shown in Table I. Significant main effects of O_3 were observed on the dry weights of leaf, root and the whole plant ($P < 0.05$), while significant main effects of water stress were detected on the dry weights of all organs and the whole plant ($P < 0.05$ or $P < 0.01$). There were no significant interactions between O_3 and water stresses on all dry

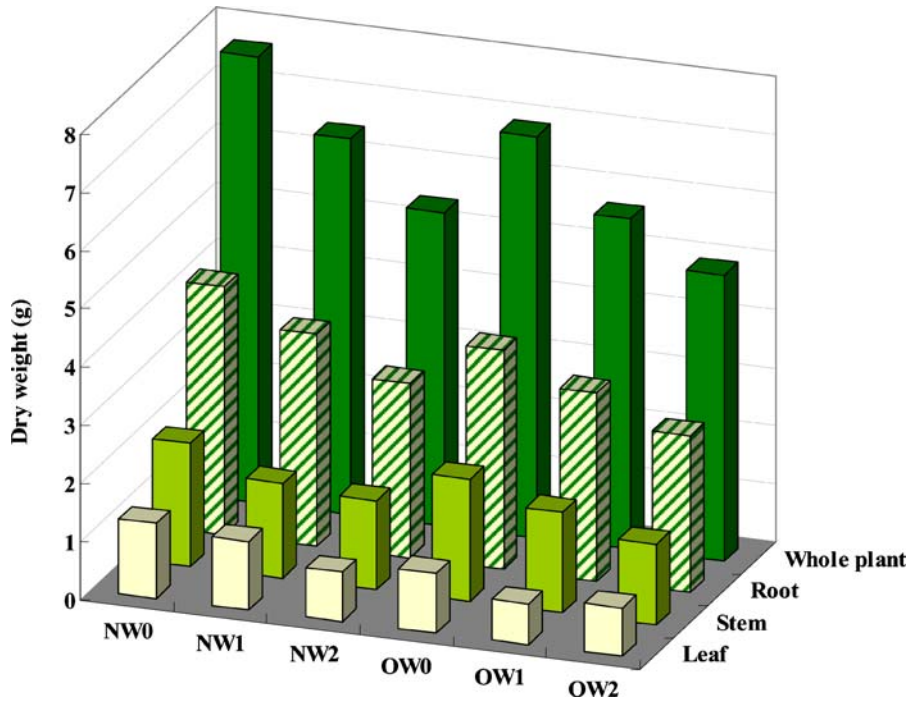

Figure 1 Effects of O_3 and/or water stresses on the dry weight growth of *Betula ermanii* Cham. The mean value of eight seedlings was shown in each treatment. *NW0* <5 ppb O_3/1.0<pF<1.8, *NW1* <5 ppb O_3/1.8<pF<2.5, *NW2* <5 ppb O_3/2.5<pF<3.0, *OW0* 50 ppb O_3/1.0<pF<1.8, *OW1* 50 ppb O_3/1.8<pF<2.5, *OW2* 50 ppb O_3/2.5<pF<3.0.

weight parameters ($P > 0.05$), which suggested that these two environmental factors reduced the growth of *B. ermanii* additively when plants were treated by O_3 and water stresses simultaneously. O_3 decreased the final dry weight of the whole plant by about 10% relative to no O_3. Mild water stress (W1), and severe water stress (W2) decreased the final dry weight of the whole plant by 17% and 29%, respectively, relative to no water stress (W0).

The results of the growth analysis of *B. ermanii* seedlings are shown in Figure 2. The average reduction in RGR by 50 ppb O_3 relative to no O_3 was 8%, and those by water stress relative to W0 were 13% in W1 and 24% in W2, respectively. The simultaneous treatment of O_3 and the severe water stress (OW2) reduced the RGR by 30% as compared with the control (NW0) treatment. NAR was also affected by O_3 and water stresses, with similar reduction tendency as RGR, whereas OW2 treatment reduced NAR by 35% as compared with NW0 treatment. Although there was not an obvious effect on LAR of *B. ermanii*, OW2 treatment slightly increased LAR relative to the other treatments. SLA was hardly reduced by water stress, whereas it was increased by O_3 exposure by about 12% relative to no O_3. The dry weight ratio of each organ to the whole plant, such as LWR, indicated that the partitioning of photosynthate was hardly affected by O_3 and/or water stresses.

3.2 Physiological/biochemical responses

Net photosynthetic rate, transpiration rate and stomatal conductance of *B. ermanii* seedlings are shown in Figure 3, and the significances of main and interactive effects of O_3 and water stresses are shown in Table II. Significant main effects of O_3 and water stresses were observed on net photosynthetic rate ($p < 0.01$ or $p < 0.05$), whereas no significant interaction between

Table I The main and interactive effects of O_3 and/or Ws on the dry weight growth of *Betula ermanii* Cham. by ANOVA

	Main effect		Interaction
	O_3	Ws	$O_3 \times$ Ws
Leaf dry weight	*	*	NS
Stem dry weight	NS	**	NS
Root dry weight	*	**	NS
Whole plant dry weight	*	*	NS

*$P<0.05$; **$P<0.01$ (significant levels).

NS Not significant, *O_3* ozone, *Ws* water stresses.

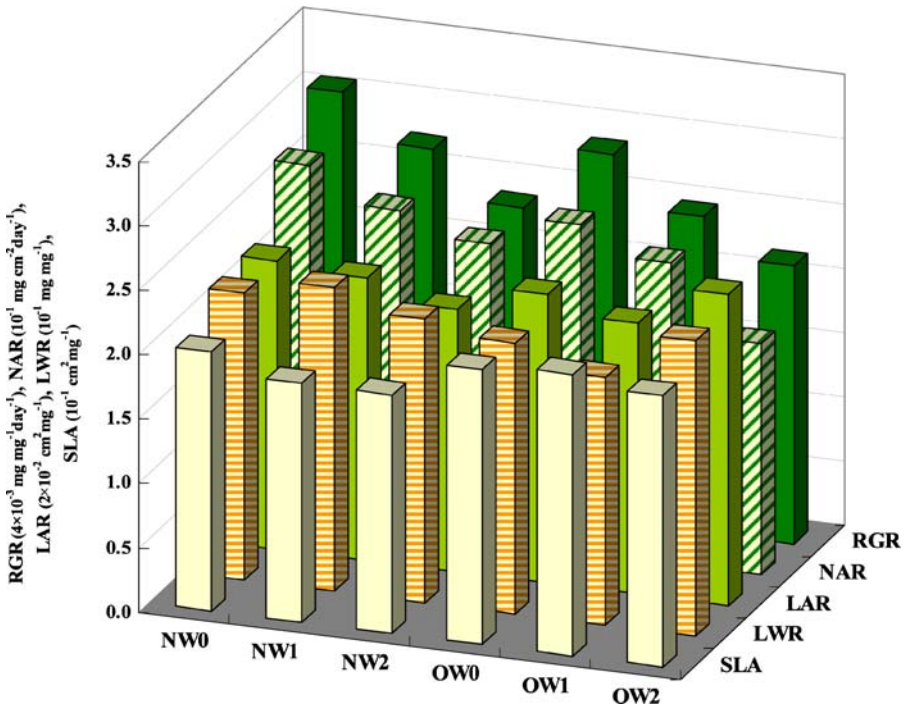

Figure 2 Effects of O_3 and/or water stresses on the growth parameters of *Betula ermanii* Cham. Each value in each treatment was calculated from the mean values of dry weights and leaf area as mentioned in Section 2. *RGR* relative growth rate, *NAR* net assimilation rate, *LAR* leaf area ratio, *LWR* leaf weight ratio, *SLA* specific leaf area, *NW0* <5 ppb O_3/1.0<pF<1.8, *NW1* <5 ppb O_3/1.8<pF<2.5, *NW2* <5 ppb O_3/2.5<pF<3.0, *OW0* 50 ppb O_3/1.0<pF<1.8, *OW1* 50 ppb O_3/1.8<pF<2.5, *OW2* 50 ppb O_3/2.5<pF<3.0.

these two factors was detected ($p > 0.05$). O_3 reduced the net photosynthetic rate by an average of 36% relative to no O_3, while water stress reduced the rate by 13% in W1 and 29% in W2, respectively, relative to W0. Then, the simultaneous treatment (OW2) severely reduced the net photosynthetic rate by 54% as compared with NW0 treatment, which suggests that O_3 exposure and water deficiency could restrict the net photosynthetic rate of *B. ermanii* leaves additively.

O_3 exposure or water deficiency reduced the transpiration rate and also the stomatal conductance more remarkably than the net photosynthetic rate

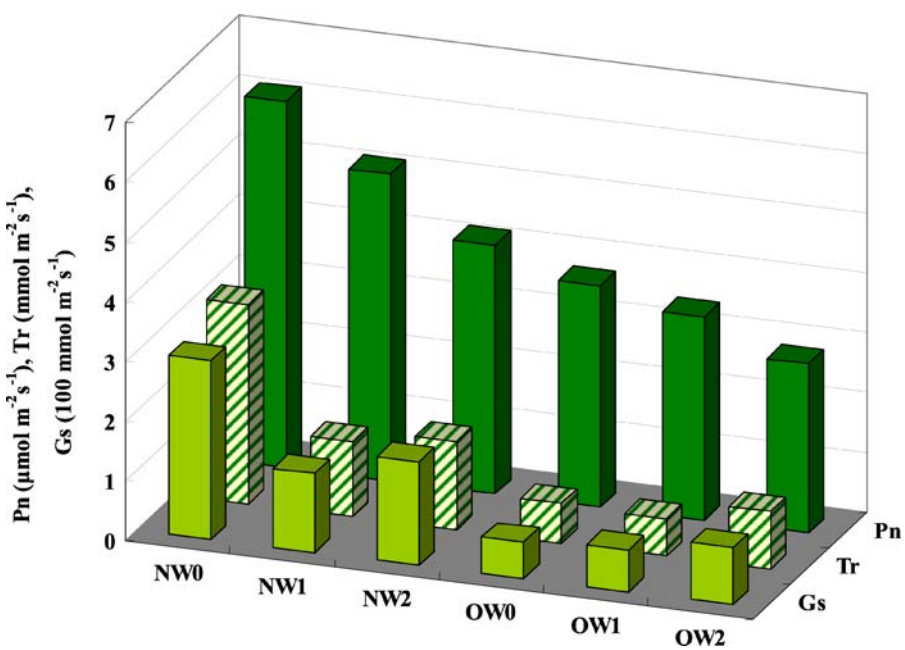

Figure 3 Effects of O_3 and/or water stresses on the physiological responses of *Betula ermanii* Cham. The mean value of six seedlings was shown in each treatment. *Pn* net photosythetic rate, *Tr* transpiration rate, *Gs* stomatal conductance, *NW0* <5 ppb O_3/1.0<pF<1.8, *NW1* <5 ppb O_3/1.8<pF<2.5, *NW2* <5 ppb O_3/2.5<pF<3.0, *OW0* 50 ppb O_3/1.0<pF<1.8, *OW1* 50 ppb O_3/1.8<pF<2.5, *OW2* 50 ppb O_3/2.5<pF<3.0.

Table II The main and interactive effects of O_3 and/or Ws on the physiological responses of *Betula ermanii* Cham. by ANOVA

	Main effect		Interaction
	O_3	Ws	O_3 × Ws
Net photosynthetic rate	**	*	NS
Transpiration rate	**	**	**
Stomatal conductance	**	**	**

*P<0.05; **P<0.01 (significant levels).

NS Not significant, *O_3* ozone, *Ws* water stresses.

(Figure 3). As compared with NW0 treatment, the mild water stress without O_3 exposure (NW1) reduced the transpiration rate and the stomatal conductance by 62% and 56%, respectively, while O_3 exposure without water stress (OW0) reduced both of them by 80%. From the results of ANOVA, we observed not only the significant main effects of both O_3 and water stresses ($P < 0.01$) but also the significant interactions (antagonistic; $P < 0.01$) between these two environmental factors (Table II).

We analyzed some important chemicals related to photosynthesis, such as chlorophyll$_{a+b}$ and Rubisco and some essential elements i.e., N, P, K, Ca and Mg in leaves (Table III). However, we could not detect any main nor interactive effects of O_3 and water stresses on all these chemicals and elements ($P > 0.05$).

4 Discussion

4.1 Effects of ozone stress

It has been widely reported that O_3 retards the growth of some sensitive tree species in the field and in laboratory exposures (Bortier, Ceulemans, & De Temmerman, 2000; Dixon et al., 1998; Feng & Shimizu, 2005; Feng et al., 2005; Matsumura, 2001; Panek, Kurpius, & Goldstein, 2002; Pye, 1988; Shimizu, Fujinuma, Kubota, Totsuka, & Omasa, 1993; Van Leeuwen et al., 2000). In the present study, the dry weight growth of *B. ermanii* was significantly reduced by O_3 exposure (Figure 1, Table I). O_3-induced reduction in RGR was mainly caused by the reduction in NAR (Figure 2). Similar results have been reported in other grass and tree species (Feng & Shimizu, 2005; Izuta, Takahashi, Matsumura, & Totsuka, 1999). The reduction in NAR by O_3 has been reported as mainly caused by the inhibition of photosynthesis (Dixon et al., 1998; Greitner, Pell, & Winner, 1994; Reich, 1983). The net photosynthetic rate in the present study decreased

Table III Effects of O_3 and/or water stresses on the biochemical responses of *Betula ermanii* Cham. with the results of ANOVA. The mean value of six seedlings was shown in each treatment

	Treatment						Two-way ANOVA[a]		
							Main effect		Interaction
	NW0	NW1	NW2	OW0	OW1	OW2	O_3	Ws	O_3 × Ws
Chlorophyll$_{a+b}$ (mg g^{-1} FW)[b]	1.75	1.86	1.81	1.52	2.00	2.21	NS	NS	NS
Rubisco (relative value)[c]	1.00	0.55	0.65	0.58	0.28	0.90	NS	NS	NS
N (mg g^{-1} DW)[d]	2.65	2.99	2.86	3.17	2.73	3.03	NS	NS	NS
P (mg g^{-1} DW)[d]	1.94	1.83	2.08	2.57	2.02	1.65	NS	NS	NS
Mg (mg g^{-1} DW)[d]	4.45	3.92	4.44	3.70	3.96	3.91	NS	NS	NS
K (mg g^{-1} DW)[d]	10.60	7.95	8.35	15.83	11.82	7.97	NS	NS	NS
Ca (mg g^{-1} DW)[d]	19.45	17.72	22.19	18.85	19.53	18.78	NS	NS	NS

[a] Significant levels of ANOVA; *NS*: not significant.

[b] The value is shown on a basis of leaf fresh weight.

[c] The value is shown as a relative value to that of NW0 treatment.

[d] Each element content is shown on a basis of leaf dry weight.

NW0 <5 ppb O3/1.0<pF<1.8, *NW1* <5 ppb O3/1.8<pF<2.5, *NW2* <5 ppb O3/2.5<pF<3.0, *OW0* 50 ppb O3/1.0<pF<1.8, *OW1* 50 ppb O3/1.8<pF<2.5, *OW2* 50 ppb O3/2.5<pF<3.0.

more remarkably in the response to O_3 than the reduction in NAR (Figures 2 and 3), which suggested that the photosynthetic damage was gradually accumulated in leaves during O_3 exposure for 123 days. This reduction in photosynthesis might mainly be caused by the O_3-induced stomatal closure (Figure 3, Table II) as reported by Wieser and Havranek (1995).

Shan, Izuta, Aoki, and Totsuka (1997) reported that the significant reduction in chlorophyll content probably occurred only at the high/acute levels of O_3 exposures. Pell, Eckardt, and Enyedi (1992) demonstrated that the O_3-induced reduction in net photosynthetic rate was related to a decrease in the quantity and activity of Rubisco. In the present study, however, chlorophyll, Rubisco and some essential elements of *B. ermanii* leaves were not significantly affected by O_3 (Table III). More research on these factors, especially on Rubisco which is a key enzyme involved in the initial capture of CO_2 in the chloroplasts, should be conducted precisely in this species.

In the present study, we did not observe any significant effects of O_3 on the photosynthate partitioning among organs, while SLA related to leaf thinness was somewhat increased by O_3 in all water stress treatments (Figure 2). This might compensate the O_3-induced reduction in net photosynthetic rate, and could result in less reduction of dry weight growth of *B. ermanii*.

4.2 Effects of soil water stress

When soil water stress was imposed, *B. ermanii* seedlings showed significant reductions in the dry weight of all organs and the whole plant, and there was a tendency toward increase in growth reduction with increasing soil water deficiency (Figure 1, Table I). RGR decreased with water stress, which was mainly caused by the reduction in NAR (Figure 2). These results confirmed the well-known observation that water stress could reduce dry matter production (Feng & Shimizu, 2005; Kramer & Boyer, 1995; Zheng & Shimizu, 2005). The reduction in NAR may be mainly induced by a significant negative effect on the net photosynthetic rate, which was mainly related to a remarkable reduction in stomatal conductance (Figure 3, Table II). In the present study, we could not detect any significant water stress effects on the contents of chlorophyll$_{a+b}$, Rubisco and essential elements in *B. ermanii* leaves (Table III). These results suggested that water stress could cause stomatal closure, which reduced the uptake of atmospheric CO_2 through stomata. This water stress-induced reduction in net photosynthetic rate could result in the reduction of dry weight growth of *B. ermanii*. Our results agreed well with those of Beyers et al. (1992) and Dixon et al. (1998).

4.3 Combined effects of ozone and water stresses

In the present study, we found no significant interactions between O_3 and water stresses on the growth of *B. ermanii* seedlings (Table I). It suggested that these two environmental stress factors were independent and impacted additively. Several studies have shown similar results in *F. sylvatica* (Dixon et al., 1998), *Populus tremuloides* Michx. (Greitner et al., 1994) and *P. ponderosa* (Beyers et al., 1992). The severe dry weight reduction of *B. ermanii* seedlings treated simultaneously with O_3 and water stresses should be caused by the reduction in net photosynthetic rate, which was also affected additively by both environmental stresses (Table II).

Beyers et al. (1992) suggested that drought might protect trees from O_3 damage via drought-induced stomatal closure, which would reduce the O_3 flux into the foliage. Seedlings growing in dry conditions might be protected to some degree from O_3 damage, but their growth would be limited via stomatal closure-induced reduction of photosynthesis (Matyssek et al., 2006). In the present study, however, we just found the combined effects (significant antagonistic interactions) of O_3 and water stresses on transpiration rate and stomatal conductance in *B. ermanii* leaves (Table II). Each treatment of O_3 exposure or water deficiency induced a marked stomatal closure, while a relative increase in stomatal conductance with simultaneous treatments of these two stresses suggested that the physiological mechanism of stomatal movement might be sluggish or uncontrolled in the leaves of *B. ermanii* as reported in Paoletti (2005). In the condition of O_3 exposure with water deficiency, drought-induced stomatal closure might be partially released, which would increase the O_3 flux into the foliage and could result in more growth reduction. Therefore, *B. ermanii* seedlings growing under water stressed conditions may be more damaged by O_3 exposure.

4.4 Consideration on the forest decline observed at Oku-Nikko region

The present experiments confirmed that a serious growth reduction was induced by the simultaneous treatments of O_3 exposure and water deficiency in seedlings of *B. ermanii*. The levels of O_3 concentration and water deficiency treated in the experiments were not so far from those measured at a declined area of *B. ermanii* forest at Mt. Mae-Shirane, Oku-Nikko, Japan. However, it might not be easy to apply the present results using juvenile seedlings directly to the declined adult trees in the field (Oksanen, 2005).

As compared with old trees, young seedlings seemed to be more sensitive to O_3 (*F. sylvatica*, Oksanen, 2005) or to water stress (*Acea rubrum* L. and *Cornus florida* L., Hanson, Todd, & Amthor, 2001), even if growth reductions were observed in old trees but not in young seedlings of *Betula pendula* Roth in response to O_3 (Oksanen, 2003). Furthermore, some tree species which were not affected by O_3 exposure for a few years, showed significant growth reductions by consecutive exposures for several years (Karnosky et al., 2005; Matsumura, 2001). Damage to trees may be influenced by many factors including seedling age and/or size, exposure dose and complex environmental conditions.

However, our previous field surveys at Mt. Mae-Shirane in Oku-Nikko region showed a significant lower soil water content, a significant higher mean O_3 concentration and no marked difference on soil acidification, toxic metals release and essential elements deficiency in soil at the declining area (the Tokyo metropolitan direction) as compared with a healthy area (Feng et al., 2002, 2005; Shimizu et al., 2002; Tamura et al., 2002). Based on the results of the present study, of a previous study on plant sensitivity to O_3 under similar experimental conditions (Shimizu et al., 1993) and of field surveys mentioned above, O_3 and water stresses are suggested among the factors of *B. ermanii* forest decline at Mt. Mae-Shirane, Oku-Nikko, Japan. It is generally accepted that a forest decline could not be induced by a single factor but should be related to two or more environmental stress factors (Oksanen, 2005; Van Leeuwen et al., 2000).

In order to clarify the observed forest decline at Oku-Nikko region, continuous measurements of O_3 concentrations and other environmental factors, including soil water content, and a long-term monitoring on tree growth in the field should be necessary, as well as precise experiments using older trees of *B. ermanii* under similar environmental conditions in the field.

Acknowledgments We would like to thank Mr. N. Ohta and Ms. H. Komatsuzaki, Ms. C. Nakazaki, Ms. C. Matsubara, and Ms. H. Wakatsuki in our laboratory for supporting harvests, data analyses and preparation of graphs and tables. We also express our thanks to the staffs of Kawakami Farm Co, Ltd, Sumitomo Seika Chemicals Co, Ltd and Koito Industries, Ltd for supporting to breed plant materials and to operate environment-controlled growth cabinets and gas exposures. We would like to express our appreciations to Drs. Y. Kohno, T. Izuta and T. Hakamata for their valuable comments, discussion and encouragement. We also wish to express our gratitude to Drs. E. Paoletti and Y. Zheng for checking the contents and English in this paper. This study was financially supported by the Global Environment Research Fund, Ministry of the Environment, Japan.

References

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology*, *24*, 1–15.

Ashmore, M., Bell, N., & Rutter, J. (1985). The role of ozone in forest damage in West Germany. *Ambio*, *14*, 81–87.

Beyers, J. L., Riechers, G. H., & Temple, P. J. (1992). Effects of long-term ozone exposure and drought on the photosynthetic capacity of ponderosa pine (*Pinus ponderosa* Laws.). *New Phytologist*, *122*, 81–90.

Bortier, K., Ceulemans, R., & De Temmerman, L. (2000). Effects of ozone exposure on growth and photosynthesis of beech seedlings. *New Phytologist*, *146*, 270–280.

Dixon, M., Thiec, D. L., & Garrec, J. P. (1998). Reactions of Norway spruce and beech trees to 2 years of ozone exposure and episodic drought. *Environmental and Experimental Botany*, *40*, 77–91.

Dobbertin, M., Mayer, P., Wohlgemuth, T., Feldmeyer-Christe, E., Graf, U., Zimmermann, N. E., et al. (2005). The decline of *Pinus sylvestris* L. forests in the Swiss Rhone valley—A result of drought stress? *Phyton*, *45*(4), 153–156.

Evans, G. C. (1972). *The quantitative analysis of plant growth*. London: Clowes.

Feister, U., & Warmbt, W. (1987). Long-term measurements of surface ozone in the German Democratic Republic. *Journal of Atmospheric Chemistry*, *5*, 1–21.

Feng, Y. W., & Shimizu, H. (2005). Effects of ozone and/or water stress on the growth of *Abies veitchii* seedlings. *Phyton*, *45*(4), 591–594.

Feng, Y. W., Ohta, N., & Shimizu, H. (2005). Decline of *Betula ermanii* with special reference to ozone concentration at Mt. Mae-Shirane, Oku-Nikko, Japan. *American Journal of Applied Sciences*, *2*(3), 701–706.

Feng, Y. W., Shimizu, H., Ohta, N., & Izuta, T. (2002). Research on decline of *Betula ermanii* related to some environmental factors. In M. Saito & T. Hakamata (Eds.), *Quantitative*

analysis of terrestrial ecosystem decline related to acidic/ oxidative substances, Final Report of Global Environment Research Fund (pp. 141–156). Tokyo: MOE, Japan.

Fujinuma, Y. (1991). Concerning the weather and atmospheric environments at Okunikko region. In T. Iwakuma (Ed.), *Environments and Biota of the Nikko Area*, Reports from Okunikko Field Station (1988–1990), F-29-'91/NIES, Japan (pp. 1–11). Tsukuba: NIES.

Gorissen, A., Joosten, N. N., Smeulders, S. M., & Van Veen, J. A. (1994). Effects of short-term ozone exposure and soil water availability on the carbon economy of juvenile Douglas-fir. *Tree Physiology*, *14*, 647–657.

Greitner, S., Pell, E., & Winner, W. (1994). Analysis of aspen foliage exposed to multiple stresses: Ozone, nitrogen deficiency and drought. *New Phytologist*, *127*, 579–589.

Hanson, P. J., Todd D. E., & Amthor, J. S. (2001). A six-year study of sapling and large-tree growth and mortality responses to natural and induced variability in precipitation and throughfall. *Tree Physiology*, *21*, 345–358.

Hatakeyama, S. (1999). Forest decline and acidic/oxidative substances at Oku-Nikko region. *Environmental Science*, *12*(2), 227–232.

Herman, F., Smidt, S., Loibl, W., & Bolhar-Nordenkampf, H. R. (2005). Evaluation of the ozone-related risk for Austrian forests. In K. Omasa, I. Nouchi & L. J. De Kok (Eds.), *Plant responses to air pollution and global change* (pp. 53–61). Tokyo: Springer.

Izuta, T., Takahashi, K., Matsumura, H., & Totsuka, T. (1999). Cultivar difference of *Brassica campestris* L. in the sensitivity to O_3 based on the dry weight growth. *Journal of Japan Society for Atmospheric Environment*, *34*(2), 137–146.

Johnson, A. H., & Siccama, T. G. (1983). Acid deposition and forest decline. *Environmental Science and Technology*, *17*, 294–305.

Karnosky, D. F., Pregitzer, K. S., Zak, D. R., Kubiske, M. E., Hendrey, G. R., Weinstein, D., et al. (2005). Scaling ozone responses of forest trees to the ecosystem level in a changing climate. *Plant, Cell and Environment*, *28*, 965–981.

Kramer, P. J., & Boyer, J. S. (1995). *Water Relations of Plants and Soils*. New York: Academic.

Matsumura, H. (2001). Impacts of ambient ozone and/or acid mist on the growth of 14 tree species: An open-top chamber study conducted in Japan. *Water, Air, and Soil Pollution*, *130*, 959–964.

Matyssek, R., Le Thiec, D. Löw, M., Dizengremel, P., Nunn, A. J., & Häberle, K.-H. (2006). Interactions between drought and O_3 stress in forest trees. *Plant Biology*, *8*, 11–17.

McLaughlin, S. B. (1985). Effects of air pollution on forests—A critical review. *Journal of the Air Pollution Control Association*, *35*, 512–534.

Nakane, K., Sakugawa, H., & Igawa, M. (2000). Air pollution and tree damage in the pine or fir forest decline area at Hiroshima or Kanagawa Prefecture, Japan. *Japanese Journal of Ecology*, *50*, 319–324.

Oksanen, E. (2003). Responses of selected birch (*Betula pendula* Roth.) clones to ozone change over time. *Plant, Cell and Environment*, *26*, 875–886.

Oksanen, E. (2005). Northern conditions enhance the susceptibility of birch (*Betula pendula* Roth) to oxidative stress caused by ozone. In K. Omasa, I. Nouchi & L. J. De Kok (Eds.), *Plant responses to air pollution and global change* (pp. 29–35). Tokyo: Springer.

Oltmans, S. J., Lefohn, A. S., Scheel, H. E., Harris, J. M., Levy II, H., Galbally, I. E., et al. (1998). Trends of ozone in the troposphere. *Geophysical Research Letters, 25*, 139–142.

Pääkkönen, E., Vahala, J., Pohjola, M., Holopainen, T., & Kärenlampi, L. (1998). Physiological, stomatal and ultrastructural ozone responses in birch (*Betula pendula* Roth.) are modified by water stress. *Plant, Cell and Environment*, *21*, 671–684.

Panek, J. A., Kurpius, M. R., & Goldstein, A. H. (2002). An evaluation of ozone exposure metrics for a seasonally drought stressed ponderosa pine ecosystem. *Environmental Pollution*, *117*, 93–100.

Paoletti, E. (2005). Ozone slows stomatal response to light and leaf wounding in a Mediterranean evergreen broadleaf, *Arbutus unedo*. *Environmental Pollution*, *134*, 439–445.

Pearson, M., & Mansfield, T. A. (1994). Effects of exposure to ozone and water stress on the following season's growth of beech. *New Phytologist*, *126*, 511–515.

Pell, E. J, Eckardt, N., & Enyedi, A. J. (1992). Timing of ozone stress and resulting status of ribulose bisphosphate carboxylase/oxygenase and associated net photosynthesis. *New Phytologist*, *123*, 351–358.

Pye, J. M. (1988). Impact of ozone on the growth and yield of trees: A review. *Journal of Environmental Quality*, *17*, 347–360.

Reich, P. B. (1983). Effects of low concentrations of ozone on net photosynthesis, dark respiration and chlorophyll contents in aging hybrid poplar leaves. *Plant Physiology*, *73*, 291–296.

Sandermann, H., Wellburn, A., & Heath, R. L. (Eds.) (1997). *Forest decline and ozone: A comparison of controlled chamber and field experiments.* Berlin: Springer.

Shan Y. F., Izuta, T., Aoki, M., & Totsuka, T. (1997). Effects of O_3 and soil acidification, alone and in combination, on growth, gas exchange rate and chlorophyll content of red pine seedlings. *Water, Air and Soil Pollution*, *97*, 355–366.

Shimizu, H., Feng, Y. W., Tobe, K., Kohno, Y., & Izuta, T. (2002). Evaluation on the effects of environmental factors on eco-physiological characteristics of plants grown in the forest declined region. In M. Saito & T. Hakamata (Eds.), *Quantitative analysis of terrestrial ecosystem decline related to acidic/oxidative substances*, Final Report of Global Environment Research Fund (pp. 59–89). Tokyo: MOE, Japan.

Shimizu, H., Fujinuma, Y., Kubota, K., Totsuka, T., & Omasa, K. (1993). Effects of low concentrations of ozone (O_3) on the growth of several woody plants. *Journal of Agricultural Meteorology*, *48*(5), 723–726.

SPSS Japan Inc. (2002). *SPSS® Base 11.5J User's Guide* (pp. 279–287). Tokyo: SPSS.

Suda, R., Sugi, Y., Utsunomiya, A., Oishi, O., & Hamamura, K. (1992). Decline of Japanese fir trees in Mt. Homan, Fukuoka Prefecture. *Journal of Resources and Environment*, *28*, 1344–1348.

Tamura, T., Yonekura, T., Nakaji, T., Feng, Y. W., Shimizu, H., & Izuta T. (2002). Field survey on phenological characteristics and leaf components of *Betula ermanii* Cham. and soil chemical property around Mt. Mae-Shirane, Oku-Nikko, Japan. *Journal of Japan Society for Atmospheric Environment*, *37*(5), 320–330.

Tanimoto, T., Liu, Y., Satomichi, T., Ohkubo, T., & Nihei, K. (1996). Forests damage and its environmental condition in the Okunikko, Ashio, and Akagi mountainous region, northern Kanto district, central Japan. *Japanese Journal of Forest Environment*, *38*(1), 1–12.

Temple, P., Riechers, G., & Miller, P. (1992). Foliar injury responses of Ponderosa pine seedlings to ozone, wet and dry acidic deposition, and drought. *Environmental and Experimental Botany*, *32*(2), 101–113.

Van Leeuwen, E. P., Hendriks, C. M. A., de Joung, E., Erisman, J. W., Klap, J. M., & de Vries, W. (2000). Effects of environmental stress on forest crown condition in Europe. Part 2: Estimation of stress induced by meteorology and air pollutants. *Water, Air and Soil Pollution*, *119*, 335–362.

Wieser, G., & Havranek, W. (1995). Environmental control of ozone uptake in *Larix deciduas* (Mill): A comparison between different altitudes. *Tree Physiology*, *15*, 253–258.

Zheng, Y., & Shimizu, H. (2005). Plant growth and water use efficiency of four Chinese conifer tree species under different air humidity. *Phyton*, *45*(4), 575–582.