Trees (2004) 18: 581–588
DOI 10.1007/s00468-004-0345-8

ORIGINAL ARTICLE

Tetsushi Yonekura · Masatoshi Yoshidome · Makoto Watanabe · Yukie Honda · Isao Ogiwara · Takeshi Izuta

Carry-over effects of ozone and water stress on leaf phenological characteristics and bud frost hardiness of *Fagus crenata* seedlings

Received: 21 November 2003 / Accepted: 5 May 2004 / Published online: 10 July 2004

Abstract We examined the carry-over effects of ozone (O_3) and/or water stress on leaf phenological characteristics and bud frost hardiness of *Fagus crenata* seedlings. Three-year-old seedlings were exposed to charcoal-filtered air or 60 nl l^{-1} O_3, 7 h a day, from May to October 1999 in naturally-lit growth chambers. Half of the seedlings in each gas treatment received 250 ml of water at 3-day intervals (well-watered treatment), while the rest received 175 ml of water at the same intervals (water-stressed treatment). All the seedlings were moved from the growth chambers to an experimental field on October 1999, and grown until April 2000 under field conditions. The exposure to O_3 during the growing season induced early leaf fall and reduction in leaf non-structural carbohydrates concentrations in the early autumn, as well as resulting in late bud break and reduction in the number of leaves per bud in the following spring. However, O_3 did not affect bud frost hardiness in the following winter. On the contrary, water stress did not affect leaf phenological characteristics, leaf and bud non-structural carbohydrates concentrations and bud frost hardiness. There were no significant synergistic or antagonistic effects of O_3 and water stress on leaf phenological characteristics, concentrations of leaf and bud non-structural carbohydrates and bud frost hardiness of the seedlings. These results show that the carry-over effects of O_3 can be found on the phenological characteristics and leaf non-structural carbohydrates concentrations, although there are almost no carry-over effects of water stress on phenological characteristics and winter hardiness of the seedlings.

Keywords *Fagus crenata* · Ozone · Water stress · Phenology · Frost hardiness

T. Yonekura
Center for Environmental Science in Saitama,
Kamitanadare 914, Kisai,
Saitama, 347-0015, Japan

M. Yoshidome · Y. Honda
Graduate School of Agriculture, Tokyo University of Agriculture and Technology,
Fuchu,
Tokyo, 183-8509, Japan

M. Watanabe
United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology,
Fuchu,
Tokyo, 183-8509, Japan

I. Ogiwara · T. Izuta (✉)
Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology,
Fuchu,
Tokyo, 183-8509, Japan
e-mail: izuta@cc.tuat.ac.jp
Tel.: +81-42-3675728
Fax: +81-42-3675728

Introduction

Tropospheric ozone (O_3) is recognized as a widespread phytotoxic air pollutant (Chameides et al. 1994). Many experimental studies have shown that O_3 causes reductions in photosynthetic activity and dry matter production of forest tree species, and may be closely related to tree dieback and forest decline in the USA and Europe (Sandermann et al. 1997; Chappelka and Samuelson 1998; Skärby et al. 1998). In Japan, relatively high concentrations of O_3 have been detected not only in urban areas, but also in mountainous areas (Hatakeyama and Murano 1996; Totsuka et al. 1997). Recently, O_3 has been shown to be one of the important stress factors relating to tree dieback and forest decline in Japan, because relatively high concentrations of O_3 above 100 nl l^{-1} have frequently been detected in mountainous areas in spring and summer (Totsuka et al. 1997). However, limited information is available on the effects of ambient levels of O_3 on growth and physiological functions of Japanese forest tree species (Izuta et al. 1996; Yonekura et al. 2001a, b; Nakaji and Izuta 2001; Matsumura 2001).

In general, forest trees are adversely affected by several simultaneous stresses including gaseous air pollutants such as O_3 and climatic stresses (Smith 1990). Because the periods with relatively high concentrations of atmospheric O_3 often occur in combination with low soil moisture, there is the possibility that many tree species are simultaneously affected by O_3 and water stress (Morgan 1984; Miller 1992; Grulke 1999). In Japan, O_3 and/or water stress are suggested to be important factors relating to tree dieback and forest decline (Yuasa 1989; Matsumoto et al. 1992; Totsuka et al. 1997). To protect Japanese forest ecosystems from environmental stresses, therefore, we must clarify the interactive effects of O_3 and water stress on Japanese forest tree species and their mechanisms. However, very limited information is available on the combined effects of O_3 and water stress on Japanese forest tree species (Yonekura et al. 2001a, b).

Japanese beech (*Fagus crenata*), one of the most representative broad-leaved deciduous tree species native to Japan, is relatively sensitive to ambient levels of O_3 and soil water status as compared with other Japanese tree species (Maruyama and Toyama 1987; Izuta et al. 1996; Matsumura and Kohno 1997). Forest decline and tree dieback of *F. crenata* can be seen in the Tanzawa Mountains in Kanagawa Prefecture, and the Amagi Mountains and Mt. Fuji in Shizuoka Prefecture, central Japan (Murai et al. 1991; Tamaki 1997). Moreover, O_3 and/or water stress are considered to be environmental stresses relating to the decline of *F. crenata* (Yuasa 1989; Totsuka et al. 1997).

For deciduous tree species such as *F. crenata*, timing of bud burst in spring and leaf fall in autumn are very important in relation to the period of carbon fixation and growth during the growing season. Several researchers have reported carry-over effects of O_3 on frost hardiness in winter and the following season's phenological characteristics such as timing of needle or leaf flush in European coniferous and broad-leaved tree species (Pearson and Mansfield 1994; Wellburn and Wellburn 1994; Langebartels et al. 1998; Oksanen and Saleen 1999). Because *F. crenata* is classified as a fixed-growth-type species (Kikuzawa 1983), there is a possibility that O_3 and/or water stress in spring and summer may adversely affect phenological characteristics in the following autumn and winter, as well as in the next year's growing season. Additionally, *F. crenata* lives in cool temperate forests and is exposed to extremely low temperatures in winter. Therefore, winter hardiness of buds in *F. crenata* is regarded as important for dry matter production in the next year. Because it was reported that O_3 alters storage, formation and translocation of carbohydrates in leaves and accelerates leaf senescence of American and European forest tree species (McLaughlin et al. 1982; Landolt et al. 1994; Lux et al. 1997), O_3 exposure during the growing season may detrimentally affect winter hardening of buds. However, there is no information on the carry-over effects of O_3 and/or water stress on phenological characteristics and winter hardiness of Japanese forest tree species.

In this study, we investigated the effects of elevated O_3 and chronic mild water stress, singly and in combination, on leaf phenological characteristics, concentrations of non-structural carbohydrates in the attached leaves and buds, concentrations of carbon and nitrogen in the attached leaves and litter, and bud frost hardiness in *F. crenata* seedlings in order to clarify the carry-over effects of O_3 and water stress on phenology and winter hardiness of Japanese broad-leaved tree species.

Materials and methods

Plant materials and stress treatments

Three-year-old seedlings of *F. crenata* Blume were transplanted into 5.3 l pots (40 cm in depth ×13 cm in diameter) filled with brown forest soil collected from a mixed deciduous forest at the University Forest of Tokyo University of Agricultural and Technology (Kusaki, Gunma Prefecture, Japan). All the seedlings (32–40 cm in height) were randomized immediately before the initiation of the experiment, and were then grown for 156 days from 10 May to 12 October 1999 in four temperature-controlled and air humidity-controlled and atmospheric CO_2 concentration-controlled growth chambers (3.2 m^2 of growth space and 2 m in height; Koito S-180-SP type, Koito, Japan) located at an experimental field of Tokyo University of Agriculture and Technology (Fuchu, Tokyo, Japan). During the growth period of the seedlings, air temperature in the growth chambers was maintained at 20.0±1.0°C in the daytime from 0600 to 1700 hours and 15.0±1.0°C in the nighttime from 1800 to 0500 hours, and was gradually increased from 15.0°C to 20.0°C between 0500 and 0600 hours, and was gradually decreased from 20.0°C to 15.0°C between 1700 and 1800 hours. Relative air humidity and atmospheric CO_2 concentration in the growth chambers were regulated at 70±5% and 350±10 $\mu l\ l^{-1}$, respectively.

In the present study, the four treatments described above were designated as CF–WW (charcoal-filtered air and well-watered soil), CF–WS (charcoal-filtered air and water-stressed soil), O_3–WW (ozone and well-watered soil) and O_3–WS (ozone and water-stressed soil). In each treatment, the 15 seedlings per chamber were randomly assigned to each O_3-water-chamber combination for a total of 120 seedlings.

The seedlings were divided into two charcoal-filtered (CF) growth chambers with O_3 concentration below 5 nl l^{-1} and two elevated O_3 chambers, where the plants were exposed to 60±5 nl l^{-1} O_3 for 7 h (from 1100 to 1800 hours) and to CF air for 17 h (from 1800 to 1100 hours). The concentration and exposure duration of O_3 were determined based on the data of atmospheric O_3 obtained at the Tanzawa Mountains in Kanagawa Prefecture where dieback of mature *F. crenata* is observed and relatively high concentrations of O_3 are detected from May to October with an average O_3 concentration in the afternoon of approximately 60 nl l^{-1} (Kanagawa Prefecture, unpublished data). During the fumigation period of 156 days between 10 May and 12 October 1999, the accumulated exposure to O_3 above a threshold of 40 nl l^{-1} (AOT40) was 22.6 $\mu l\ l^{-1}$ h. The O_3 was generated from charcoal-filtered air with an electrical discharge O_3 generator (MO-5A, Nippon Ozone, Japan), and was then injected into the two growth chambers for O_3-exposure through a water trap to remove nitrogen by-products produced by the O_3 generator (Brown and Roberts 1988). The concentrations of O_3 at the plant canopy height in the four growth chambers were continuously monitored at 6-min intervals with UV absorption O_3 analyzer (Model 1100, Dylec, Japan).

Half of the seedlings within each growth chamber were randomly selected and grown in well-watered soil (WW). In the WW treatment, the seedlings received 250 ml of water per pot at 3-day intervals during the growth period of 156 days between 10 May and 12 October 1999, which corresponds to 1,193 mm of water supply to the potted soil surface. The amount of irrigated water in the WW treatment was determined based on the annual mean precipitation at many forested areas of Japanese deciduous broad-leaved tree species such as *F. crenata* (Murai et al. 1991; National Astronomical Observatory 1997). The remaining seedlings were grown in water-stressed soil (WS). In the WS treatment, the seedlings received

175 ml of water per pot at 3-day intervals during the same growth period, which corresponds to 835 mm of water supply to the potted soil surface. This quantity is equivalent to 70% of the WW treatment. During the treatment period, the weekly average value of soil water tensions (pF-value) in the WW treatment and WS treatment were 1.8 and 2.2, respectively (Yonekura et al. 2001b).

On 13 October 1999, all the seedlings were moved from the four growth chambers to the experimental field of Tokyo University of Agriculture and Technology (Fuchu, Tokyo, Japan) and were then grown in well-watered soil until 30 April 2000 under field conditions. Average daily O_3 concentration at the experimental field between October 1999 and April 2000 was 11.8 nl l^{-1}. Average daily air temperatures and average daily lowest air temperatures were 12.5°C and 7.6°C in November 1999, 6.0°C and 1.3°C in December, 6.4°C and 1.8°C in January 2000, 4.1°C and −1.5°C in February, 8.2°C and 2.0°C in March, and 13.4°C and 8.2°C in April, respectively. The minimum air temperature between November 1999 and April 2000 was −4.3°C on 17 February 2000.

Phenological observation

The date on which leaf fall and bud break were first observed were recorded for each seedlings about 1 or 2 days interval to evaluate phenological changes of *F. crenata* seedlings (Pearson and Mansfield 1994). Same seedlings were observed leaf fall and bud break. The number of buds was counted in January 2000, and leaf number per bud was determined after bud break on 30 April 2000.

Measurements of leaf element concentrations

The attached leaves were collected on 12 October 1999 (156th day after the initiation of gas and soil watering treatments), and litter from each seedling were collected at 1or 2 day intervals between November and December 1999. Total carbon (C) and nitrogen (N) concentrations in the attached leaves and litters were analyzed with a C/N analyzer (MT-500, Yanagimoto, Japan).

Measurements of non-structural carbohydrate concentrations

To determine the concentrations of carbohydrates, the attached leaves and buds were harvested on 12 October 1999 (156th day after the initiation of gas and soil watering treatments), and on 12 October 1999 and 28 February 2000 from 0900 to 1100 hours. The concentrations of soluble carbohydrates (glucose, fructose and sucrose) were determined by the modified method of Ogiwara et al. (1999). The soluble carbohydrates were extracted from the samples of leaves (2 g fresh weight) and buds (0.5 g fresh weight), using 30 ml of 80% (v/v) ethanol (pH 7.0) for 30 min at 80°C. The extract was centrifuged at 1,660*g* for 10 min. The ethanol in the supernatant was excluded using a rotary evaporator at 40 rpm and 40°C. The remaining pellets were reconstituted in 25 ml of distilled water, and then the solution was passed through a Sep-Pak C_{18} cartridge (Waters, USA) for excluding pigments, two types of ion-exchange cartridges (Sep-Pak QMA and CM, Waters, USA) and a 0.45 µm filter (Ultrafree-MC 0.45 µm filter Unit, Millipore, Japan). After these procedures, the concentrations of glucose, fructose and sucrose were analyzed by high performance liquid chromatography [HPLC, HPLC systems: Pump (LC-9A, Shimadzu, Japan), Detector (RDI-6A, Shimadzu, Japan), Column (Shim-pack SCR-101N, Shimadzu, Japan)]. Starch concentration was determined from the residual pellet after ethanol extraction and centrifugation. The dried residual pellet samples (20 mg) were homogenized with 4 ml of dimethylsulfoxide and 1 ml of HCl (8 mol l^{-1}) at 60°C for 30 min. After these procedures, the concentration of starch in the sample solutions was determined by using F-Kit Starch (Boehringer-Mannheim K.K., Japan) (Lux et al. 1997).

Evaluation of bud winter hardiness

Winter hardiness of buds was evaluated by the electrolyte leakage method (Murray et al. 1989). On 28 February 2000, branches (3–4 cm in length) with bud were subjected to each of four temperature regimes: an unfrozen control (4°C), and minimum temperatures of −5, −20 and −30°C. The buds in the unfrozen control treatment were kept at 4°C for 3 h. The buds in the remaining three temperature regimes were placed in a temperature-controlled medical freezer (MDF-U536, Sanyo Electric, Japan). The buds were sprayed with deionized water, placed in polypropylene vials, and then cooled to the desired temperature at a rate of 5°C h^{-1}. Each temperature was held for 3 h and then was increased at 10°C h^{-1} back to 4°C. After the frost treatment, each bud was cut from the branch. Fifty milliliters of deionized water was added to each vial containing two buds, and the vials were stored at 4°C. After 24 h and 120 h, electrical conductivity of the distilled water in each vial was measured using a digital conductivity meter (D-24, Horiba, Japan). At the end of measurements, the vials containing two buds and solution were autoclaved at 105°C for 5 min to destroy all the cells. After the autoclaved solution was stored at 4°C for 24 h, total conductivity value of the solution was measured. The electrolyte leakage rate (*k*) was calculated by the following equation:

$$K = \{-\ln[1 - (C_{120} - C_{24})/(C_{\mathrm{auto}} - C_{24})]\}/96,$$

where C_{auto} is electrical conductivity after autoclaving, C_{24} is electrical conductivity after infiltration for 24 h, and C_{120} is electrical conductivity after infiltration for 120 h.

Table 1 Effects of O_3 and/or soil water stress on the concentrations of soluble carbohydrates and starch in leaves of *F. crenata* seedlings on 12 October 1999 (156th day after the initiation of gas and soil watering treatments). The seedlings were grown in well-watered (WW) or water-stressed (WS) soil, and exposed to charcoal-filtered air (CF) or 60 nl l^{-1} O_3 (O_3). The *TSC* and *TNC* show total soluble carbohydrate (sucrose + glucose + fructose) and total non-structural carbohydrate (sucrose + glucose + fructose + starch), respectively. Each value is the mean of 6 determinations (3 seedlings per chamber), and the standard deviation is shown in parentheses. ANOVA: $^{**}P<0.01$; *NS* not significant

Parameter (mg g^{-1} DW)	Treatment				ANOVA		
	CF–WW	O_3–WW	CF–WS	O_3–WS	O_3	WS	O_3×WS
Sucrose	43.56 (4.28)	36.16 (10.40)	44.03 (2.66)	36.83 (6.84)	**	NS	NS
Glucose	3.98 (1.05)	1.89 (0.82)	3.06 (1.03)	3.65 (1.31)	NS	NS	NS
Fructose	7.17 (1.38)	4.47 (1.01)	5.30 (1.27)	5.01 (1.89)	**	NS	NS
TSC	54.82 (5.09)	41.63 (10.24)	51.81 (5.66)	45.49 (5.69)	**	NS	NS
Starch	6.82 (1.90)	3.49 (0.79)	4.66 (2.60)	3.35 (0.55)	**	NS	NS
TNC	61.65 (4.14)	45.13 (10.48)	56.48 (5.44)	47.90 (5.46)	**	NS	NS

Experimental design and statistical analysis

The present experiment was a factorial, split-plot in randomized blocks with O_3 concentration as the whole-plot treatment. The whole-plot treatment comprised two levels of O_3 replicated two times for a total of four chambers. The sub-plot treatment consisted of two levels of water supply to the potted soil in each chamber. The statistical analyses of variance (ANOVA) were performed with SPSS statistical package (SPSS 10.0.5J, SPSS, Japan). Two replicated chambers were randomly assigned to each O_3 treatment (*n*=2).

Results

Carbohydrate concentration in attached leaves and buds

Table 1 shows the concentrations of soluble carbohydrates and starch in the leaves of *F. crenata* seedlings on 12 October 1999. The concentrations of sucrose, fructose, total soluble carbohydrate (*TSC*), starch and total non-structural carbohydrate were significantly reduced by the exposure to O_3. There were no significant effects of water stress on the concentrations of soluble carbohydrates and starch. In addition, no significant interactions between O_3 and water stress were detected on the concentrations of these carbohydrates.

Table 2 indicates the concentrations of soluble carbohydrates and starch in buds in the early autumn (12 October 1999) and in the following winter (28 February 2000). In all the treatments, the concentration of sucrose in the following winter was greater than that in the early autumn, but the concentration of starch in the following winter was less than that in the early autumn. In the early autumn and the following winter, no significant effects of O_3 and water stress, alone and in combination, were found on the concentrations of soluble carbohydrates and starch of the buds.

Timing of leaf fall

Figure 1 shows the percentage of *F. crenata* seedlings showing leaf fall. The first day on which leaf fall began was 25 October 1999 in O_3–WW treatment, and the leaf fall of all the seedlings had started within 27 days. The day on which leaf fall had begun for over 50% of the seedlings (FL_{50}) in O_3–WW treatment was 8 days earlier than that in CF–WW treatment, and the FL_{50} in O_3–WS treatment was 2 days earlier than that in CF–WW treatment. By contrast, the FL_{50} in CF–WS treatment was the same as that in CF–WW treatment. Furthermore, considering the well-watered and water-stressed seedlings together, linear regression analysis revealed that the seedlings which had experienced the O_3 treatment began leaf fall approximately 7 days earlier than the seedlings in the charcoal-filtered air treatment. In contrast, considering the charcoal-filtered air and O_3 treated seedlings together, no differences were detected in the timing of leaf fall between well-watered and water-stressed seedlings.

Element concentration in attached leaves and litter

Table 3 shows the concentrations of carbon (C) and nitrogen (N) in attached leaves in the early autumn and litter in winter. The concentrations of C and N in the leaves were significantly reduced by the exposure to O_3. However, C/N ratio in the leaves was significantly increased by O_3. On the other hand, no significant effects of water stress or interactive effects of O_3 and water stress were detected on the concentrations of C and N, and C/N ratio in the leaves. In the litter, no significant effects of O_3 and water stress, singly or in combination, were found on the concentrations of C and N, and C/N ratio.

Table 2 Effects of O_3 and/or soil water stress on the concentrations of soluble carbohydrates and starch in buds of *F. crenata* seedlings. The buds were harvested on 12 October 1999 and 28 February 2000. The *TSC* and *TNC* show total soluble carbohydrate (sucrose + glucose + fructose) and total non-structural carbohydrate (sucrose + glucose + fructose + starch), respectively. Each value is the mean of 6 determinations (3 seedlings per chamber), and the standard deviation is shown in parentheses. ANOVA: *NS* not significant

Parameter (mg g^{-1} DW)	Treatment				ANOVA		
	CF–WW	O_3–WW	CF–WS	O_3–WS	O_3	WS	O_3×WS
156 days after treatment (12 October 1999)							
Sucrose	21.54 (3.41)	16.94 (3.47)	20.70 (4.62)	20.63 (4.16)	NS	NS	NS
Glucose	8.87 (1.97)	10.34 (1.99)	10.73 (1.24)	9.87 (1.40)	NS	NS	NS
Fructose	8.00 (1.75)	10.26 (1.66)	9.74 (0.95)	8.89 (1.44)	NS	NS	NS
TSC	38.41 (5.79)	37.53 (4.81)	41.17 (4.88)	39.38 (5.40)	NS	NS	NS
Starch	18.64 (1.65)	16.87 (2.22)	16.09 (1.33)	17.33 (3.61)	NS	NS	NS
TNC	57.05 (6.27)	54.41 (8.75)	57.26 (4.77)	56.72 (7.33)	NS	NS	NS
Following winter (28 February 2000)							
Sucrose	44.50 (3.78)	46.22 (1.68)	47.71 (3.17)	44.47 (5.07)	NS	NS	NS
Glucose	10.32 (1.35)	9.29 (1.69)	7.84 (0.68)	11.13 (3.44)	NS	NS	NS
Fructose	9.13 (2.14)	9.42 (1.31)	9.05 (3.12)	9.97 (2.07)	NS	NS	NS
TSC	63.95 (4.74)	64.93 (2.91)	64.65 (5.56)	65.57 (7.12)	NS	NS	NS
Starch	8.96 (1.35)	7.18 (1.89)	6.87 (1.46)	9.36 (2.09)	NS	NS	NS
TNC	72.91 (5.50)	72.11 (4.72)	70.91 (6.72)	74.93 (5.73)	NS	NS	NS

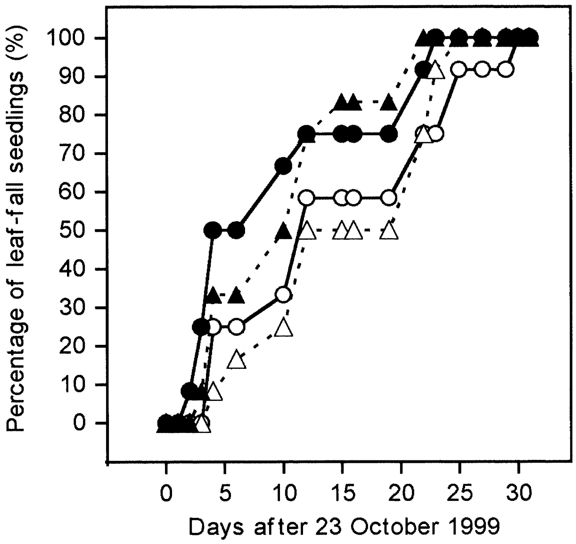

Fig. 1 Effects of O_3 and/or soil water stress on the percentage of *F. crenata* seedlings in each treatment for which leaf fall had begun. The seedlings were grown in well-watered (WW) or water-stressed (WS) soil, and exposed to charcoal-filtered air (CF) or 60 nl l^{-1} O_3 (O_3). Treatments are CF–WW (*open circle*), O_3–WW (*filled circle*), CF–WS (*open triangle*) and O_3–WS (*filled triangle*). The total number of the seedlings in each treatment was 14 (7 seedlings per chamber)

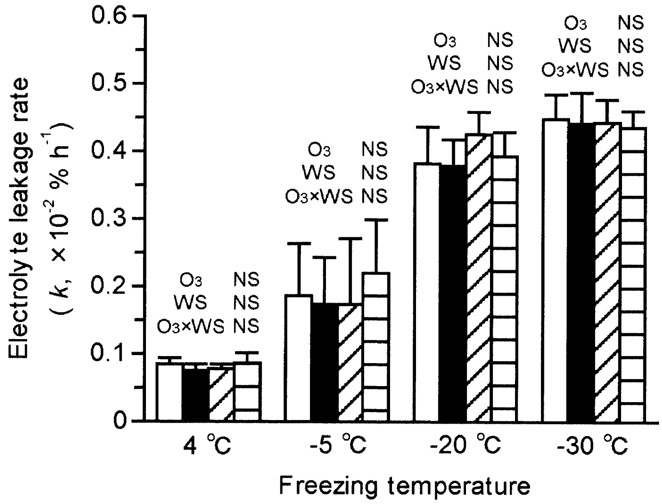

Fig. 2 Effects of O_3 and/or soil water stress on winter hardiness in buds of *F. crenata* seedlings on 28 February 2000. The winter hardiness is evaluated by electrolyte leakage rate (k) at each freezing temperature. Treatments are CF–WW (*open square*), O_3–WW (*filled square*), CF–WS (*cross-hatched square*) and O_3–WS (*hatched square*). Each bar is the mean of 6 determinations (3 seedlings per chamber). The standard deviation is given by a vertical bar. The results of ANOVA are shown in this figure

Forest hardiness of buds

Figure 2 illustrates frost hardiness of buds in the winter (28 February 2000), which was evaluated by the electrolyte leakage rate (k). The value of k in the buds increased with decreasing the freezing temperature in all the treatments. There were no significant effects of O_3 and water stress, singly or in combination, on the k value at each freezing temperature.

Timing of bud break, and the number of leaves after bud break

Figure 3 shows the effects of the previous year's O_3 and/or water stress on the percentage of *F. crenata* seedlings showing bud break. The bud break was first observed on 14 April 2000 in CF–WS treatment, and that of all the seedlings had started within 9 days. The day on which bud break had begun for over 50% of the seedlings (BB_{50}) in CF–WS treatment was 2 days earlier than that in CF–WW treatment. In contrast, the BB_{50} in O_3–WW treatment and that in the O_3–WS treatment were 3 days and 1 day later than that in CF–WW treatment, respectively. Furthermore, considering the well-watered and water-stressed seedlings together, linear regression analysis revealed that the seedlings which had experienced the previous growing season's O_3 treatment attained a theoretical 100% bud break approximately 8 days later than the seedlings in the charcoal-filtered air treatment. In contrast, considering the charcoal-filtered air and O_3 treated seedlings together, the seedlings which had experienced the previous growing season's water stress attained a theoretical 100% bud burst

Table 3 Effects of O_3 and/or soil water stress on the total carbon (C) and nitrogen (N) concentrations and C/N ratio in the leaf and the litter of *F. crenata* seedlings. The leaves were harvested on 12 October 1999, and the litter was sampled between November and December 1999. Each value is the mean of 8 determinations (4 seedlings per chamber), and the standard deviation is shown in parentheses. ANOVA: *P<0.05; *NS* not significant

Parameter	Treatment				ANOVA		
	CF–WW	O_3–WW	CF–WS	O_3–WS	O_3	WS	O_3×WS
Leaf (12 October 1999)							
C (mg g^{-1} DW)	446.6 (27.2)	426.9 (24.1)	441.6 (35.5)	437.5 (27.6)	*	NS	NS
N (mg g^{-1} DW)	21.3 (3.1)	18.5 (1.1)	19.6 (2.8)	18.1 (2.3)	*	NS	NS
C/N ratio	21.0 (2.7)	23.1 (1.1)	22.6 (2.3)	24.1 (2.5)	*	NS	NS
Litter (November and December 1999)							
C (mg g^{-1} DW)	389.2 (24.0)	386.3 (17.3)	372.7 (16.9)	393.9 (22.0)	NS	NS	NS
N (mg g^{-1} DW)	6.30 (2.2)	6.96 (3.0)	7.71 (2.1)	7.29 (2.3)	NS	NS	NS
C/N ratio	67.5 (20.0)	63.4 (22.0)	51.6 (13.9)	58.7 (17.8)	NS	NS	NS

about 1 day earlier than well-watered seedlings. However, the duration between the bud break was first observed and all buds were flushed in each seedling (data not shown).

Table 4 indicates the effects of O_3 and/or water stress during the previous year's growing season (1999) on the bud number per seedling, leaf number per seedling after bud break and leaf number per bud on 30 April 2000. The leaf number per seedling and leaf number per bud were significantly reduced by the previous growing season's exposure to O_3. In contrast, no significant effects of water stress or interaction of both stresses on the bud number per seedling, leaf number per seedling and leaf number per bud were detected.

Discussion

In the present study, the concentrations of soluble carbohydrates, starch, total carbon and nitrogen in the leaves of *F. crenata* seedlings were significantly reduced by the exposure to O_3. The effects of O_3 on foliar non-structural carbohydrate concentrations were complicated. For example, the concentrations of non-structural carbohydrates in the leaves were found to be reduced by exposure to O_3 in *Populus nigra*, *Picea abies* and *Pinus taeda* (Meier et al. 1990; Barnes et al. 1995; Fialho and Bücker 1996), whereas the carbohydrate concentrations were increased or not affected by O_3 in *Populus × euramericana*, *Pinus sylvestris* and *P. abies* (Landolt et al. 1994; Holopainen et al. 1996; Utriainen and Holopainen 2000). In our previous studies, it was reported that net photosynthetic rate, starch grain size in chloroplasts and whole-plant dry mass of *F. crenata* seedlings were reduced by the exposure to O_3 (Yonekura et al. 2001a, b). The exposure to O_3 caused the partition of assimilated carbon into organic acid rather than into starch (Friend and Tomlinson 1992). Therefore, the O_3-induced reductions in the concentrations of carbohydrates and elements in the leaves of *F. crenata* seedlings are considered to be mainly due to the reduction of net photosynthesis and CO_2 fixation, and the inhibition in the amount of assimilated carbon partitioned into starch and protein. Temple and Riechers (1995) reported that *Pinus ponderosa* seedlings affected by O_3 increased resorption of N from older needles. Thus, translocation rate of N from the leaves to the other organs in accordance with aging of the leaves might be increased by exposure to O_3. As a result, C/N ratio in attached leaves of O_3-injured seedlings was increased. In contrast, the concentrations of C and N in the litters were not significantly affected by O_3 and/or water stress treatments. Since the amounts of C and N of the leaves were reduced in O_3 exposed seedlings, it is likely that the overall quantity of translocation of C and N from the older leaves to the other organs might be decreased by exposure to O_3.

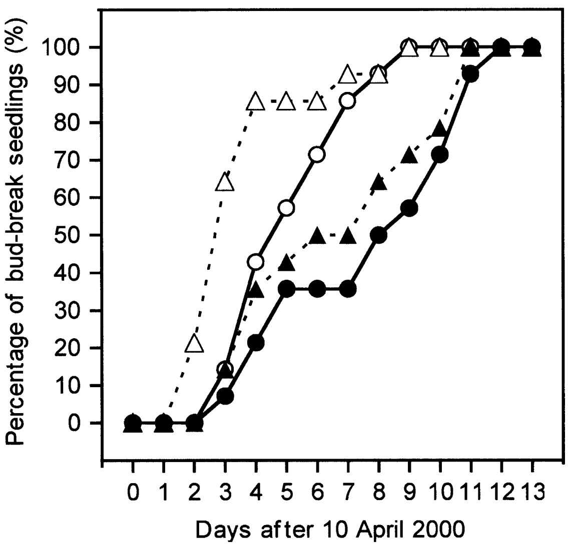

Fig. 3 Effects of O_3 and/or soil water stress during the previous year's growing season (1999) on the percentage of *F. crenata* seedlings in each treatment which had begun to break bud in April 2000. Treatments are CF–WW (*open circle*), O_3–WW (*filled circle*), CF–WS (*open triangle*) and O_3–WS (*filled triangle*). The total number of the seedlings in each treatment was 14 (7 seedlings per chamber)

In contrast to the result of leaf non-structural carbohydrate concentrations, there were no significant effects of O_3 and/or water stress on the concentrations of soluble carbohydrates and starch in the buds in early autumn and winter. These results suggest that carbohydrate allocation to buds from other organs was not affected by O_3 and water stress. The buds may have the priority for getting the accumulated carbohydrates as compared with the other organs, because the dry mass per one bud was not affected, but the dry mass of the other organs were significantly reduced by O_3 and water stress (Yonekura et al. 2001a).

In the present study, non-structural carbohydrate concentrations of buds in early autumn was less than those in winter, and sucrose concentration in winter was greater than that in early autumn. In contrast, starch concentration in winter was less than that in early autumn, and TSC concentration in buds increased from early autumn to winter. The starch-to-sugar conversion following the reduction of air temperature between autumn and winter is observed in many woody plants (Sakai and Larcher 1987; Sauter et al. 1996). In general, carbohydrate composition and change in the concentrations of non-structural carbohydrates in buds between autumn and winter are closely related to the freezing tolerance (Levitt 1980). The increase in the concentration of sucrose parallels to the development of freezing tolerance (Sakai and Yoshida 1968), and the frost hardiness more closely follows the increasing level of sucrose than TSC concentration (Sauter et al. 1996), supporting the result that the freezing resistance of buds was not significantly affected by O_3 and water stress in the present study. Furthermore, the minimum air temperature in winter was approximately −20°C at Kuromatsunai in Hokkaido (42°40′N, 140°23′E), the northernmost area of naturally occurring *F. crenata* in Japan (Murai et al. 1991, Japan Meteorology Agency 2000). Although extrapolation of these results to mature trees contains risks, our results suggest that bud frost

Table 4 Effects of O_3 and/or soil water stress applied during the growing season of 1999 on bud number per seedling, leaf number per seedling after bud break in 2000 and leaf number per bud of *F. crenata* seedlings. Each value is the mean of 12 determinations (6 seedlings per chamber), and the standard deviation is shown in parenthesis. ANOVA: *$P<0.05$; **$P<0.01$; *NS* not significant

Parameter	Treatment				ANOVA		
	CF–WW	O_3–WW	CF–WS	O_3–WS	O_3	WS	O_3×WS
Bud number	34.8 (4.1)	34.2 (4.1)	31.3 (2.9)	30.0 (4.3)	*	NS	NS
Leaf number	209.6 (40.1)	165.6 (38.1)	178.0 (36.4)	149.1 (35.8)	*	NS	NS
Leaf number/Bud	6.0 (0.7)	4.8 (0.8)	5.8 (1.4)	5.0 (1.1)	*	NS	NS

hardiness of *F. crenata* in Japan is not affected by our experimental levels of O_3 and water stress.

In the present study, the timing of leaf fall in the O_3 treatments occurred earlier than that in the charcoal-filtered air treatments, but there was no difference between the well-watered and water-stressed treatments. These results suggest that O_3 stress accelerated leaf senescence of *F. crenata* seedlings. The acceleration of leaf senescence of trees affected by O_3 is a well known symptom (e.g., Chappelka and Samuelson 1998). O_3-induced symptoms of accelerating leaf senescence were also detected in net photosynthetic rate and leaf ultrastructural characteristics in our previous study (Yonekura et al. 2001b).

Although the timing of bud break in the O_3 treatments occurred later than that in the charcoal-filtered air treatments, that in the water-stressed treatments was slightly earlier than that in the well-watered treatments. The timing of bud break may be related to changes in the duration of sunlight, air temperature during the winter and spring and the levels of phytohormones such as abscisic acid (Thomas 2000). Therefore, there is the possibility that the previous year's O_3 altered or confused the levels of phytohormones in the buds, and affected the bud condition of *F. crenata* during the period of bud formation. According to the results of the timing of leaf fall and bud break, the duration of leaf retention would be shortened by the exposure to O_3 resulting in a reduced yield in the long term. In the present study, however, we were unable to clarify the reason for the change in the timing of bud break by the previous year's O_3 treatment.

The number of flushed leaves per bud was significantly reduced by the previous growing season's exposure to O_3. The O_3-induced reduction in the number of flushed leaves per bud was mainly due to the reduction in the number of leaves per bud. *F. crenata* is classified as a fixed-growth-type species, and the number of leaves in the following season is generally determined in the previous year (Kikuzawa 1983; Maruyama 1983; Eschrich et al. 1989). Therefore, the previous year's O_3 exposure may affect bud condition of *F. crenata* during the period of bud formation. In addition, if the result that bud carbohydrate concentration was not affect by O_3 is taken into consideration, it might be that the seedlings affected by O_3 tried to maintain the normal metabolic balance in the next year's buds and leaves at the expense of the number of flushed leaves. There were no significant interactions between the previous year's exposure to O_3 and water stress on the timing of leaf fall and bud break, and the number of leaves after bud break of *F. crenata* seedlings. These results indicate that the synergistic or antagonistic effects of both stresses were not revealed in leaf phenological characteristics of the seedlings.

Water stress treatment in this study did not cause any immediate or carry-over changes in phenological characteristics or winter hardiness of *F. crenata* seedlings, although previously significant reductions in leaf water potential, net photosynthetic rate, stomatal conductance to water vapor, transpiration and whole-plant dry mass has been reported (Yonekura et al. 2001a, b).

In conclusion, although there were not any carry-over effects of water stress on phenological characteristics and winter hardiness of *F. crenata* seedlings, the persistent effects of O_3 could be found in phenological characteristics and concentrations of non-structural carbohydrates in the leaves. The exposure of the seedlings to O_3 from spring to autumn has an impact on phenological characteristics from autumn to the following winter such as early leaf fall, late bud break and reduction of the leaf number per bud. Therefore, the long-term exposure to ambient levels of O_3 is likely to have a negative impact on the productivity of trees.

References

Barnes JD, Pfirrmann T, Steiner K, Lütz C, Busch U, Küchenhoff H, Payer H-D (1995) Effects of elevated CO_2, elevated O_3 and potassium deficiency on Norway spruce [*Picea abies* (L.) Karst.]: seasonal changes in photosynthesis and non-structural carbohydrate content. Plant Cell Environ 18:1345–1357

Brown KA, Roberts TM (1988) Effects of ozone on foliar leaching in Norway spruce (*Picea abies* L. Karst): confounding factors due to NO_x production during ozone generation. Environ Pollut 55:55–73

Chameides WL, Kasibhatla PS, Yienger J, Levy H (1994) Growth of continental-scale metro-agro-plexes, regional ozone pollution, and world food production. Science 64:74–77

Chappelka AH, Samuelson LJ (1998) Ambient ozone effects on forest trees of the eastern United States: a review. New Phytol 139:91–108

Eschrich W, Burchardt R, Essiamah S (1989) The induction of sun and shade leaves of European beech (*Fagus sylvatica* L.): anatomical studies. Trees 3:1–10

Fialho RC, Bücker J (1996) Changes in levels of foliar carbohydrates and myoinosotol before premature leaf senescence of *P. nigra* induced by a mixture of O_3 and SO_2. Can J Bot 74:965–970

Friend AL, Tomlinson PT (1992) Mild ozone exposure alters ^{14}C dynamics in foliage of *Pinus taeda* L. Tree Physiol 11:215–227

Grulke NE (1999) Physiological responses of ponderosa pine to gradients of environmental stressors. In: Miller PR, McBride JR (eds) Oxidant air pollution impacts in the montane forests of southern California: a case study of the San Bernardino Mountains. Springer, Berlin Heidelberg New York, pp 126–163

Hatakeyama S, Murano K (1996) High concentration of ozone observed in Mt. Maeshirane in Oku-Nikko (in Japanese with English summary). J Jpn Soc Atmos Environ 31:106–110

Holopainen T, Anttonnen S, Palomäki V, Kainulainen P, Holopainen JK (1996) Needle ultrastructure and starch content in Scots pine and Norway spruce after ozone fumigation. Can J Bot 74:67–76

Izuta T, Umemoto M, Horie K, Aoki M, Totsuka T (1996) Effects of ambient levels of ozone on growth, gas exchange rates and chlorophyll contents of *Fagus crenata* seedlings. J Jpn Soc Atmos Environ 31:95–105

Japan Meteorology Agency (2000) Monthly report of Japan Meteorology Agency (February 2000). CD-ROM version (in Japanese). JMA, Tokyo

Kikuzawa K (1983) Leaf survival of woody plant in deciduous broad-leaved forests. 1. Tall trees. Can J Bot 61:2133–2139

Landolt W, Gunthard-Georg MS, Pfenninger I, Scheidegger C (1994) Ozone-induced microscopical changes and quantitative carbohydrate contents of Hybrid poplar (*Populus* × *euramericana*). Trees 8:183–190

Langebartels C, Heller W, Führer G, Lippert M, Simons S, Sandermann HJ (1998) Memory effects in the action of ozone on conifers. Ecotoxicol Environ Saf 41:62–72

Levitt J (1980) Responses of plants to environmental stresses. Academic, New York

Lux D, Leonardi S, Müller J, Wiemken A, Flückiger W (1997) Effects of ambient ozone concentrations on contents of non-structural carbohydrates in young *Picea abies* and *Fagus sylvatica*. New Phytol 137:399–409

Maruyama K (1983) Shoot characteristics as a function of the bud length on Japanese beech trees (in Japanese with English summary). J Jpn For Soc 65:43–51

Maruyama K, Toyama Y (1987) Effects of water stress on photosynthesis and transpiration in tree tall deciduous trees. J Jpn For Sci 69:165–170

Matsumoto Y, Maruyama Y, Morikawa Y, Inoue T (1992) Some negative results of simulative acid mist and ozone treatments to *Cryptomeria japonica* D. Don seedlings in explanation of mature *C. japonica* decline in the Kanto plains in Japan (in Japanese). Jpn J For Environ 34:85–97

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 Soil Pollut 130:959–964

Matsumura H, Kohno Y (1997) Effects of ozone and/or sulfur dioxide on tree species. In: Kohno Y (ed) Proceedings of CRIEPI international seminar on transport and effects of acidic substances. Central Research Institute of Electric Power Industry, Japan, pp 190–205

McLaughlin SB, McConathy RK, Duvick D, Mann LK (1982) Effects of chronic air pollution stress on photosynthesis, carbon allocation and growth of white pine trees. For Sci 28:60–70

Meier S, Grand LF, Schoeneberger MM, Reinert RA, Bruck RI (1990) Growth, ectomycorrhizae and nonstructural carbohydrate of loblolly pine seedlings exposed to ozone and soil water deficit. Environ Pollut 64:11–27

Miller PR (1992) Mixed conifer forests of the San Bernardino Mountain, California. In: Olson PK, Binkley D, Bohm M (eds) The response of western forests to air pollution. Springer, Berlin Heidelberg New York, pp 461–497

Morgan JM (1984) Osmoregulation and water stress in higher plants. Annu Rev Plant Physiol 35:299–319

Murai H, Yamatani K, Kataoka Y, Yui M (1991) Natural environment and its conservation on Buna (*Fagus crenata*) forest. Soft Science, Tokyo, pp 142–144 (In Japanese)

Murray MB, Cape JN, Fowle D (1989) Quantification of frost damage in plant tissues by rates of electrolyte leakage. New Phytol 113:307–311

Nakaji T, Izuta T (2001) Effects of ozone and/or excess soil nitrogen on growth, needle gas exchange rates and Rubisco contents of *Pinus densiflora* seedlings. Water Air Soil Pollut 130:971–976

National Astronomical Observatory (1997) RIKA NENPYO (Chronological Scientific Tables 1998) (in Japanese). Maruzen, Tokyo, pp 210–213

Ogiwara I, Ohtuka Y, Yoneda Y, Sakurai K, Hakoda N, Shimura I (1999) Extraction method by water followed by microwave heating for analyzing sugars in strawberry. J Jpn Soc Hortic Sci 68:949–953

Oksanen E, Saleen A (1999) Ozone exposure results in various carry-over effects and prolonged reduction in biomass in Birch (*Betula pendula* Roth). Plant Cell Environ 22:1401–1411

Pearson M, Mansfield TA (1994) Effects of exposure to ozone and water stress on the following season's growth of Beech (*Fagus sylvatica* L.). New Phytol 126:511–515

Sakai A, Larcher W (1987) Frost survival of plants. Springer, Berlin Heidelberg New York

Sakai A, Yoshida S (1968) The role of sugar and related compounds in variations of freezing resistance. Cryobiology 5:160–174

Sandermann H, Wellburn AR, Heath RL (1997) Forest decline and ozone: a comparison of controlled chamber and field experiments. Springer, Berlin Heidelberg New York

Sauter JJ, Wisniewaki M, Witt W (1996) Interrelationships between ultrastructure, sugar levels, and frost hardiness of ray parenchyma cells during frost acclimation and deacclimation in Poplar (*Poplus* × *canadensis* Moench ⟨robusta⟩) wood. J Plant Physiol 149:451–461

Skärby L, Ro-Poulsen H, Wellburn FAM, Sheppard LJ (1998) Impacts of ozone on forests: a European perspective. New Phytol 139:109–122

Smith WH (1990) Air pollution and forests. Springer, Berlin Heidelberg New York

Tamaki M (1997) Present state of acid deposition surveillance in forested region over (in Japanese). Jpn Environ Conserv Eng 26:3–12

Temple PJ, Riechers GH (1995) Nitrogen allocation in ponderosa pine seedlings exposed to interacting ozone and drought stresses. New Phytol 130:97–104

Thomas P (2000) Trees: their natural history. Cambridge University Press, Cambridge

Totsuka T, Aoki M, Izuta T, Horie K, Shima K (1997) Comparison of the air pollutant concentration and soil conditions on south-slope of damaged beech stands and north-slope of healthy beech stands on the submit of Mt. Hinokiboramaru, Kanagawa Prefecture. Report of Mt. Tanzawa Range Environment Studies (in Japanese). Kanagawa Prefecture, Japan, pp 93–96

Utriainen J, Holopainen T (2000) Impact of increased springtime O_3 exposure on Scots pine (*Pinus sylvestris*) seedlings in central Finland. Environ Pollut 109:479–487

Wellburn FAM, Wellburn AR (1994) Atmospheric ozone affects carbohydrate allocation and winter hardiness of *Pinus halepensis* (Mill.). J Exp Bot 45:607–614

Yonekura T, Dokiya Y, Fukami M, Izuta T (2001a) Effects of ozone and/or soil water stress on growth and photosynthesis of *Fagus crenata* seedlings. Water Air Soil Pollut 130:965–970

Yonekura T, Honda Y, Oksanen E, Yoshidome M, Watanabe M, Funada R, Koike T, Izuta T (2001b) The influences of ozone and soil water stress, singly and in combination, on leaf gas exchange rates, leaf ultrastructural characteristics and annual ring width of *Fagus crenata* seedlings. J Jpn Soc Atmos Environ 36:333–351

Yuasa Y (1989) Ecological study of debilitated beech forests in the Amagi Mountains. In: Shizuoka University Environment Study Society (ed) Conservation of rhododendrons and beech in the Amagi mountains (in Japanese with English summary). Shizuoka University of Environmental Study Society, Shizuoka, pp 9–29