

Effects of Elevated Carbon Dioxide and Ozone on Foliar Proanthocyanidins in *Betula platyphylla*, *Betula ermanii*, and *Fagus crenata* Seedlings

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Abstract Proanthocyanidins (PAs) or condensed tannins are a major group of phenolic compounds in the leaves of birch trees and many other woody and herbaceous plants. These compounds constitute a significant allocation of carbon in leaves and are involved in plant responses to environmental stress factors, such as pathogens or herbivores. In some plants, PA concentrations are affected by atmospheric carbon dioxide (CO₂) and ozone (O₃) levels that may influence, for example, species fitness, community structure, or ecosystem nutrient cycling. Therefore, a study on the quantitative response of PAs to elevated concentrations of carbon dioxide (CO₂) and ozone (O₃) was undertaken in seedlings of *Betula platyphylla*, *Betula ermanii*, and *Fagus crenata*. Seedlings were exposed to ambient or elevated O₃ and CO₂ levels during two growing seasons in the Kanto district in

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Japan. Ten open-top chambers were used for five different treatments with two replicates: filtered air (FA), ambient air (ambient O₃, 43 ppb; ambient CO₂, 377 ppm), elevated O₃ (1.5 × ambient O₃, 66 ppb), elevated CO₂ (1.5 × ambient CO₂, 544 ppm), and elevated O₃ and CO₂ combined. In addition, seedlings growing in natural conditions outside of chambers were studied. Leaf samples were analyzed for total PA concentrations by butanol–HCl assay and for polymeric PA concentrations by normal-phase high-performance liquid chromatography. Total PA concentrations in leaves of all species were similarly affected by different treatments. They were significantly higher in seedlings treated with elevated CO₂ and O₃ combined, and in seedlings growing outside chambers compared with the FA controls. *F. crenata* contained only traces of polymeric PAs, but significant species × treatment interaction was observed in the polymeric PA concentrations in *B. ermanii* and *B. platyphylla*. In *B. platyphylla*, leaves treated with elevated CO₂ + O₃ differed significantly from all other treatments. It was suggested that the strongest effect of elevated CO₂ and O₃ combined on leaf PA contents resulted from the additive effect of these environmental factors on phenolic biosynthesis.

Keywords Beech · Birch · Butanol–HCl assay · Carbon dioxide · HPLC · Ozone · Proanthocyanidins

Introduction

Background levels of greenhouse gases, ozone (O₃) and carbon dioxide (CO₂), have continued to increase because of human activities. The total amount of tropospheric O₃ has been estimated to have increased by 36% since 1750. Atmospheric CO₂ has increased more than 30% over the same time (IPCC, 2001). O₃ inhibits plant growth by reduction in net photosynthesis (inhibition of carbon assimilation), causes an imbalance of carbon allocation to plant organs (decreasing yield and nutritive quality of biomass), and increases sensitivity to other environmental stresses such as plant diseases (Reich, 1983; Darral, 1989; Manning and Tiedemann, 1995; Fuhrer and Booker, 2003). CO₂ has direct effects on plant photosynthesis and growth, and enriched CO₂ can alter the concentrations of primary and secondary metabolites in leaves (Lindroth et al., 1993). Furthermore, O₃ stimulates phenylpropanoid metabolism and thus induces accumulation of phenolic compounds, such as flavonoids and tannins in leaf tissues (Jordan et al., 1991; Kangasjärvi et al., 1994; Eckey-Kaltenbach et al., 1994; Booker and Miller, 1998).

Proanthocyanidins (PAs) (also known as condensed tannins) form a class of natural polyphenolic compounds. They are oligomeric and polymeric end products of the biosynthetic flavonoid pathway consisting of chains of flavan-3-ol units linked together mainly by carbon–carbon bonds at C4→C8 or C4→C6. PAs can be classified to six subclasses (procyanidins, prodelphinidins, proflisetinidins, propelargonidins, prorobinetinidins, and proguibourtinidins) based on the substitution pattern of the single flavan-3-ol unit. Birch leaf PAs are procyanidins and prodelphinidins (Ossipova et al., 2001). PAs are the main phenolic compounds in the leaves of certain woody plants and in herbaceous plants. For example, in the mature leaves of mountain birch, PA concentration can be as high as 120 mg g⁻¹ of dry mass that corresponds to approximately 80% of their total phenolic concentration (Ossipova et al., 2001).

PA concentrations typically increase in response to enriched CO₂. For example, enriched CO₂ increased total PA concentrations in *Populus tremuloides*, *Quercus rubra*, and *Acer saccharum* leaves (Lindroth et al., 1993; Kinney et al., 1997). PA concentrations were also increased by elevated CO₂ in *Salix myrsinolia* (Julkunen-Tiitto et al., 1993), *Eucalyptus tereticornis* (Lawler et al., 1997), *Pinus palustris* (Entry et al., 1998), *Pinus taeda* (Gebauer et al., 1998; Booker and Maier, 2001), *Lotus cornutus* (Carter et al., 1999), and *Gossypium hirsutum* (Booker, 2000; Booker et al., 2000). The effect of elevated CO₂ or O₃ levels on total PA concentration in *Betula* sp. leaves has also been investigated (Lavola et al., 1994, 2000; Traw et al., 1996; Lindroth et al., 2001). Experiments with *B. allegheniensis*, *B. populifolia*, *B. pendula*, and *B. papyrifera* seedlings showed that total PA concentrations in leaves of seedlings growing under elevated concentration of CO₂ were significantly higher than those in control leaves (Traw et al., 1996; Lavola et al., 2000; Lindroth et al., 2001; Kuokkanen et al., 2003). In contrast, enriched O₃ did not affect total PA concentrations in birch leaves (Lavola et al., 1994; Lindroth et al., 2001). The strongest effect was observed with combined CO₂ and O₃ treatments. However, in *Gossypium hirsutum*, O₃ treatment did not have a significant effect on leaf PA concentration, but a CO₂–O₃ interaction was observed (Booker, 2000). Jordan et al. (1991) and Booker et al. (1996) have reported that treatment with high O₃ concentrations increases PA concentration in *Pinus taeda* needles.

PAs influence plant–herbivore interactions. They bind and precipitate proteins and polysaccharides and may reduce the suitability of foliage of woody plants for herbivores (Haslam, 1988; Herms and Mattson, 1992). Concentrations of PAs are generally used to quantify resistance levels of woody plants to insects and other herbivores. The total PA concentrations in birch leaves correlate negatively with growth rates in later larval stages of *Epirrita autumnata* (Kause et al., 1999; Ossipov et al., 2001). Therefore, induction of phenolic biosynthesis by elevated levels of CO₂ and O₃, single or combined, may also regulate plant–herbivore relationships.

PAs are commonly quantified as total PA concentrations by using colorimetric methods. These methods are inexpensive and fast, but lack specificity (Rohr, 2002). The butanol–HCl (BH) and vanillin assays are the most commonly used methods. The BH assay uses hydrochloric acid catalyzed depolymerization of PAs in butanol to yield colored anthocyanidins that can be detected spectrophotometrically (Waterman and Mole, 1994). Various limitations for this method have been reported (see, for example, Porter et al., 1986; Scalbert, 1992; Waterman and Mole, 1994; Schofield et al., 2001). The most important are the proportion of water in the reaction mixture and the choice of PA standard. The BH assay should be used with caution for quantification (Schofield et al., 2001). The vanillin method uses the condensation reaction of PAs with vanillin (Rohr, 2002). The reliability of this method can be affected by many factors as listed in Schofield et al. (2001): the type of solvent used, the nature and concentration of acid, the vanillin concentration, the reaction time and temperature, and the PA standard used. Chromatographic methods enable more specific determination and quantification of PAs. We have used reversed-phase high-performance liquid chromatography (RP-HPLC) for analysis of individual shorter-chain PAs (less than tetramers) and normal-phase HPLC (NP-HPLC) for analysis of higher PAs separated by their degree of polymerization (DP; Karonen et al., 2004). PAs in birch leaves are mainly in polymeric form (DP >10), and their concentrations can be easily determined with NP-HPLC (Karonen et al., 2006).

The principal aim of this research was to test some biochemical mechanisms involved in the adaptations of woody plants to an atmospheric pollutant (such as tropospheric O₃) possibly involved in forest decline and to increasing concentrations of atmospheric CO₂. The separate and combined effect of CO₂ and O₃ on the concentrations of total and polymeric PAs in leaves of three woody plant species, *Betula platyphylla*, *Betula ermanii*, and *Fagus crenata*, were evaluated and compared.

Methods and Materials

Study Site

Experiments were carried out at the Akagi Testing Center of the Central Research Institute of Electric Power Industry (100 km north–northwest of Tokyo, Seta county, Gunma prefecture, Japan), 540 m above sea level on the south slope of Mt. Akagi.

Plant Material and Experimental Design

In May 2002, 2-yr-old *B. platyphylla* Sukatchev var. *japonica* seedlings, 3-yr-old *B. ermanii* Cham. seedlings, and 4-yr-old *F. crenata* Blume seedlings were planted individually in pots filled with 12 l of a volcanic ash soil. Trees were grown outdoors at the experimental site until transfer into the chambers. Twenty-four seedlings per species were put into each open-top chamber or ambient plot (AP; outdoor) at the beginning of July 2002. To reduce positional effects, each chamber was divided into four square sections, and seedlings were placed in a fully randomized design within each of these sections. Each spring and summer, all pots were fertilized at a rate of 80–80–80 kg/ha of N–P–K. Seedlings in chambers received subterranean water (pH 7.7) by drip irrigation to the soil, as well as deionized water to the top of plants via mist generating systems.

Exposure to O₃ and CO₂ was conducted by using modified square-greenhouse-type open-top chambers (3.6 × 3.6 m). Advantages of the open-top chamber systems are the ability to control exposure dynamics, an easy definition of the experimental unit, and the possibility to exclude a proportion of ambient O₃ by charcoal filtering of ambient air (Reich, 1987). Chamber walls were transparent glass. Each open-top chamber was equipped with a fan box. Fan boxes were fitted with two fans and two filters containing activated charcoal. Filtered air and treatment gases were fed into a 7.8-m³ space beneath the chamber floor where a thorough mixing of gases occurred before injection into the chamber. Treatment air was introduced into the chamber through holes in the interior. Air supply was sufficient to achieve four changes per min. Injected air was passed through activated charcoal filters. Chamber fans operated continuously (24 hr per day) over the experimental period. Wind velocity at a height of 1.0 m above the floor of chamber ranged from 0.06 to 0.21 m/sec and averaged 0.15 m/sec. Air temperature and gas concentrations were not uniformly distributed within chambers, but deviations were small (about 5%). During the experiment, increases in annual average of mean air temperature inside the chambers as compared with outside were <1°C. Photosynthetic photon flux density inside the chambers was 75% of full sunlight.

Ten open-top chambers were used for five different treatments (two replicates of each): filtered air was passed through activated charcoal (FA), ambient air ($1.0 \times$ ambient O_3), elevated O_3 ($1.5 \times$ ambient O_3), elevated CO_2 ($1.5 \times$ ambient $CO_2 + 1.0 \times$ ambient O_3), and elevated O_3 and CO_2 combined. In addition, two non-chambered APs were established outdoors to test the effect of the chamber. Treatments were conducted from 12 July to 31 October in 2002 and from 26 March to 31 October in 2003. Mean O_3 concentration was 43 ppb in ambient air and 66 ppb in the elevated O_3 treatment during the entire exposure time. Mean CO_2 concentration was 377 ppm in ambient air and 544 ppm in the elevated CO_2 treatment. Additional experimental parameters are shown in Table 1. Ozone was added to the chambers in the $1.0 \times O_3$ and $1.5 \times O_3$ treatments 24 hr per day. Ozone was generated by O_3 generator (model OZ-24-UA; Ebara Corp., Kanagawa, Japan) that used oxygen-enriched dry air as the source gas. Ozonated air was passed through a water scrubber to remove highly reactive by-products such as N_2O_5 before introduction into the air-mixing space. Gas cylinders containing pure liquid CO_2 were used as a source of CO_2 gas. The supply of CO_2 to the chambers in the $1.5 \times CO_2$ treatment was interrupted during the night. Ozone and CO_2 concentrations inside the chambers and in the ambient air were monitored at 3-min intervals by O_3 analyzers (Model 9810; Monitor Labs, Inc., USA) and infrared gas analyzer (Model ZRH; Fuji Electric Corp., Tokyo, Japan), respectively. The air sampling tube was 0.8 m above the floor in the center of the chamber.

Table 1 Average daily CO_2 and O_3 concentrations, mean daily and maximum air temperatures, and mean daily photosynthetic photon flux densities (PPFD) from 21 July to 31 October 2002 and from 26 March to 31 October 2003

	2002	2003
CO_2 concentration (ppm) ^a		
Amb	362	384
$1.5 \times$ Amb	534	549
AP	375	383
O_3 concentration (ppb) ^b		
FA	16	16
Amb	41	44
$1.5 \times$ Amb	61	68
AP	46	46
Mean daily temperature ($^{\circ}C$)		
In the open-top chambers	21	18
Outside the chambers	20	17
Mean maximum temperature ($^{\circ}C$)		
In the open-top chambers	26	23
Outside the chambers	24	21
Mean PPFD ($mol\ m^{-2}\ day^{-2}$)		
In the open-top chambers	17	15
Outside the chambers	22	20

^a Daily 12 hr (0600–1800) average CO_2 concentration in open-top chambers: in ambient CO_2 concentration (Amb) and in the elevated $1.5 \times CO_2$ concentration ($1.5 \times$ Amb); and in an ambient plot outside of the chambers (AP).

^b Daily 12 hr (0600–1800) average O_3 concentration in open-top chambers: in charcoal-filtered air (FA), in ambient O_3 concentration (Amb), and in the elevated $1.5 \times O_3$ concentration ($1.5 \times$ Amb); and in the ambient plot outside of the chambers (AP). O_3 was added to nonfiltered air for 24 hr daily.

Leaf Sampling

Mature leaves were sampled 70 d after the end of bud break in all individual trees in summer 2003: short shoot leaves of *B. ermanii* on 13 June and of *B. platyphylla* var. *japonica* on 20 June, and first-flushed leaves of *F. crenata* seedlings on 8 August. Leaves were collected from two or three lateral branches at the upper part of the seedling from six seedlings per species chamber for a total of 12 samples (seedlings) per treatment. Leaves were frozen in liquid nitrogen and lyophilized. Dry leaf material was ground to powder with a Retsch MM200 Mixer mill and stored at -20°C .

Extraction

Leaf material (30 mg) was extracted in Eppendorf tubes for 30 min with 0.65 ml of 80% aqueous acetone in a planar shaker. The homogenate was centrifuged for 10 min at $8800 \times g$, the clear supernatant was collected, and the insoluble residue was re-extracted once more with 0.65 ml of 80% aqueous acetone and twice with 100% acetone. Extracts were combined, and acetone was removed in a vacuum concentrator (Concentrator 5301, Eppendorf AG, Germany) prior to lyophilization. Lyophilized extracts were dissolved in 1.0 ml of chloroform/80% aqueous methanol mixture (1:1, v/v) and shaken for 25 min in a planar shaker. Following this treatment, 0.5 ml of 20% aqueous methanol was added, and the sample was mixed again for 20 min and centrifuged for 10 min at $8800 \times g$. The aqueous methanol fraction containing polar phenolic compounds was collected, and the methanol was removed by vacuum concentrator prior to lyophilization. The lyophilized fraction was dissolved in 0.7 ml of water, centrifuged for 10 min at $8800 \times g$, filtered through a 0.45- μm PTFE filter, and kept frozen until PA analyses.

Analysis of Proanthocyanidins

Total PA concentrations were analyzed as previously described (Ossipova et al., 2001). A 0.1-ml sample of birch leaf extract and 0.6 ml of water were added to 6 ml of 1-butanol/hydrochloric acid (95:5, v/v) solution. The reaction mixture was heated at 95°C for 2 hr and then cooled to room temperature. Absorbance was measured at 555 nm on Perkin-Elmer Lambda 12 UV/VIS spectrophotometer (Norwalk, CT, USA).

Polymeric PA concentrations were analyzed by using an HP 1090 Liquid Chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an autosampler, binary pump, column oven, diode array detector, and HP ChemStation data system. The column and chromatographic conditions were previously described (Karonen et al., 2004, 2006). A Merck LiChrospher Si 60 column (250 \times 4 mm i.d., 5 μm) equipped with a precolumn at 37°C was used. Two solvents, dichloromethane–methanol–water–acetic acid (82:14:2:2, v/v) (A) and methanol–water–acetic acid (96:2:2, v/v) (B), were used. An injection volume of 5 μl and a flow rate of 1 ml/min were used. The elution profile was as follows: 0–30 min, 0–18% B in A (linear gradient); 30–45 min, 18–31% B in A; 45–50 min, 31–88% B in A; 50–60 min, 88% B (isocratic). The UV trace was recorded at 280 nm.

Total and polymeric PA concentrations were quantified against purified PA standard, which was isolated and purified from mountain birch leaf extract by column chromatography on Sephadex LH-20, as previously described (Ossipova et al., 2001).

Statistical Methods

The effects of treatments on total and polymeric PA concentrations were tested with mixed analyses of variance where treatments and tree species were included as fixed effects and growth chamber as a random effect. *F. crenata* contained only traces of polymeric PAs and was omitted from the analysis. Mixed models were analyzed with PROC MIXED in SAS 8.2 (SAS Inst. Inc. 1999–2001). The Kenward-Roger Method was used for degrees of freedom and variance components for the type of variance-covariance matrix. The desired pairwise comparisons were made with contrast statements according to the significance of the effects in the model. With significant species \times treatment interaction, the differences between treatments within species and the differences between species within treatments were tested. The normal distribution of model residuals was checked visually and with *Kolmogorov-Smirnov* test. Both TPA and PPA concentrations were log-transformed to meet the assumption of normal distribution of model residuals. Back-transformed least-squares means and their 95% confidence limits were used in the figures.

Results and Discussion

Betula platyphylla, *B. ermanii*, and *F. crenata* seedlings were exposed to ambient or elevated O₃ or/and CO₂ during two growing seasons using the open-top chambers.

Table 2 Effect of tree species and treatment on total proanthocyanidin concentration using mixed model analysis of variance and the required pairwise comparisons

	<i>df</i>	<i>F</i>	<i>P > F</i>
Effect			
Spec	2	0.43	0.654
Treat	5	5.56	<0.001
Spec \times Treat	10	1.33	0.216
Pairwise comparisons			
AP vs. Amb	1	9.71	0.002
AP vs. CO ₂	1	3.61	0.059
AP vs. FA	1	8.44	0.004
AP vs. O ₃	1	5.61	0.019
AP vs. CO ₂ + O ₃	1	0.96	0.328
Amb vs. CO ₂	1	1.48	0.225
Amb vs. FA	1	0.04	0.833
Amb vs. O ₃	1	0.56	0.455
Amb vs. CO ₂ + O ₃	1	16.79	<0.001
CO ₂ vs. FA	1	1.01	0.313
CO ₂ vs. O ₃	1	0.22	0.640
CO ₂ vs. CO ₂ + O ₃	1	8.30	0.004
FA vs. O ₃	1	0.29	0.592
FA vs. CO ₂ + O ₃	1	15.11	<0.001
O ₃ vs. CO ₂ + O ₃	1	11.22	0.001

Amb, ambient air; AP, outside of the chamber; CO₂, elevated CO₂; CO₂ + O₃, elevated levels of combined CO₂ and O₃; FA, air filtered through the activated charcoals; O₃, elevated O₃; Spec, species; Treat, treatment.

Leaves of seedlings were collected and extracted, and the soluble PAs were analyzed with two different methods, i.e., colorimetric and chromatographic. Total PA concentrations were analyzed by the colorimetric BH assay. This assay reveals the presence of PAs reliably, but should be used with caution for quantification (see above). There was a significant difference in total PA concentrations between treatments, but no difference between species or significant species \times treatment interaction (Table 2). Total PA concentration was highest in CO₂ + O₃ treatment that differed significantly from all other treatments except AP. Total PA concentration was 29% higher in CO₂ + O₃ treatment compared with the FA control. The AP, in turn, had significantly higher total PA concentrations compared to Amb (24% higher), FA (23% higher), and elevated O₃ (19% higher; Table 2, Fig. 1). Earlier, it was noticed that shading of mountain birch trees reduced the concentrations of soluble PAs in leaves (Henriksson et al., 2003). In this study, light intensity inside the chambers was only 75% of the full sunlight, and the reduced levels of PAs in control seedling leaves inside the chambers (Amb and FA) compared to controls outside the chambers (AP) could be a result of the shading effect. Thus, AP seedlings cannot be used as a control for determining effects of elevated CO₂ or O₃ levels on PA concentrations in the leaves.

The chromatographic methods enable more specific determination and quantification of PAs. In birch species, which have grown in Finland, PAs are mainly in the polymeric form (DP > 10), and only traces of oligomeric PAs can be detected in mature leaves (Karonen et al., 2006). Polymeric PA concentrations can be easily determined with NP-HPLC. The clear peak of polymeric PAs was detected in NP-HPLC traces of *B. ermanii* and *B. platyphylla* extracts (Fig. 2) corresponding to our previous studies (Karonen et al., 2006). Beech leaves contained high amounts of oligomeric PAs, but only traces of polymeric PAs (Fig. 2). The amount of polymeric PAs in beech leaves was below the quantification limit of the NP-HPLC method, and, thus, quantification could not be conducted. The effects of tree species and treatment on polymeric PA concentrations are presented in Table 3 and Fig. 1. Significant species \times treatment interactions were observed. In *B. ermanii*, the polymeric PA concentration was highest in the AP seedlings growing outside of chambers, presumably because of the shading effect of the chamber (see above). The polymeric PA concentration was 13% higher in AP seedlings compared with the FA control. In *B. platyphylla*, the highest polymeric PA concentration was found in the leaves of seedlings exposed to elevated CO₂ + O₃. These leaves differed significantly from all other treatments including AP seedlings. Polymeric PA concentration was 27% higher in CO₂ + O₃ treatment compared with the FA control. In comparison of species within treatments, *B. platyphylla* had significantly higher polymeric PA concentration in seedling leaves exposed to elevated CO₂ + O₃ (27% higher) than *B. ermanii*, whereas in the leaves of AP seedlings, the opposite was true (40% lower; Table 3 and Fig. 1). TPA concentrations were always higher compared to PPA concentrations (Fig. 1). This can be a result of different factors. Various limitations have been reported for the BH assay (see above). PA polymers theoretically produce more anthocyanidins than PA oligomers, but the yield does not stay constant when the chain lengthens. It is likely that even if the birch leaf PAs were mainly PPAs, the leaves also contained shorter-chain PAs. These oligomeric PAs were included in TPA concentrations quantified with the BH assay, but not in PPA concentrations quantified with the normal-phase HPLC method.

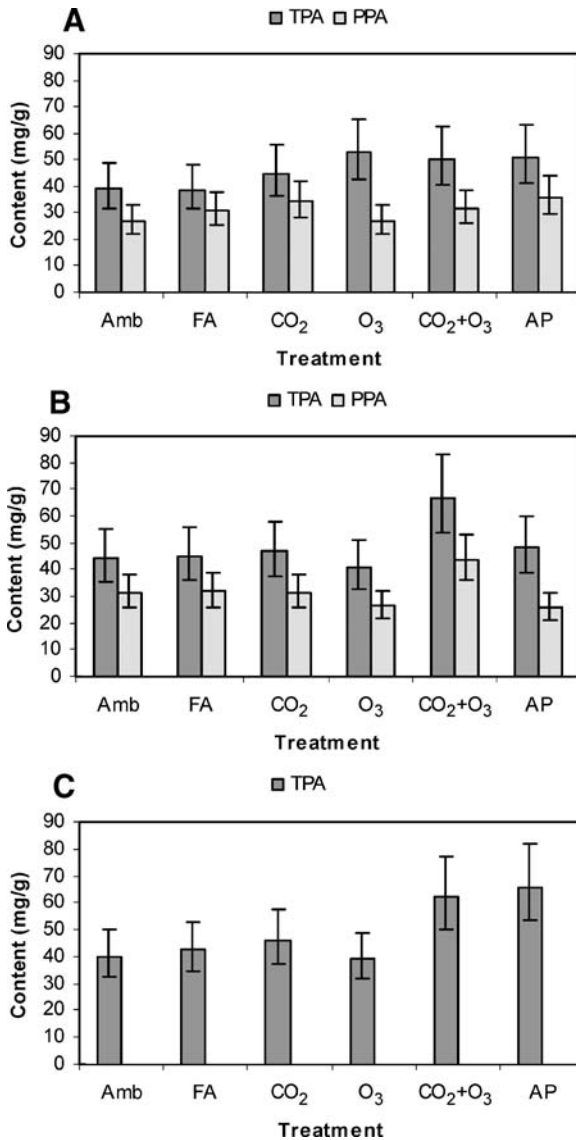


Fig. 1 Total (TPA) and polymeric (PPA) proanthocyanidin concentrations (mg g⁻¹ of dry weight) in the *Betula ermanii* (A), *B. platyphylla* (B), and *Fagus crenata* (C) leaves under atmospheres of ambient air (Amb), air filtered by activated charcoal (FA), elevated CO₂ (CO₂), elevated O₃ (O₃), and elevated levels of combined CO₂ and O₃ (CO₂ + O₃) in open-top chambers, as well as in the seedlings growing outside of the chamber (AP). Data are presented as means with 95% confidence interval

Previously, Lavola et al. (1994, 2000) and Lindroth et al. (2001) reported that total PA concentrations were higher in CO₂-exposed leaves than in control leaves in *B. pendula* and *B. papyrifera*, but fumigation with O₃ did not affect total PA concentrations. However, in our study, only combined CO₂ + O₃ treatment increased PA levels significantly. In the leaves of *B. ermanii*, the positive effect of CO₂

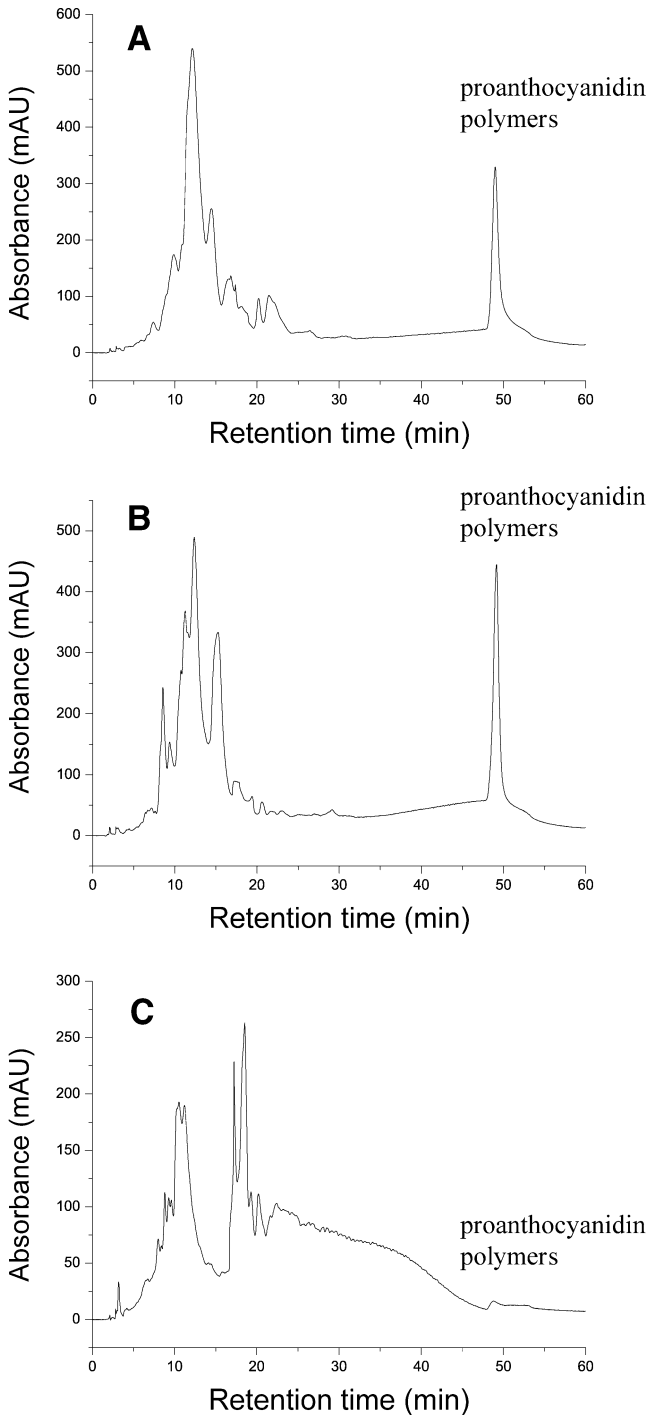


Fig. 2 Normal-phase HPLC traces of soluble phenolics for leaf extracts of *B. ermanii* (A), *B. platyphylla* (B), and *F. crenata* (C)

Table 3 Effect of tree species and treatment on polymeric proanthocyanidin concentration using the mixed model analysis of variance and the required pairwise comparisons

	<i>df</i>	<i>F</i>	<i>P > F</i>
Effect			
Spec	1	0.02	0.876
Treat	5	2.55	0.031
Spec × Treat	5	2.48	0.035
Pairwise comparisons			
<i>Betula ermanii</i>			
AP vs. Amb	1	4.17	0.043
AP vs. CO ₂	1	0.11	0.744
AP vs. FA	1	0.97	0.326
AP vs. O ₃	1	4.18	0.043
AP vs. CO ₂ + O ₃	1	0.78	0.379
Amb vs. CO ₂	1	2.94	0.089
Amb vs. FA	1	1.11	0.293
Amb vs. O ₃	1	0.00	0.997
Amb vs. CO ₂ + O ₃	1	1.34	0.249
CO ₂ vs. FA	1	0.43	0.511
CO ₂ vs. O ₃	1	2.95	0.088
CO ₂ vs. CO ₂ + O ₃	1	0.31	0.579
FA vs. O ₃	1	1.12	0.292
FA vs. CO ₂ + O ₃	1	0.01	0.918
O ₃ vs. CO ₂ + O ₃	1	1.35	0.248
<i>Betula platyphylla</i>			
AP vs. Amb	1	2.06	0.154
AP vs. CO ₂	1	2.20	0.140
AP vs. FA	1	2.33	0.129
AP vs. O ₃	1	0.07	0.792
AP vs. CO ₂ + O ₃	1	14.29	<0.001
Amb vs. CO ₂	1	0.00	0.961
Amb vs. FA	1	0.01	0.927
Amb vs. O ₃	1	1.37	0.244
Amb vs. CO ₂ + O ₃	1	5.50	0.021
CO ₂ vs. FA	1	0.00	0.967
CO ₂ vs. O ₃	1	1.49	0.225
CO ₂ vs. CO ₂ + O ₃	1	5.27	0.023
FA vs. O ₃	1	1.59	0.209
FA vs. CO ₂ + O ₃	1	5.08	0.026
O ₃ vs. CO ₂ + O ₃	1	12.36	<0.001
Difference between species within treatments			
Be vs. Bp (AP)	1	5.75	0.018
Be vs. Bp (Amb)	1	1.16	0.283
Be vs. Bp (CO ₂)	1	0.34	0.558
Be vs. Bp (FA)	1	0.01	0.909
Be vs. Bp (O ₃)	1	0.01	0.929
Be vs. Bp (CO ₂ + O ₃)	1	5.13	0.025

Amb, ambient air; AP, outside of the chamber; Be, *B. ermanii*; Bp, *B. platyphylla*; CO₂, elevated CO₂; CO₂ + O₃, elevated levels of combined CO₂ and O₃; FA, air filtered through the activated charcoals; O₃, elevated O₃; Spec, species; Treat, treatment.

treatment on polymeric PA concentration was close to significant. These differences could be a result of experimental conditions (open-top chambers instead of field and greenhouse experiments), different sensitivity of birch species to elevated concentrations of CO₂ and O₃, or different methods used for PA quantification. Furthermore, leaf dry mass can be influenced by starch levels in tissues, especially when plants are treated with elevated CO₂, and PA concentrations are calculated on dry mass basis. This so-called dilution effect might partly explain why PA concentrations were not increased at elevated CO₂ levels. Moreover, these data were obtained with small individuals grown under almost optimal light and moisture conditions, and plant stresses other than elevated O₃ or CO₂ were minimized. Therefore, the experimental seedlings were not exposed to a variety of interacting stresses, as is the case in nature. As Reich (1987) stated, we do not know if, or how, water stress, herbivores, pathogens, plant–plant competition, nutrition, shading, temperature fluctuations, or other factors influence the O₃-dose or O₃-uptake relations.

Elevated atmospheric CO₂ levels stimulate photosynthesis and increase carbohydrate availability for synthesis of carbon-based secondary metabolites such as phenolics (Booker, 2000). In contrast to CO₂, O₃ affects primary metabolism by inhibition of photosynthesis, which decreases carbohydrate availability for phenolic biosynthesis (Booker, 2000). However, O₃ may exhibit more specific and positive effects on phenolic metabolism by activating the signaling pathways of defensive mechanisms (Rao et al., 2000). Treatment of plants with elevated O₃ increases the biosynthetic activity of the phenylpropanoid pathway, which plays a significant role in plant defense responses by synthesizing compounds potentially protective against phytopathogens and herbivores (Kangasjärvi et al., 1994). Therefore, both elevated CO₂ and O₃ can activate or affect phenolic biosynthesis in different branches of a plant's metabolic network (both primary and secondary metabolism). Thus, the combined influence of elevated CO₂ and O₃ on the PA level in woody plants was the strongest.

Even if the enriched CO₂ or O₃ level alone did not affect PA concentrations, they still affected the growth of *B. platyphylla*, *B. ermanii*, and *F. crenata* seedlings. It was noticed that at ambient CO₂ concentration with increasing level of O₃, the shoot, root, and total biomass of *F. crenata* were decreased. Decreases in shoot or total biomass were also observed in *B. platyphylla* and *B. ermanii*. Predictably, at ambient O₃ concentration, the increased level of CO₂ caused positive effects on the growth of all three species. The total biomass of *B. platyphylla* and *F. crenata* increased significantly and likewise for the shoot biomass of *B. ermanii*. The interactive effects of enriched O₃ and CO₂ on growth of these tree species were species-specific. Significant interaction was found on the biomass of *B. platyphylla*, but not on the biomass in *B. ermanii* or *F. crenata* (Matsumura et al., 2005). Elevated CO₂ is known to increase biomass production, whereas elevated O₃ suppresses it. Increase or decrease of biomass because of the various gas treatments will affect PA inputs to the soil and thus decomposition and nutrient-cycling processes (Booker et al., 2005).

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