

Foliar chemical composition of two oak species grown in a free-air enrichment system with elevated O₃ and CO₂

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Abstract

Ground level ozone (O₃) is rapidly increasing in Asia and penetrates plants via stomata. Atmospheric carbon dioxide (CO₂) has also been increasing in global and in short term may promote plant growth via photosynthetic enhancement. Oaks are representative deciduous broadleaved trees native to northeastern Asia. In this study, we focused on the effects of elevated O₃ and/or CO₂ on leaf nutrients status (phosphorous, nitrogen, potassium, magnesium, etc.) of two oak species: Konara oak (*Quercus serrata*) and Mizunara oak (*Quercus mongolica* var. *crispula*) to assess their function. We investigated foliar starch and sugar contents and nutrients composition of leaves of 2-year-old oak seedlings grown under elevated O₃ and/or CO₂ in a free air enrichment system. From elements concentration, nitrogen and magnesium may become the major indices in assessing the O₃ effects on these species, and investigation of both of them would be of use in field studies to discriminate O₃ and CO₂ effects, especially under the projected elevated CO₂ levels.

Key words: Elevated ozone, Elevated carbon dioxide, Free-air enrichment, Nutrient elements, Oak.

1. Introduction

Recently, tropospheric ozone or ground level ozone (O₃) is continuously increasing in northeast Asia and reduces vigor and health of forests (e.g. Izuta, 2006; Takigawa *et al.*, 2009; Yamaguchi *et al.*, 2011; Koike *et al.*, 2013). In Asian countries, the emission of precursors for O₃ had been increasing and it is expected to continually increase in the foreseeable future (Solberg *et al.*, 2005; Karnosky *et al.*, 2007; Ohara *et al.*, 2007; Watanabe *et al.*, 2007). As a result, O₃ in Asia, including China and Japan, will be also increasing for at least several decades (e.g. Koike *et al.*, 2013). Thus, O₃ has been regarded as one of the most serious environmental stresses for plants (Matyssek *et al.*, 2012; Agathokleous *et al.*, 2015), concerning with forest decline and tree dieback in East Asia (Izuta and Nakaji, 2003; Izuta, 2006; Watanabe *et al.*, 2005). O₃ concentration usually shows the highest value from spring to summer in northeast Japan (Yamaji *et al.*, 2006).

On the other hand, atmospheric carbon dioxide (CO₂) concentration has been yearly increasing and reached to nearly 400 μmol mol⁻¹ from May 2013 (NOAA, 2014). Elevated CO₂ concentration (eCO₂) may have combined effects with elevated O₃ concentration (eO₃) on the growth and photosynthetic capabilities of representative forest species in Japan (e.g. Matsumura *et al.*, 2005). It is said that eCO₂ can somehow compensate the negative effect of high O₃ on growth of deciduous trees related to stomatal closure (Watanabe *et al.*, 2010; Koike *et al.*, 2012). Some researches indeed were carried out on foliar chemical compositions

in eO₃ or eCO₂; most of them however mainly focus on the concentrations of nitrogen (N) and carbon (C) (e.g. Baldantoni *et al.*, 2011). Therefore, few data are available for understanding eO₃ and/or eCO₂ effects on woody species in terms of specific nutrient analysis, even though many physiological and morphological functions are directly regulated by nutrients (e.g. Kohno *et al.*, 2005; Izuta, 2006; Kitao *et al.*, 2015).

Konara (*Quercus serrata*: *Qs*) and Mizunara (*Q. mongolica* var. *crispula*: *Qm*) are deciduous oaks, economically and ecologically important in the rural region of Japan (Maruyama and Miyaura, 2007; Watanabe *et al.*, 2014). These are ectomycorrhizal (ECM) trees (e.g. Oh *et al.*, 1995) and flush 1 to 2 times their shoots in the growing season. Although oaks are regarded as O₃ stress tolerant species among 18 woody species in Japan, they are declining in southern Japan (Kohno *et al.*, 2005). Among the 18 species, *Qs* is relatively sensitive to O₃ compared with *Qm* (Yamaguchi *et al.*, 2011).

At eCO₂, stomatal conductance (Gs) is reduced, accompanied with an enhanced photosynthetic rate because plants do not need widely open stomata for getting CO₂ (Paoletti and Grulke, 2005; Fares *et al.*, 2014). A reduction in Gs would decrease xylem sap flow via a reduced transpiration. Conversely, eO₃ induces a reduction in Gs but accompanied with a decline in photosynthetic function (Paoletti and Grulke, 2005; Hoshika *et al.*, 2012; 2013). It has also been suggested that eO₃ can affect carbohydrate metabolism and hence to reduce their contents (Slid *et al.*, 2002); yet remains unclear. On the contrary, carbohydrates (e.g. starch and sugar) contents may be increased under eCO₂ treatment mainly due to the elevated starch level (e.g. Keutgen and Chen, 2001; Koike, 2006).

In general, leaf mineral content is determined by the balance between influx via xylem and phloem, and efflux via phloem (e.g. Larcher, 2003). In this context, eO₃ and/or eCO₂ would modify

Received; June 4, 2014.

Accepted; October 22, 2015.

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DOI: 10.2480/agrmet.D-14-00018

leaf mineral contents such as macro-elements: phosphorous (P), nitrogen (N), potassium (K), magnesium (Mg) and calcium (Ca) as well as micro-elements: manganese (Mn) via the changes in xylem flow and phloem loading. For instance, concentrations of K, Mg and Mn in leaves are significantly increased under eCO₂ (Fangmeier *et al.*, 2002) whereas photosynthetic performances will be depressed under eO₃ exposure with decreasing of Mg and Mn (Saul, 1964; Paoletti *et al.*, 2002).

From these, it is hypothesized that eO₃ and/or eCO₂ may alter the balance of water-soluble nutrients such as K, Mg, Mn, through change in Gs. It was also expected that foliar nutrients balance in 2 oaks would be altered due to eO₃ effects. Especially, *Qs* may have big changes in some nutrient elements due to relatively higher O₃ sensitivity as compared with *Qm* (Yamaguchi *et al.*, 2011).

To address these hypotheses, we focused on the nutrients composition and also carbohydrate accumulation in 2 oak species grown under free-air enrichment of eO₃ and/or eCO₂. Based on findings of the changes in starch and sugar as well as 6 nutrient elements (P, N, K, Mg, Mn and Ca), we discuss the representative elements and starch in relation to growth traits in the 2 oaks grown under eO₃ and/or eCO₂ and hence investigate indices for understanding the effects of eO₃ and eCO₂ in these oaks in such future environmental conditions.

2. Materials and methods

2.1 Plant Materials

Two-year-old seedlings of Konara oak (*Quercus serrata* Murray: *Qs*) and Mizunara oak (*Quercus mongolica* Fisch. ex Ledeb. var. *crispula* (Blume) Ohashi: *Qm*) were obtained from near Tsukuba, and planted in the nursery (soil pH= about 5.5) of Forestry and Forest Products Research Institute (FFPRI) in Tsukuba, near Tokyo. They were planted at 50 cm interval from each other. Generally, these deciduous oak species cover most parts of Japan and their varieties are also dominant in cool temperate regions of China and Korean peninsula (Menitsky, 2005). Konara oak is one of the most common and widely distributed oak tree species in East Asian temperate forests; Mizunara oak is also typical species in the mixed broadleaf/conifer forests native to the cool-temperate of northeastern Asia (Nakashizuka and Iida, 1995).

2.2 Free-air enrichment system

The free-air enrichment system was located at the experimental nursery of FFPRI in Tsukuba, near Tokyo, Japan (36°00'N, 140°08'E, 20 m a.s.l.). The mean annual precipitation and temperature was 1396 mm yr⁻¹ and 14.0°C. The system was installed with 4 factorial combination treatments of elevated O₃ (eO₃) and/or elevated CO₂ (eCO₂) as follow: ambient (control), eO₃, eCO₂, eO₃ and eCO₂ (e(O₃+ CO₂)) (each frame size was 3.0 m × 3.0 m × 2.0 m) constructed by DALTON Ltd. Co. (Tokyo). Totally 12 plots were installed with 3 replicates for each treatment. Elevated O₃ (eO₃) concentration was 2 times higher than the ambient level and eCO₂ concentration was controlled at about 550 μmol mol⁻¹ in order to simulate 2060 projections (IPCC, 2007). Mean 10h daytime (from 7:00-17:00) O₃ concentration during the experimental period was 30.2 ± 0.4 and 52.2 ± 2.1 nmol mol⁻¹ (mean ± SD) for ambient and eO₃, respectively. Meanwhile, mean 10h daytime (from 7:00-17:00) CO₂ concentration during the experiment period was 377 ± 2.9 and 546 ± 21.3 μmol mol⁻¹

for ambient and eCO₂, respectively (Kitao *et al.*, 2015).

O₃ was generated from an ozone generator (Model PZ2A; Kofloc, Kyoto, Japan) and eO₃ (2 times of ambient) was released from a tubing system suspended throughout the plot. CO₂ was provided by AIR WATER INC. (Osaka, Japan) from liquid CO₂. An O₃ analyzer (Model EG-3000F; Ebara Jitsugyo Co. Ltd., Kanagawa, Japan) and an O₃ monitor (Model 205; 2B Technologies, Boulder, Colorado) were used to control and monitor the O₃ concentration. In addition, CO₂ concentration was monitored with both a Carbon Dioxide Probe (Model GMP343; Vaisala, Helsinki, Finland) and an infrared CO₂ analyzer (Model LI-820; LI-COR Inc., Lincoln, Nebraska) (Kitao *et al.*, 2015). The 2 oak seedlings were grown for one growing season, from beginning of April to the end of October in 2011 under the treatment combinations. Each plot had 6 seedlings of each oak species (*i.e.* 6 *Qs* and 6 *Qm*, totally 12 seedlings).

2.3 Measurements

After 2 to 4 months' treatment with eO₃ and/or eCO₂, 3rd or 4th leaves (counted from the shoot tip) were sampled. As a shoot developmental patterns, either *Qm* or *Qs* was classified as a flush type shoot growth (*e.g.* Kikuzawa, 1983). However, oaks normally flush 2-3 times in one growing season. Therefore, first-flush leaves in May and the first- and second-flush leaves in August were sampled from 2 to 4 different plants in each plot in order to be chemically analyzed. After harvest, leaf greenness was assessed for the fresh sampled leaves by a greenness meter (SPAD 502, Minolta, Osaka, Japan). SPAD readings are closely related to total amount of chlorophyll and nitrogen (N) condition of a leaf (Uddling *et al.*, 2007). Hereafter, leaves were dried at 80°C for at least 5 days.

N concentration was determined by the combustion method with a NC analyzer (NC-900, Sumica, Osaka, Japan).

Sugar content was determined by the phenol-sulfuric acid method (Dubois *et al.*, 1956).

The dried leaf samples were digested to glucose with amyloglucosidase solution (Kabeya and Sakai, 2003) and the starch concentration was determined with Wako Autokit Glucose afterwards (439-90901, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Since leaf starch content is significantly increased in eCO₂ treatment (*e.g.* Koike, 2006), concentrations of the 6 elements are shown on the basis of leaf dry matter subtracting non-structural carbohydrates (starch + sugar) as net values.

Moreover, the prepared dried leaf samples were then digested by nitric acid, hydrogen peroxide method and the concentrations of projected elements (P, K, Mg, Mn and Ca) of the treated leaf samples were measured with an Inductively Coupled Plasma Mass Spectrometry (ICP-MS; IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., Massachusetts, U.S.A.) analysis.

2.4 Statistical analysis

The defined threshold level of significance for rejection of the null hypothesis was set at $\alpha=0.05$. The values of each species and for each treatment were averaged in order to provide the sample estimate for the actual replicate. The data of each parameter were standardized to have a mean equal to 0 and a standard deviation equal to 1; this procedure allows a fair inter-comparison and minimizes the heterogeneity (Saitanis *et al.*, 2014; 2015). All the standardized data were subjected to split-plot general linear model

Table 1. Summary of the GLM results for the value of concentration of starch and sugar, P, N, K, Mg, Mn, Ca and SPAD readings in leaves of 2 oaks. The values represent the F values and the number in the parentheses the *p*-values. Bold text shows significance of the factors. Leaf is consisted of the three types of leaves: a) first-flushed leaves sampled in late May (leaf age was c.a. 40 days old), b) first-flushed sampled in August, and c) second-flushed sampled in August. Gas treatment includes the ambient air, elevated O₃, elevated CO₂, elevated O₃ and CO₂. The two species subjected to the treatments were *Q. mongolica* and *Q. serrata*. Three replications were used for each treatment. Analysis was conducted using standardized data.

	Leaf	Gas	Species	Leaf × Gas	Leaf × Species	Gas × Species	Leaf × Gas × Species
Starch	87.59 (<0.001)	39.45 (<0.001)	1.74 (=0.193)	3.25 (<0.010)	1.59 (=0.215)	3.36 (<0.050)	1.15 (=0.351)
Sugar	9.39 (<0.001)	0.13 (=0.940)	2.89 (=0.095)	0.61 (=0.721)	0.49 (=0.124)	0.69 (=0.560)	0.30 (=0.932)
P	13.95 (<0.001)	1.79 (=0.161)	3.89 (=0.054)	0.59 (=0.734)	0.91 (=0.408)	0.50 (=0.687)	0.62 (=0.715)
N	6.63 (<0.010)	12.91 (<0.001)	5.24 (<0.050)	0.62 (=0.716)	7.16 (<0.010)	1.28 (=0.293)	0.65 (=0.693)
K	9.55 (<0.001)	3.30 (<0.050)	3.18 (=0.081)	0.68 (=0.664)	3.13 (=0.053)	3.36 (<0.050)	0.54 (=0.773)
Mg	37.66 (<0.001)	5.17 (<0.010)	136.11 (<0.001)	1.20 (=0.319)	16.66 (<0.001)	2.20 (=0.101)	1.20 (=0.322)
Mn	27.87 (<0.001)	6.35 (<0.010)	72.05 (<0.001)	1.45 (=0.216)	8.22 (<0.001)	3.35 (<0.050)	1.23 (=0.306)
Ca	67.25 (<0.001)	4.97 (<0.010)	0.88 (=0.354)	2.57 (=0.051)	3.14 (=0.055)	0.95 (=0.424)	0.39 (=0.881)
SPAD	20.56 (<0.001)	1.85 (=0.151)	18.93 (<0.001)	0.62 (=0.714)	9.10 (<0.001)	1.94 (=0.136)	0.69 (=0.657)

(GLM) randomized by blocks (Crawley, 2005), and if there were significant effects, Tukey range, post-hoc test was followed.

To the purpose of the presentation of the data, we present the net values (as shown in above); the results of the GLM are shown in Table 1. Since the responses to the gas treatments were not depended on the leaf types (except starch contents) or the combination of “leaf type” and “species”, the data are represented only for the interactions: Gas × Species and Leaf × Species (Tables 2 and 3, respectively); the differences were marked based on the Tukey range, post-hoc tests. The Cohen’s *d* (Cohen, 1977) was calculated for the concentrations of starch, sugar, P, N, K, Mg, Mn and Ca, independently from leaf types and species.

A principal component analysis (PCA) with varimax rotation (Kaiser, 1958), based on the Cohen’s *d*, was conducted using the pooled data of the 2 species. The gas treatments were used as the classification criteria in assessing the relationships among them and the nutrients. PCA was conducted in order to reduce the dimensionality of the data matrix, avoid redundancy, and highlight relationships (Abdi and Williams, 2010). The continuity of the variables and the number of elements observed were bigger than the number of the original variables. Data were processed and analyzed using MS EXCEL 2010 and STATISTICA v.10 software.

3. Results

Table 1 indicates that the 3 leaf types (“Leaf”) (first-flushed in May and first- and second-flushed in August) had significantly affected carbohydrates and nutrient element contents and SPAD. Leaf starch and N, K, Mg, Mn and Ca contents were significantly affected by the gas treatments (“Gas”) which included ambient air, eO₃, eCO₂, and e(O₃+CO₂). Moreover, species (“Species”) as well as interaction of Leaf × Species were found to have significant effects on leaf N, Mg, Mn and SPAD. Meanwhile, the inter-

actions of Gas × Species, starch and leaf K, Mn were found to be significant. As for the species difference in non-structural carbohydrates and these elements, Table 1 shows that N, Mg and Mn were significantly different between species. No significant effect was observed on either Leaf × Gas or Leaf × Gas × Species interactions for mineral nutrients; only starch was observed to be significantly affected by the interaction of Leaf × Gas.

Starch concentrations were significantly decreased by eO₃ treatments as compared with control (Table 2). Meanwhile, starch was significantly increased by both eCO₂ and e(O₃+CO₂). For *Qm*, although there was no significant difference on starch at eO₃, we observed that starch was 14% lower at eO₃ but significantly recovered by 78% from eO₃ to e(O₃+CO₂). This recovery was smaller than that of *Qs* (which is 110%). Apparently, with eCO₂ and e(O₃+CO₂) treatments in both oaks, N concentration was significantly decreased while starch content was significantly increased. Sugar concentrations were significantly decreased in the 2nd flush of both oaks in August (Table 3). Starch and P concentrations were significantly decreased; however leaf N and K concentrations were significantly increased in August at the 1st or 2nd flush for both oaks.

Foliar Mg, Mn and Ca were significantly decreased by eO₃ treatments (Table 2). The decrease in Mg was clearly found in *Qs*. We found that Mg concentration in both types of leaves of *Qs* was higher than that of *Qm* (Table 3). For 2 oaks, the 1st flush leaves had higher Mg concentration compared with the 2nd flush leaves. For *Qm*, Mg was decreased from May to August at the 1st flush, and also it was significantly lower at the 2nd flush. For *Qs*, Mg was not changed from May to the 1st flush in August, but was significantly lower at the 2nd flush.

In the contrast, for both *Qm* and *Qs*, Mn concentration was significantly increased at the 1st flush in August and had significantly higher value than at the 2nd flush. As for foliar Ca concentration

Table 2. Mean net values (\pm SD) of the concentrations (mg g⁻¹ dry mass) of starch and sugar, P, N, K, Mg, Mn, Ca and the readings of SPAD (arbitrary units) under the 4 gas treatments (Gas \times Species). The values are shown separately for the species *Q. mongolica* (*Qm*) and *Q. serrata* (*Qs*) and pooled for both species (pooled = main effects of the treatment). Comparisons were made within each line of the table (*i.e.* each variable). The lowercase letters above the mean values indicate the significant differences among the 4 gas treatments of the pooled data while the uppercase indicate the differences among the 4 gas treatments for the *Qm* and *Qs*. The results of each variable obtained by one GLM analysis or a Tukey range, post-hoc test, after significant results of the GLM analysis, based on standardized data. Means within each variable marked with different letter differ statistically significantly at a level of significance $\alpha=0.05$.

	Ambient			Elevated O ₃		
	<i>Qm</i>	<i>Qs</i>	Pooled	<i>Qm</i>	<i>Qs</i>	Pooled
Starch	42.87 ^A (26.10)	56.93 ^{AB} (17.90)	49.90 ^a (22.89)	36.64 ^A (21.48)	37.62 ^A (20.91)	37.13 ^b (20.57)
Sugar	69.39 ^A (9.42)	66.94 ^A (6.75)	68.17 ^a (8.05)	68.29 ^A (12.97)	68.98 ^A (9.11)	68.64 ^a (10.88)
P	1.17 ^A (0.53)	1.07 ^A (0.47)	1.12 ^a (0.49)	1.41 ^A (0.43)	1.22 ^A (0.38)	1.32 ^a (0.41)
N	2.01 ^A (0.28)	1.77 ^{ABC} (0.19)	1.89 ^a (0.26)	1.87 ^{AB} (0.20)	1.80 ^{ABC} (0.21)	1.84 ^a (0.20)
K	7.59 ^{AB} (1.22)	7.20 ^{AB} (1.19)	7.39 ^{ab} (1.19)	8.05 ^{AB} (1.46)	7.87 ^{AB} (1.24)	7.96 ^b (1.32)
Mg	2.44 ^{AC} (0.31)	4.03 ^B (1.36)	3.24 ^a (1.26)	2.03 ^A (0.53)	3.12 ^{CD} (1.03)	2.57 ^b (0.97)
Mn	0.32 ^A (0.13)	0.17 ^{CD} (0.04)	0.25 ^a (0.12)	0.23 ^{BC} (0.08)	0.14 ^D (0.02)	0.18 ^b (0.07)
Ca	21.33 ^A (7.82)	18.67 ^{AB} (4.82)	20.00 ^a (6.45)	16.11 ^{AB} (4.20)	14.99 ^B (2.79)	15.55 ^b (3.50)
SPAD	34.16 ^{AB} (4.27)	34.90 ^A (2.75)	34.53 ^a (3.51)	29.72 ^B (4.80)	34.89 ^A (4.38)	32.31 ^a (5.19)

	Elevated CO ₂			Elevated (O ₃ +CO ₂)		
	<i>Qm</i>	<i>Qs</i>	Pooled	<i>Qm</i>	<i>Qs</i>	Pooled
Starch	88.88 ^D (37.21)	77.45 ^{BCD} (37.23)	83.17 ^c (36.59)	65.37 ^{BC} (35.16)	79.34 ^{CD} (19.97)	72.36 ^c (28.65)
Sugar	72.39 ^A (5.97)	65.66 ^A (6.65)	69.03 ^a (7.04)	69.57 ^A (8.10)	65.30 ^A (7.04)	67.44 ^a (7.68)
P	1.30 ^A (0.42)	1.20 ^A (0.54)	1.25 ^a (0.47)	1.21 ^A (0.68)	0.81 ^A (0.43)	1.01 ^a (0.59)
N	1.61 ^{BC} (0.23)	1.60 ^C (0.19)	1.60 ^b (0.21)	1.64 ^{BC} (0.17)	1.57 ^C (0.15)	1.60 ^b (0.16)
K	6.63 ^B (0.96)	7.12 ^{AB} (1.13)	6.88 ^a (1.05)	8.51 ^A (1.06)	6.79 ^B (1.28)	7.65 ^{ab} (1.44)
Mg	2.11 ^A (0.33)	3.56 ^{BD} (1.21)	2.83 ^{ab} (1.14)	2.11 ^A (0.55)	4.14 ^B (1.23)	3.13 ^a (1.39)
Mn	0.26 ^{AB} (0.07)	0.21 ^{BCD} (0.06)	0.23 ^a (0.07)	0.25 ^B (0.07)	0.16 ^{CD} (0.04)	0.21 ^{ab} (0.07)
Ca	19.55 ^{AB} (9.21)	18.63 ^{AB} (6.18)	19.09 ^a (7.62)	18.03 ^{AB} (8.03)	19.47 ^{AB} (7.38)	18.75 ^{ab} (7.52)
SPAD	32.06 ^{AB} (5.24)	34.32 ^A (2.43)	33.19 ^a (4.13)	30.54 ^{AB} (5.04)	34.81 ^A (2.40)	32.68 ^a (4.41)

Note: Since foliar starch contents were significantly increased in elevated-CO₂-grown seedlings, the concentrations of P, N, K, Mg, Mn, and Ca are shown on the basis of leaf dry matter subtracting non-structural carbohydrates (starch + sugar).

for both oaks, it was significantly increased at the 1st flush but was significantly lower at the 2nd flush in August. No difference in Ca was found between species.

SPAD values did not significantly differ among the 4 treatments (Table 2), whereas SPAD values of *Qs* were significantly higher than those of *Qm* at eO₃. Given that they were not different at ambient air, it can be stated that eO₃ had higher effect on leaf greenness of *Qm* than that of *Qs*. Based on Table 3, SPAD values were significantly increased at both the 1st and 2nd flush in August. At the 1st flush in August, SPAD value of *Qs* was significantly increased while at the 2nd flush, *Qm*'s was significantly higher.

Principal component analysis (PCA) was conducted in order to identify elements associated with gas treatments and screen the studied variables as indices in assessing the gas treatment effects on the 2 oaks (Fig. 1). PC1 and PC2 explained 100% of variance and PC1 can be considered as the major axis, with 54% explanation of the total variance. Factor loading is a coefficient which is related to the correlation with the principal components. Loadings range from -1 to 1, and values close to -1 or 1 indicate that the factor strongly affects the variable. Values close to 0 indicate

weak effect of the factor on the variable. High loading reflect the high correlation of the relevant variables with the principal component.

Highest loading on PC1 had the variables starch, N, Mn and Ca (whose factor loading are 0.980, 0.977, 0.962 and 0.907, respectively), explaining 22.3, 22.2, 21.5 and 19.1%, respectively, of the variance of this factor. On the other hand, high loading on PC2 had the variables sugar, P, K and Mg (whose factor loading are 0.993, 0.989, 0.902 and 0.761, respectively), explaining 26.7, 26.5, 22.0 and 15.7%, respectively. Thus, the other highly inter-correlated group of variables is that of sugar, P, K and Mg. eO₃ and e(O₃+ CO₂) contributes 58.9 and 39.1% to PC1, respectively, while eCO₂ and e(O₃+ CO₂) contribute 64.7 and 27.6%, respectively, to PC2.

4. Discussion

As Gs is usually reduced by eCO₂ (*e.g.* Norby and Zak, 2011), plants have higher water use efficiency and lower O₃ uptake (*e.g.* Koike *et al.*, 2012), which may change the absorption of water-soluble elements with aging of leaves (Hoshika *et al.*, 2013).

Table 3. Mean net values (\pm SD) of the concentrations (mg g^{-1} dry mass) of starch and sugar, P, N, K, Mg, Mn, Ca and the readings of SPAD (arbitrary units) in the 3 types of leaves, independently of the gas treatment (Leaf \times Species). The values are shown separately for the species *Q. mongolica* (*Qm*) and *Q. serrata* (*Qs*) and pooled for both species (pooled = main effects of the leaf type). Comparisons were made within each line of the table (*i.e.* each variable). The lowercase letters above the mean values indicate the significant differences among the 3 types of leaves of the pooled data while the uppercase indicate the differences among the 3 types of leaves for the *Qm* and *Qs*. The results of each variable obtained by one GLM analysis or a Tukey range, Post-hoc test, after significant results of the GLM analysis, based on standardized data. Means within each variable marked with different letter differ statistically significantly at a level of significance $\alpha=0.05$.

	1 st flush leaves - May			1 st flush leaves - August			2 nd flush leaves - August		
	<i>Qm</i>	<i>Qs</i>	Pooled	<i>Qm</i>	<i>Qs</i>	Pooled	<i>Qm</i>	<i>Qs</i>	Pooled
Starch	93.60 ^A (27.43)	89.61 ^A (27.57)	91.60 ^a (26.97)	37.56 ^B (19.19)	46.57 ^B (23.33)	42.07 ^b (21.40)	44.17 ^B (30.91)	52.33 ^B (17.33)	48.25 ^b (24.86)
Sugar	73.94 ^A (6.09)	71.45 ^A (3.55)	72.70 ^a (5.04)	72.22 ^A (7.79)	66.48 ^A (8.71)	69.35 ^a (8.60)	63.57 ^A (10.15)	62.23 ^A (5.78)	62.90 ^b (8.11)
P	1.60 ^A (0.15)	1.42 ^A (0.12)	1.51 ^a (0.17)	1.34 ^A (0.64)	0.96 ^A (0.55)	1.15 ^b (0.61)	0.88 ^A (0.33)	0.84 ^A (0.42)	0.86 ^b (0.37)
N	1.64 ^A (0.19)	1.76 ^{AB} (0.18)	1.70 ^a (0.19)	1.73 ^A (0.25)	1.58 ^A (0.19)	1.66 ^a (0.23)	1.97 ^B (0.28)	1.71 ^A (0.22)	1.84 ^b (0.28)
K	7.12 ^{AB} (0.98)	6.30 ^B (0.57)	6.71 ^a (0.89)	8.18 ^A (1.52)	7.21 ^{AB} (1.18)	7.69 ^b (1.42)	7.79 ^A (1.35)	8.22 ^A (1.01)	8.01 ^b (1.19)
Mg	2.61 ^A (0.21)	4.36 ^B (0.43)	3.48 ^a (0.96)	2.03 ^{AC} (0.44)	4.36 ^B (1.25)	3.19 ^a (1.50)	1.89 ^C (0.32)	2.41 ^{AC} (0.57)	2.15 ^b (0.52)
Mn	0.22 ^A (0.03)	0.14 ^B (0.02)	0.18 ^a (0.05)	0.36 ^C (0.10)	0.20 ^{AB} (0.05)	0.28 ^b (0.11)	0.22 ^A (0.07)	0.16 ^{AB} (0.05)	0.19 ^a (0.06)
Ca	11.59 ^A (2.55)	13.18 ^A (1.91)	12.38 ^a (2.35)	26.56 ^C (5.84)	22.88 ^C (5.64)	24.72 ^c (5.93)	18.11 ^B (3.79)	17.76 ^B (3.59)	17.94 ^b (3.62)
SPAD	27.75 ^A (2.12)	32.47 ^{BC} (1.24)	30.11 ^a (2.95)	30.89 ^{AB} (5.26)	36.68 ^D (3.68)	33.78 ^b (5.33)	36.21 ^D (2.42)	35.05 ^{CD} (1.79)	35.63 ^b (2.17)

Note: Since foliar starch contents were significantly increased in elevated-CO₂-grown seedlings, the concentrations of P, N, K, Mg, Mn, and Ca are shown on the basis of leaf dry matter subtracting non-structural carbohydrates (starch + sugar).

According to Yamaguchi *et al.* (2011) and Kitao *et al.* (2015), Gs was relatively higher in *Qm* than in *Qs*. The decrease of Mg, Mn and Ca in eO₃ in both species, maybe because of declined photosynthetic function and low Gs which were induced by eO₃ (Yamaguchi *et al.*, 2011; Kitao *et al.*, 2015), as was also found in Siebolds' beech sapling during young stage of leaf until late-July (Hoshika *et al.*, 2012; 2013).

Carbohydrates (*e.g.* starch and sugar) are produced by green plants served as storage products of energy (*e.g.* Keutgen and Chen, 2001; Koike, 2006). An increase of starch at eCO₂ has also been observed in our study in coincidence with many researches' results (*e.g.* Norby and Zak, 2011). This may be attributed to a feedback mechanism which reduces the sucrose synthesis in the cytosol; as a result, triose phosphate is exported from the chloroplast, finally stimulating starch accumulation in the chloroplast (Sharkey, 1985; Katny *et al.*, 2005). Net photosynthetic rate was decreased by eO₃ but this reduction was slightly recovered by e(O₃+ CO₂) at the 1st flush and by eCO₂ at the 2nd flush for *Qm*; while it was increased by e(O₃+ CO₂) for *Qs* during the entire flush time (Kitao *et al.*, 2015). These trends have suggested that *Qs* may have higher ability for the recover from O₃ damages and may be more tolerant to eO₃ than *Qm* under eCO₂ based on the

summary made by Kohno *et al.* (2005) and Yamaguchi *et al.* (2011).

No significant effects were observed in foliar P concentration at eO₃ for both species at the present study, which was in agreement with the results of Wang *et al.* (2015). Although we hypothesized that leaf P would be increased under eCO₂ since the acquisition of P is strongly affected by physiological condition of host plants through the morphological and the physiological properties of rhizosphere (Adalsteinsson *et al.*, 1994; Ruiz *et al.*, 1996; Kayama *et al.*, 2005; Kayama and Yamanaka, 2014; Wang *et al.*, 2015), there was no difference in leaf P at eCO₂. This was also supported by Conroy (1992), indicating the view that eCO₂ increased the P demand of leaves. As one of the most actively sources for carbohydrates, leaves may prefer the photosynthetic carbon reduction cycle at eCO₂ (Fangmeier *et al.*, 1997).

On the other hand, P concentration was significantly decreased in August for both oaks at the 1st flush leaves and the values continued to be significantly decreased at the 2nd flush of leaves most likely due to P consumption by the leaf flushing. This change is also supported by general trend: P is an essential component of cellular membranes and nucleic acid and is recognized as a significant element for energy storage and transfer (Raghothama, 1999;

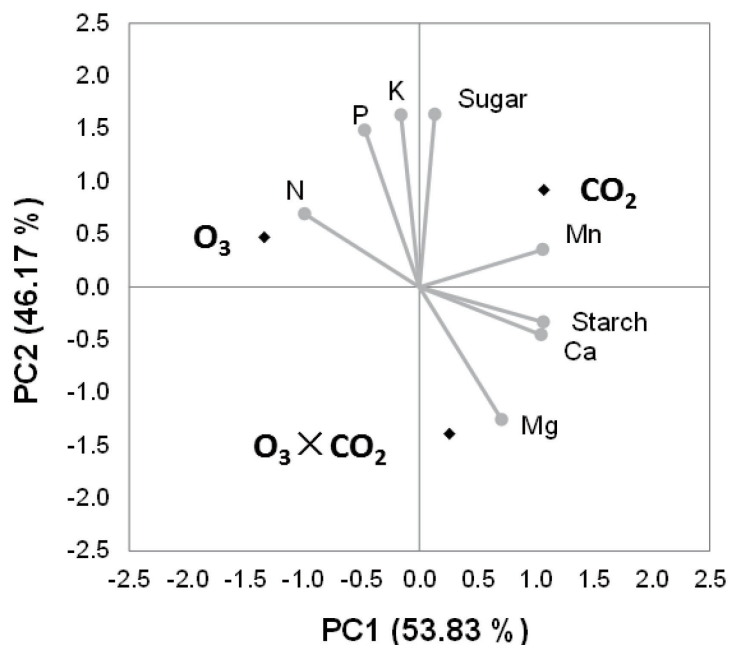


Fig. 1. Biplot of the PCA ordination (with varimax rotation) of the 3 gas treatments, based on their Cohen's *d* of the gas-induced changes on the concentrations of starch and sugar, P, N, K, Mg, Mn, Ca. The circular dot points represent the scores of each response variable, while rhombus dot points represent the scores of the gas treatments, all after Cohen's *d*.

Singh *et al.*, 2013).

As one of the most important macro-nutrients, N is the major component of protein and various enzymes, which importantly affects not only energy reserves but also the process of photosynthesis (Marschner, 2012). eO₃ did not significantly affect leaf N for both *Qm* and *Qs*. This result is consistent with previous results from former researches with other tree species like holm oak (*e.g.* Baldantoni *et al.*, 2011; Matyssek and Sandermann, 2003). Foliar N concentration is decreased due to the reduced demand for N in eCO₂ (*e.g.* Rogers *et al.*, 1993). At eCO₂ and e(O₃+ CO₂) treatments, oaks did not require to accumulate much N because of enriched CO₂ environment (Norby and Zak, 2011). This result was supported by some other researches; such as a potato variety (Fangmeier *et al.*, 2002) and larch species (Koike *et al.*, 2012). The reduced N concentration under eCO₂ may be also attributed to the limitation in nutrients supplies from soil. In addition, the markedly increased leaf N of *Qm* at the 2nd flush in August could be due to the physiological or morphological enhancement of roots (Eguchi *et al.*, 2008) driven by relatively higher net photosynthetic rate (Kitao *et al.*, 2015), which promote N absorption.

Although K is not a direct component of a plant body, it regulates the translocation of photosynthates in sap of plants. High level of K may accelerate the various functions in photosynthesis, such as net carbon exchange, phloem loading, metabolic conversion of sucrose, etc. (Conti and Geiger, 1982). Despite the fact that some previous researches indicated eO₃ and eCO₂ treatment decreased foliar K in potato and beech (*e.g.* Fangmeier *et al.*, 2002; Thomas *et al.*, 2006), what we found in this study (Table 2) is in consistence with Wang *et al.* (2015) where leaf K was not clearly different at eO₃ and eCO₂ from that grown under ambient. It was possibly because the decreased photosynthetic rate

(Kitao *et al.*, 2015) was somehow offset by the enhanced phytohormones induced by eO₃ or eCO₂ (Yong *et al.*, 2000; Winwood *et al.*, 2007; Matyssek *et al.*, 2012), thereby regulating the foliar K independent with the specific gas treatments. Moreover, the high leaf K in August, both the 1st and 2nd flush, was likely connected with remobilisable stored water or with succulence so that K may be accumulated in vacuoles (Von Willert *et al.*, 1992; Cornelissen *et al.*, 1997).

P, K and Mg are known to be water soluble mobile in the phloem and easily re-translocated from older to younger leaves (Marschner, 2012). Mg is the central atom of chlorophyll pigment and essential for the ribosomes aggregation. Therefore, Mg is usually considered as an indicator of leaf greenness and many physiological functions (Shaul, 2002; Teklic *et al.*, 2009). In the present study, the lower result of leaf Mg at eO₃ may also be associated with eO₃ accelerated leaf senescence (*e.g.* Fuhrer *et al.*, 1990; Fangmeier *et al.*, 2002) and reduction of water use efficiency induced by stomatal sluggishness at eO₃ (Hoshika *et al.*, 2015). For the eO₃ treatment and the 1st flush leaves in both May and August, SPAD readings of *Qs* were significantly higher than that of *Qm*, which are consistent with the higher leaf Mg observed in the leaves of *Qs*. Leaf Mg of *Qs* was significantly decreased by eO₃, which may result from the relatively higher susceptibility of *Qs* to eO₃ (Yamaguchi *et al.*, 2011).

Ca is a cellular-level second messenger in signal transduction between the environment and plant responses in terms of development and growth (Marschner, 2012). Mn as one of the co-factors together with Ca and chlorine, promote the water oxidation occurrence in the photosynthetic oxygen evolution (Yachandra *et al.*, 1993; Kitao *et al.*, 1998). Foliar Mn showed the same trend as found in Ca. Leaf mineral content is determined by the balance

between influx via xylem and phloem, and efflux via phloem (e.g. Larcher, 2003). As Mn and Ca are transported from roots to leaves through xylem but poorly mobile in phloem (e.g. Riesen and Feller, 2005; Marschner, 2012), cumulative transpiration flux through stomata would be proportional to leaf Mn concentration (Kitao *et al.*, 2001). In addition, as new flush leaves were developed on the old leaves axles, the nutrient retranslocation of newly flushed leaves were likely great influenced by the nutrient content of old leaves (e.g. Dickson, 1989). Leaf Mn and Ca at the 1st flush in August were significantly higher than those in May, which is consistent with the low mobility of Mn and Ca (Kitao *et al.*, 2001). Leaf Mn and Ca at the 2nd flush, however, had significantly lower values compared with the 1st flush in August. One of the possible reasons is that stomata may become fairly tough until late August as stomata closed (lower Gs) at eO₃ by mid-August for the beech seedlings (Hoshika *et al.*, 2012; 2013). Furthermore, the higher leaf Mn in *Qm* might be due to the higher Gs of *Qm*.

Due to the facts that (a), N was reduced by eCO₂ and e(CO₂ + O₃) but it was not affected by eO₃, while Mg was decreased by eO₃ but not affected by eCO₂ and e(CO₂ + O₃) (Table 2); and (b) one of the highest loading on PC1 (58.9% contribution of eO₃) was that of N and that Mg had a high loading on PC2 (64.7% contribution of eCO₂), N and Mg could be a good pair of indexes for assessing the response to the gas treatments on these species. The two elements are also negatively correlated, which means that both of them will simultaneously respond to the gas treatments. Thus, the effect (or no effect) of the dominant pollutant will be observed. If N is reduced then there is a dominant effect of CO₂, whereas if Mg is decreased then there is a dominant effect of O₃. Consequently, investigation of both N and Mg would be of help in field studies to discriminate O₃ and CO₂ effects, especially under the projected high CO₂ levels. A positive association was indicated between eCO₂ and e(O₃ + CO₂), which were negatively associated with eO₃ (PC1).

Eventually, to understand the dynamics of mineral nutrients mobility in the phloem, demands from young developing tissues and nutrient allocation as a whole plant should also be taken into account.

Acknowledgements

We thank the financial support in part by the Grant-in-aid (JSPS-TypeB 26292075, HokREST and ERTDF-B1105 to T.K.) and Dr. Toshihiro Watanabe of Plant Nutrient Laboratory of Hokkaido University for the guidance of elements analysis with ICP. Thanks are also due to Dr. Masahiro Yamaguchi for invaluable discussion on species difference.

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