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Physiological and stoichiometry study on foliar nutrients and defensive characteristics of representative deciduous broad-leaved tree species in northern Japan under environmental changes

(変動環境における北日本の落葉広葉樹の葉成分と防御特性に関する生理的・化学量論的研究)

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List of abbreviation

A/Amb	ambient ozone
Al	aluminum
AlRE	retranslocation rate of aluminum
ATP	adenosine triphosphate
B	brown forest
Bp	*Betula platyphylla* var. *japonica* (Japanese white birch)
C	carbon
Ca	calcium
CaRE	retranslocation rate of calcium
CNBH	carbon nutrient balance hypothesis
CO_2	carbon dioxide
ConTan	condensed tannins
Cr	chrome
CrRE	retranslocation rate of chrome
E	elevated ozone in chapter 3&4
ECM	ectomycorrhizal
eCO_2	elevated carbon dioxide concentration
eO_3	elevated ozone concentration
$e(O_3+ CO_2)$	combination of elevated ozone and carbon dioxide concentration
ERE	element resorption efficiency (retranslocation rate)
Fc	*Fagus crenata* (Siebold's beech)
Fe	iron
FeRE	retranslocation rate of iron
GDBH	growth differentiation balance hypothesis
Gs	stomatal conductance
H_2O_2	hydrogen peroxide
HCl	hydrochloride acid
HNO_3	nitric acid
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
K	potassium
KRE	retranslocation rate of potassium
LMA	leaf mass per unit area

Mg	magnesium
MgRE	retranslocation rate of magnesium
Mn	manganese
MnRE	retranslocation rate of manganese
N	nitrogen
Ni	nickel
NiRE	retranslocation rate of nickel
N_{mature}	nitrogen contents in mature leaves
NO_x	nitrogen oxides
NRE	retranslocation rate of nitrogen
$N_{senescing}$	nitrogen contents in senescing leaves
O_3	ozone
ODH	optimal defense hypothesis
P	phosphorus
PCA	principle component analysis
PRE	retranslocation rate of phosphorus
Qs	*Quercus serrata* (Konara oak)
Qm	*Quercus mongolica* var. *crispula* (Mizunara oak)
S	serpentine
SM	secondary metabolites
ToPhe	total phenolics
V	volcanic ash

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Summary

Physiological and stoichiometry study on foliar nutrients and defensive characteristics of representative deciduous broad-leaved tree species in northern Japan under environmental changes

（変動環境における北日本の落葉広葉樹の葉成分と防御特性に関する生理的・化学量論的研究）

The global environment has been dramatically changing due to human activities, especially elevated atmospheric carbon dioxide (CO_2), ozone (O_3), and nitrogen (N) as a precursor for O_3. Atmospheric CO_2 has been globally increasing and promotes plant growth via photosynthetic enhancement for an extent. At the same time, ground-level O_3, as one of the serious environmental stresses for plants, has also been continuously increasing in Asia. As O_3 has strong oxidative characteristics, O_3 via stomata penetrating plants and induces morphological and chemical changes in leaves. This can accelerate foliar senescence and negatively affect the vigor and health of forests. Moreover, soil conditions (e.g. nutrient availability) also affect the physiological function of both nutrient retranslocation and plant defense.

Representatives of deciduous broad-leaved tree species native to the northern forests of Japan, such as, Japanese white birch (*Bp*: *Betula platyphylla* var. *japonica* Hara), Siebold's beech (*Fc*: *Fagus crenata* Blume), Mizunara oak (*Qm*: *Quercus mongolica* Fisch. ex Ledeb. var. *crispula* (Blume) Ohashi) and Konara oak (*Qs*: *Quercus serrata* Murray) are considered to be the essential components. Specific O_3 sensitivity have been estimated by former experiments with potted plants in open top chambers for the beech, birch and oak, which have different shoot developmental patterns as determinate, indeterminate and semi-determinate, respectively.

Therefore, my research concerning the physiological and stoichiometry study on these trees will be of help for their adaption investigation to environmental changes and will be utilized on developing appropriate strategies of afforestation in the future. To assess the responses of the above species on various environmental changes, three researches have been carried out with a free-air enrichment system to simulate forest ecosystems.

Firstly, I investigated the physiological effects of elevated CO_2 (eCO_2) and/or elevated O_3 (eO_3) on leaf nutrient status of oak species. I chose two oak (*Qm* and *Qs*)

species as the Japanese representative of *Quercus* species that more tolerant to O_3. Secondly, I examined the stoichiometry of foliar element concentrations and retranslocation of three species to eO_3 under three different soils (brown forest: B, volcanic ash: V, and serpentine: S) over one or two growing seasons and I also discussed on the plausible understanding of physiological functions. Finally, I studied foliar defensive characteristics of three species in responses to eO_3 under two soil conditions (B and V) in relation to protection capacity of the species against biological stresses via eO_3.

1. Foliar nutrients chemical composition of oak species to elevated O_3 and CO_2

Oaks are regarded as O_3 stress tolerant species among 18 woody plants tested in Japan while *Qm* is considered to be more tolerant to O_3 compared to *Qs*. At eCO_2, stomatal conductance is low, which results in suppression of O_3 absorption via stomata. As eCO_2 may have combined effects with eO_3 on growth and photosynthetic capabilities of the two oak species grown under B soil, I investigated foliar nutrients composition (P, N, K, Mg, Mn, Ca) as well as the foliar carbohydrates (starch and sugar) amount of 2-year-old oak seedlings (*Qm* and *Qs*) exposed to eCO_2 and/or eO_3 with a free-air enrichment system. From the results of element concentration, it was found that N and Mg may have the potential to be major indicators in assessing the effects of O_3 on two oaks. I also found that *Qs* may have a higher ability of recovering from O_3 damages and likely become more tolerant to eO_3 than *Qm* under eCO_2 independent of sugar and starch concentration.

2. Foliar element concentrations, the retranslocation and seasonal changes of three species to elevated O_3 under three different soil types

This study was divided into two sections. Retranslocation is the amount of an element that is depleted from aged plant components and is provided for new growth. As leaf senescence is usually accelerated at eO_3 and leaf shedding is also influenced by soil nutrient availability (and acidification), 2 year-old (as of 2014) seedlings of *Bp*, *Fc*, and *Qm* were planted in a free-air O_3 enrichment system under 3 soil types (B, V and S).

For the first section (chapter 3), I focused on the net retranslocation of foliar nutrients (major elements: N, P, K, minor elements: Ca, Fe, Mg, Mn, and non-essential element: Al) to discuss potential effects of eO_3 on seedlings in relation to different soil

conditions via retranslocation traits. I also found that the retranslocation rate of P was increased by eO_3 in *Bp* and by soil treatment in *Qm*; but constant across treatments in *Fc*. Retranslocation of N was affected by soil in *Qm*. Retranslocation of other elements was most sensitive to both eO_3 and soil in *Fc*. I could not detect molybdenum because of too small value.

For the second section (chapter 4), in addition to foliar elements studied above, I added foliar Ni and Cr as an additional minor essential and non-essential element, respectively. This was to further estimate the effects of eO_3 alone and together with different soil conditions on seasonal changes and physiological understanding of stoichiometry of the foliar elements of three species. Relationships among the foliar elements within each species were also investigated. I found that *Fc*, with a determinate shoot growth pattern, was relatively more sensitive to O_3 stress on foliar contents, meanwhile *Qm* was possibly susceptible to O_3 concerning dynamics of immobile elements. Soil nutrients had distinct impacts on retranslocation rate of K, Fe, and P. Principal component analysis revealed that Mn and K can become indicators in assessing O_3 and soil effects in both short and long term growth monitoring of these tree species.

3. Foliar defense characteristics of three species to elevated O_3 under two different soil types

Plants defend themselves against herbivores by employing mainly physical and chemical defense mechanisms. As leaf defense depends on the strong influence of both genotype and environmental conditions, I investigated the leaf chemical defense traits by analyzing the C/N ratio and amount of defensive compounds (lignin, secondary metabolites production: total phenolics and condensed tannin) in response to eO_3 under B and V soils for *Bp*, *Fc* and *Qm* species as components of Fagaceae. In this study, foliar defensive traits were affected by eO_3 for *Bp;* N and C/N were influenced by soils but defensive chemicals were by eO_3 for *Fc*; *Qm* as a tolerant species, it was able to survive under various environment changes.

In conclusion, my results provide the evidences that physiological explanation on stoichiometry of foliar elements and their dynamic variation as well as foliar defensive traits are varied upon environmental changes for each species and they are also species specific. Although *Qm* is regarded as a O_3 tolerant species, after making comparisons among species, it can be susceptible to O_3 concerning dynamics of immobile elements.

My findings are essential in further comprehension to nutrient conservation mechanism in the nutrient dynamics of cool-temperate forests of Japan.

Chapter 1: General introduction

1.1 General description of foliar elements and defense characteristics

1.1.1 Foliar nutrients

In natural forest, nutrients cycling as an essential process that drives significant quantities of nutrients return to soils via decomposition of litter fall and become available for next nutrients life cycling (Salehi et al., 2013). Utilities of nutrients are varied specifically. Some participate in physiological responses, others being storage in different organs, or return to soil again and then partially absorbed by roots for next cycle. Therefore, the uptake and release of nutrients are important aspects due to the representative of primary fluxes through the ecosystem (e.g. Miller and Alpert, 1984).

Leaf stoichiometry, especially for various foliar nutrients, plays essential roles in understanding forest composition, ecosystem function and nutrient limitation (Elser et al., 2000; Wu et al., 2012). Plant stoichiometry of element concentrations reflects the environment changes (Ågren and Weih, 2012) and it deals with the balance of multiple elements in ecology interactions (Rong et al., 2015). Thus, clarify the nutrients stoichiometry issues will be of help for understanding of species adaption capacity to environmental changes and will be utilized on developing appropriate strategies of afforestation in the future.

Retranslocation, as one of the stoichiometric parameters, primarily happens from foliage and fine roots. It is an important process in nutrient dynamics in most ecosystems, especially in broadleaf ecosystems. Retranslocation can be defined as amount of a certain element removal from aged plant organ into the perennial part of the plant prior to senescence and is available for reuse and production in other plant organs on next growth cycle (e.g. Salifu and Timmer, 2001; Killingbeck, 2004). The amount of retranslocation is typically dependent on mobility of element, genotype of tree species and nutrient availability of the soil status (Hagen-Thorn et al., 2004). Therefore, retranslocation as the key mechanism for improving both plant growth and nutrient competitiveness, the percentage of which usually is greater under poor-nutrient soils, reflecting the role as nutrient and species conservation mechanism (Salifu and Timmer, 2001; Uddling et al., 2006).

There are at least 16 essential elements necessary for all plant species to their growth and completion of the life cycles from seed to seed for their succession (Barak, 1999; Ågren and Weih, 2012; Marschner, 2012). Depending on the required quantities for plant growth, it can be divided as macro-nutrients, such as nitrogen (N), phosphorous (P), potassium (K), magnesium (Mg) and calcium (Ca) are required in large amounts; while micro-nutrients, namely trace elements, are those required in smaller quantities but deficiency in which can seriously inhibit plant growth, such as iron (Fe), manganese (Mn) and nickel (Ni) (Barak, 1999; Marschner, 2012). Generally, foliar element concentration is determined by the balance between upward influx via xylem, and downward efflux via phloem (e.g. Larcher, 2003).

Nutrients importance for plant development is dependent on their ability to move within the plant (Marschner, 2012; Goldy, 2013). Thus, understand their mobility is useful in diagnosing their dynamics and comprehending their physiological functions.

Known as water soluble elements, N, P, K, Mg and trace nutrient Ni, are able to be mobile in phloem and become unstable during senescence because they are prepared to retranslocate from older to younger leaves (Helmisaari, 1992; Marchner, 2012). Consequently, the concentration of them in leaves will clearly decrease during senescence (Helmisaari and Siltala, 1989). Hereafter I summarize an essential role of representative elements as follows:

Nitrogen (N) as one of the major limiting elements for plant vigorous growth, foliar N content closely links to photosynthesis, productivity, and litter decomposition, etc. Almost all the plants require N for maintain various ecosystem processes and there is no doubt that N plays a key role in plant growth and is necessary in formation of amino acids, vitamins, proteins, cell division, etc. (Field and Mooney, 1986; Vitousek, 2004; LeBauer and Tresder, 2008; Marchner, 2012).

Phosphorus (P) as a vital element promotes root development that helps strengthen plants. P is important in energy storage and transfer, cell growth and structure, seed formation, genetic materials and water use, etc. There is increasing evidence that foliar P is likely more important than foliar N in limiting plant growth and development (e.g. Reich et al., 2009; Chen et al., 2013).

Potassium (K) regulates the translocation of photosynthates in sap of the plants. It plans an important role on limiting plant growth (Lawniczak, 2011) and helps plants withstand adverse surroundings, defend against diseases and uptake of other elements. High level of K is able to help the improve the overall health of plants by accelerating

the various functions in photosynthesis, such as net carbon exchange, phloem loading, metabolic conversion of sucrose, etc. (Conti and Geiger, 1982; Pettigrew, 2008; Kanai et al., 2011).

Magnesium (Mg) as a secondary macro-nutrient and an intermediately mobile nutrient in plant (Helmissari and Siltala, 1989), it helps regulate phosphorus mobility, iron utilization and aids uptake of other plant nutrients. Since Mg is contained in chlorophyll pigment and essential for ribosomes aggregation, it is also important in leaf greenness and many physiological functions (Shaul, 2002; Teklic et al., 2009; Marchner, 2012; Goldy, 2013).

Nickel (Ni) as a micronutrient, though is needed in small amount as the name implies, it plays an essential role in nitrogen metabolism and fixation. As the metal component, Ni is essential for the structure and catalytic function of the enzyme. It helps plants become tolerant to defend various diseases (Klucas et al., 1983; Marchner, 2012).

Contrarily, Ca, Fe, Mn as well as non-essential elements aluminum (Al) and chrome (Cr) as immobile minerals are transported upward via xylem from roots to leaves but poorly mobile in phloem (Zayed and Terry, 2003; Marschner, 2012). So they are usually remained in older leaves and result in the deficiencies in younger leaves during senescence (Schulze et al., 2005; Shi et al., 2016a).

As calcium (Ca) is a component of cell walls, it helps maintenance of membrane structure and permeability and it also activates some enzymes. Ca is important for general plant vigor and promotes the growth of young roots and shoots. As a cellular-level second messenger in signal transduction between environment and plant, Ca is essential on plants various responses in terms of development and growth (Marschner, 2012; Shi et al., 2016b).

Iron (Fe) assists in the manufacture of chlorophyll and cytochromes. It also plays an important role as oxygen carrier to promote many other biochemical processes such as cell division and nitrogenase, etc. (Marschner, 2012)

Manganese (Mn) as a co-factor together with Ca and chlorine, it promotes the water oxidation occurrence in the photosynthetic oxygen evolution (Yachandra et al., 1993; Kitao et al., 1998). Mn also aids production of chlorophyll and involves in activation of certain enzymes (Marschner, 2012).

Aluminum (Al) and Chrome (Cr), both as are non-essential elements, are widely known as mineral toxic elements. Al as an abundant element in the earth's crust, it is

contained relatively more in mineral soils with lower pH. Although Al is a highly rhizo-toxic and a major stress element to most plants, low-level of which has been observed to have beneficial effects on growth of many plants such as tea and oak (especially *Quercus serrata*), etc. (Marschner, 2012).

Moreover, the metabolic activity involving Cr in plants currently remains unclear but previous study gives the evidence that accumulation of Cr inhibits the N uptake in plants (Kayama et al., 2005; Singh et al., 2013). As all mineral elements need to come from the soil, Cr is important in Hokkaido forest since it is widely known as a component of a typical infertile soil type (serpentine soil) in northern Japan (Brady et al., 2005; Kayama and Koike, 2015).

1.1.2 Defense characteristics and secondary metabolites

Unlike living organisms, plants are incapable to escape from the unfavorable surrounded environment so they have several defense mechanisms against biotic or abiotic stresses. They make appropriate bio-physiological response positively or negatively, to achieve the purpose of survival in hostile environment. Plants defend themselves against herbivores, various diseases as well as battle pathogens. Therefore, plant defense is a vital mechanism for biological conservation and improvement of which is essential on forest maintenance and biological diversification (Ohgushi et al., 2007; Kagata et al., 2005; Nakamura et al., 2005; Aoyama et al., 2010; Lindroth 2010).

Defense mechanism of plants against herbivores is physical, chemical, phonological, and other defensive strategies are adopted (Karban and Baldwin, 1997; Stamp, 2003; Novriyanti et al., 2010). Woody plants defend themselves against herbivores by employing mainly physical and chemical defense mechanisms, especially for foliage (Nabeshima et al., 2001; Haukioja, 2003; Matsuki et al., 2004).

Physical defense is usually based on morphological defense, which involves changes on leaf toughness, leaf mass per area (LMA) and some other characteristics to drive leaves become tougher and better equipped with hair or sticky substances, eventually being hard to be eaten by insects (Bauer at a. 1991; Matsuki et al., 2004; Matsuki and Koike, 2006; Koike et al., 2006a)

With consideration of chemical defense, secondary metabolites (SM) are organic chemical compounds manufactured by plants that are not directly involved in the growth processes or reproduction. But SM affect the long-term of the plants survivability and are often play an essential role in plant defense against herbivory and

other interspecies defenses. As defensive compounds, mainly SM including lignin, condensed tannins, total phenolics, etc. are considered as the stoichiometry parameters, revealing plants chemical defense characteristics. Moreover, there is a biochemical trade-off relationship in synthesis of broadleaved trees between lignin and tannin because both SM is originated from phenylalanine (Schoonhoven et al. 1998). Therefore, I should reveal the synthesis of lignin and defense chemicals under changing environment.

Lignin is a large molecular weight compounds with immobile trait in metabolism process in plant (Coley, 1988). It protects energetic products with a coating for lignified issues and makes plants defense against diseases pathogens and herbivores (Moreira-Vilar et al., 2014).

Phenolics as a major class of SM are widespread in plants (Kraus et al., 2003; Watanabe et al., 2017a). They play a major role in plant defense against herbivores (Lill and Marquis, 2001). The content of total phenolics in plant tissues varies with leaf species, age and degree of decomposition. Among the phenolics, condensed tannins are most widely distributed in many species (Gessner and Steiner, 2005) and are demonstrated to particularly have ecological effects on many functions involve binding to proteins, participating enzymes and working as antioxidatants (Zucker, 1983; Gessner and Steiner, 2005). Previous researches supported that herbivores prefer to graze leaves with low contents of tannins as they act as feeding deterrents and block digestion for insects and herbivores (Ayres et al., 1997).

There are several defense theories and hypotheses to explain the chemical defense mechanisms in plants and here I briefly make descriptions about them as follow.

Carbon-nutrient balance hypothesis (CNBH) explains the effects of soil nutrients on plant defensive chemicals via the allocation of carbon-nutrients in plants (Bryant et al., 1983). CNBH proposes that plant response to herbivores is constrained by resource availability and the plants grown under poorly fertilized soil have higher defense level (Stamp, 2003). Because resource is favored to be available for production of SM when growth condition is sufficient. For example, Aoyama et al. (2010) indicated that fertilized nutrient soil promotes plants growth and stimulates plants flush more leaves and thus loss of leaves grazed by herbivores are able to be compensated and the individual defensive ability of plant (oak and beech) is decreased.

Growth differentiation balance hypothesis (GDBH) as an advanced theory on basis of CNBH (Herms and Mattson, 1992), both indicate that growth processes dominate

over differentiation or production of carbon-based SM as long as environmental conditions are favorable for plant growth. But compared to CNBH, GDBH is associated with the temporal variation in growth activity more directly (Tuomi, 1992). GDBH emphasizes that plants with higher growth rates have less defensive chemicals (Herms and Mattson, 1992). It confirms that plants have to raise defenses effectively to gain sufficient growth and reproduction for survival after they are grazed by herbivores. As a result, the trade-off between growth processes (e.g. lignin synthesis) and production of SM (e.g. total phenolics and condensed tannin) affects resources allocations (Herms and Mattson, 1992; Novriyanti et al., 2012).

Optimal defense hypothesis (ODH) indicates that defense trends to be effective on more valuable part since leaf toughness is favored to be greater in younger leaves (Stamp, 2003; Matsuki et al., 2004; Riipi et al., 2012). As chemical defense, it is mainly relative with production of carbon-based SM, inducing foliage become harsh flavor and even poisonous (Aoyama et al., 2010). ODH with emphasis on allocation cost defense, serves as mainly for investigation of species genotype of the plant defenses (Stamp, 2003)

When taken together, CNBH, GDBH as well as ODH, all stand for the point of view that defensive mechanism in plants is linked to cost and benefit considerations by appropriate resource allocation in plants defensive strategy (Bryant et al., 1983; Herms and Mattson, 1992; Riipi et al., 2002).

1.2 Environmental changes

1.2.1 Ground-level ozone

Generally speaking, most of the atmospheric ozone (O_3) exists in the stratosphere, merely about 10% presents in troposphere. In contrast to stratospheric O_3, which protects the biota in the Earth from harmful ultraviolet but is suffering from decreasing caused by a largely rise in the concentration of chemicals such as chlorofluorocarbons, tropospheric O_3 or ground-level O_3 has been observed to increase over the 20th century (e.g. Lamarque et al., 2005; Grantz, 2014; Yonekura and Izuta, 2017) and is also recognized as one of the significant greenhouse gases since the preindustrial period in northern hemisphere (e.g. Akimoto, 2003; Paoletti et al., 2007; Lindroth, 2010; Koike et al., 2013). Lelieved and Dentener (2000) presented a model calculated ground-level

O_3 concentration over 1860-2025 in northern hemisphere, indicating that the increase of ground-level O_3 concentration will be continuously increasing in foreseeable future, especially in northeastern Asia. According to Sitch et al. (2007) and Ainsworth et al. (2012), gross primary productivity of terrestrial ecosystem will be reduced up to 30 % within this century.

Nitrogen (N) compounds as the most widely precursor to O_3 synthesis, enhancement of emission of atmospheric N is the main source of the O_3 increase (e.g. Wong et al., 2004). Ground-level O_3 is generated from the nitrogen oxides (NO_x) as precursor gases (originating to a large extent from anthropometric sources such as burning of fuels and petrochemicals) by photochemistry reactions that driven by solar radiation and suitable weather condition. In other words, O_3 is formed photochemically from the photolysis of NO_x as follows:

$$NO_x \rightarrow O + R,$$

$$O + O_2 + M \rightarrow O_3 + M,$$

where, R stands for the nitrogen or its oxides; M stands for any carrier body with mass, nitrogen as well as particles, etc. to absorb energy from the photolysis reaction (Atkinson, 2000; Lindroth, 2010; Preiss, 2015; Hatakeyama, 2017).

As an important phytotoxic air pollutant, O_3 strongly oxidizes plants and reduce vigor and health of forests (Lindroth, 2010; Matyssek et al., 2012; Koike et al., 2013). In Japan, O_3 is expected to continually increase for several decades due to the increasing emissions of precursor gases (Lelieved and Dentener, 2000; Paoletti and Manning, 2007). Moreover, O_3 concentration generally shows higher during spring and summer in northeast Japan (Yamaji et al., 2006). Compared to the urban region, O_3 concentration and O_3-induced forest damages, are frequently observed much higher and severe in rural sites as found in a poplar clonal plants (e.g. Gregg et al., 2003).

Several studies have confirmed that elevated O_3 (eO_3) concentration affects woody plants growth and defense as well as their physiological responses, concerning with forest decline and tree dieback in Eastern Asia (Watanabe et al., 2005; Izuta, 2017). Hoshika et al. (2012b) have demonstrated that elevated O_3 (eO_3) induces stomatal sluggishness that can accelerate foliar senescence, thus eO_3 affects the retranslocation and allocation dynamics of foliar nutrients in relation to different physiological responses of O_3 effect for different species.

With eO_3 exposure, N retranslocation efficiency is also declined (Kam et al., 2015), resulting in nutrient deficiencies in leaves caused by the reduction of N acquisition

(Karnosky et al. 2007). Considering the effects of eO_3 on acquisition and loss of foliar mineral elements (i.e. Ca, K, Mg, Mn and Al, Fe), effects of O_3 varied across species with different leaf developments and shoot growth patterns (Shi et al. 2016b).

Moreover, eO_3 also affects the physical and chemical defense traits of leaves (Kangasjärvi et al., 1994; Watanabe et al., 2005) and hence effects on productivity of woody plants. For instance, Watanabe et al. (2005) and Di Baccio et al. (2008) have reported that foliar secondary metabolites concentrations were increased by eO_3.

1.2.2 Carbon dioxide

At the same time, carbon dioxide (CO_2) concentration has been globally risen since pre-industrial period (Barnola et al., 1995). As a significant greenhouse gas and the essential factor for plants photosynthesis, CO_2 has been yearly increasing (NOAA, 2014). There are many studies indicated that increasing atmospheric CO_2 concentration, unlike eO_3, usually promotes photosynthesis, delays foliar senescence and stimulates plants growth in both above- and below-ground to some extent (e.g. Ghannoum et al. 2010; Kirschbaum, 2011; Koike et al. 2015).

Elevated CO_2 (eCO_2) induces a reduction in stomatal conductance (Gs) and accompanied with an enhanced photosynthetic rate since plants do not need open stomata widely for getting CO_2. There are several published data on enhancement of photosynthesis production (e.g. carbohydrates) under eCO_2 (Keutgen and Chen, 2001; Koike, 2006). Conversely, at elevated O_3 (eO_3), Gs is also reduced but accompanied with a decline in photosynthetic function because eO_3 would affect carbohydrate metabolism (Paoletti and Grulke, 2005; Slid et al. 2002). Data from several studies suggest that foliar Mg, K, Mn are increased at eCO_2 (Fangmeier et al., 2002) while foliar Mg and Mn can be also decreased by eO_3 due to the depression of photosynthesis process (Saul,1964; Paoletti et al., 2002). As a result, eCO_2 likely has combined effects with eO_3 and is able to compensate the negative effects of eO_3 on growth of deciduous trees (Matsumura et al. 2005; Koike et al. 2012).

1.2.3 Soil characteristics

Foliar nutrients absorption as well as the plant defense against herbivores are affected by plants species (genotype), soil fertility and other environmental factors such as O_3 (Hagen-Thorn et al., 2004; Koike et al., 2006b). Many previous studies provide

evidences that effects of eO_3 on plants are also highly influenced by soil conditions because plants obtain most nutrient elements from soils and fertilizers (e.g. Agathokleous et al., 2016). For instance, Yamaguchi et al. (2007) found that beech species become more sensitive to O_3 grown under higher N load soil whereas Watanabe et al. (2005) demonstrated a lower negative effect of O_3 on beech trees when seedlings grown under relatively moisture soils. Gleadow et al. (1998) and Koike et al. (2006b) reported that N deposition in soils can change the foliar defense traits due to the altered soil nutrient availability can change plants strategy on defense according to the theory of the CNBH.

Consequently, in order to evaluate the soil impacts on the plants, I applied three different soil types as brown forest soil, volcanic ash soil and serpentine soil to represent different soil fertility (or acidification) conditions throughout my study. The general information about the soil characteristics are summarized in Table 1.1.

Brown forest (B) soil originates from granite and is mostly Cambisols with average pH value of 5.3 (Shi et al., 2017). As a common soil in deciduous broad-leaved forest, it essentially contains necessary elements for plants growth (Okitsu, 2003; Kim et al., 2011). B soil represents rich fertility soil type in my study. pH value of which is in between with other two soil types.

Volcanic ash (V) soil is regarded as an acidic soil type with average lower pH value, widely distributes all over eastern Hokkaido in Japan where volcanoes are located (Takahashi and Shoji, 2002). V soil typically contains high contents of Al and Fe but lacks of many nutrient elements such as N, K, Mg, Ca and Mn (Watanabe et al., 2010; Kayama et al., 2011). In my study, I consider V soil as a medium fertility soil type with relatively acidic pH level.

Serpentine (S) soil as formed by weathering of ultramafic rocks, it lacks of several necessary nutrients for plant growth but characterized by excessive heavy metals such as Mg, Ni and Cr, which resulting in a high ratio value of Mg/Ca and relatively a higher pH value (Okitsu, 2003; Kayama et al., 2005). S soil is known to be widely distributed over northern Japan and it is deemed as a poor fertility soil type with a relatively alkaline pH value in my study.

In perspective of environmental changes, N deposition in soils has been ever-increasing in recent decades, especially for those regions developing dramatically in eastern Asia (Galloway et al., 2004). As a result, soil nutrients availability has been changed by N deposition. Previous researches indicated that forest decline and dieback

may also due to the N saturation, nutrient imbalance, soil fertility and acidification, etc. (e.g. Magani et al., 2007). Therefore, different soil fertilities (or acidification) should also be taken into account as it is an essential factor on study of environmental changes.

1.3 Tree species

As the representative deciduous tree species in northern Japan forest, Japanese white birch (hereafter *Bp*: *Betula platyphylla* var. *japonica* Hara), Siebold's beech (hereafter *Fc*: *Fagus crenata* Blume), Mizunara oak (hereafter *Qm*: *Quercus mongolica* Fisch. ex Ledeb. var. *crispula* (Blume) Ohashi) and Konara oak (hereafter *Qs*: *Quercus serrata* Murray) were selected in my study because of the essential roles they played in cool temperate forests in Japan, providing the benefits in social, economic and environment (e.g. Koike, 1988; 1995; Koike et al., 1998; Shi et al., 2016a;b; Shi et al. 2017).

Japanese white birch *Bp* is broadly distributed in cool temperate region of northeastern Asia, including northern Japan and northeast of China (Mao et al., 2010). As a typical pioneer tree species in northern forest, it can be rapidly established after large disturbances (Koike, 1995; Hoshika et al., 2013b). Matsumura et al. (2005) and Hoshika et al. (2012a) provide evidences that long-term eO_3 exposure may have adverse effects on birch *Bp* but no reduction effects are observed on short-time eO_3 exposure during summer time. Birch *Bp* as a heteophyllous shoot development with two types of leaves (early and late), early-leaves are less susceptibility to eO_3 than late-leaves because early-leaves show a higher capacity of avoidance for eO_3-induced low stomata conductance (Gs) (Koike, 1995; Hoshika et al., 2012a; 2013b). Moreover, plant defense characteristics are various with different successional traits. Matsuki and Koike (2006) reported that species with shorter leaf lifespan have more effective photosynthetic productivity but have less defense capacity and consequence of more susceptible to herbivore attack. Thus, as birch *Bp* is an early-successional species that has a shorter leaf lifespan with indeterminate shoot growth (Coley, 1988; Koike, 1988; 2004; Matsuki and Koike, 2006), the defense mechanism of *Bp* is expected as to herbivores in this study.

Siebold's beech *Fc* is the most representative deciduous broad-leaved tree species and widely distributed in cool temperate forests of Japan (Nakashizuka and Iida, 1995; Koike et al. 1998). Forest of beech *Fc*, however, recently is observed to be decline and

dieback in several mountainous regions in Japan (Aihara et al., 2004). Previous researches have reported that beech *Fc* is considered as highly sensitive species to O_3 (Kohno et al., 2005; Yamaguchi et al., 2011) and it prefers mesic and fertile soil condition for growth (Izuta et al., 2004). As a result, the sensitivity of growth of beech *Fc* seedlings to O_3 becomes higher with increasing amount of N in soil contents (Yamaguchi et al., 2007).

Konara oak *Qs* and Mizunara oak *Qm*, both are important deciduous oak species, essentially benefit for economic and ecology of cool temperate forests in Japan, especially play an important role in rural areas (Maruyama and Miyaura, 2007; Watanabe et al., 2014). However, oak trees have been declining since the 1980s and incidence of oak wilt have become gradually more conspicuous in forest of Japan (Ito and Yamada, 1998; Matsuda et al., 2010). Generally, oaks are ectomycorrhizal trees and flush 1 to 2 times during their growing season (e.g. Oh et al., 1995). Although Kohno et al. (2005) reported that oaks are usually regarded as tolerant species to eO_3 among 18 woody plants in Japan, for *Quercus* species, *Qs* as the oak species with moderate O_3 sensitivity level, is relatively more sensitive to O_3 compared with *Qm* which has the low sensitivity level to O_3 stress among 18 species tested in Japan (Yamaguchi et al., 2011; Watanabe et al., 2017a).

Effects of O_3 stress on plants is relatively widespread and species-specific (Watanabe et al., 2017b). Table 1.2 presents the classification of Japanese forest trees to O_3 sensitivity groups. Understanding of the plants sensitivity or tolerant capacity to O_3 stress is very critical to further study on physiological and ecological adaption of the specific species. According to the summary in Table 1.2, O_3 sensitivity of the concerning species in my study (which have been shown in bold) can be ranked from high to low as follows: beech *Fc* > birch *Bp* > oak *Qs* > oak *Qm*.

1.4 Hypotheses of the study

1.4.1 Foliar nutrients and elements retranslocation under environmental changes

Referring to the O_3 tolerant capacity (or sensitivity) that mentioned earlier, beech species *Fc* is the most sensitive and oak species *Qm* is the most tolerant to O_3 among the trees species applied in my study. Considering foliar nutrients and their allocation dynamics about retranslocation rate, I made hypotheses under environmental changes

as follow:

(1) Under eO_3 accompanied with eCO_2, Gs is reduced by both elevated gas conditions. Therefore, I hypothesized that eO_3 and/or eCO_2 can change the balance of water-soluble foliar nutrients via alternations in xylem flow and phloem loading that induced by reduced Gs. Moreover, photosynthesis performance is declined by eO_3 but enhanced by eCO_2, I also expected the balance of foliar chemical composition and especially photosynthesis production (e.g. carbohydrates) may be altered under eO_3 effects. Considering the different O_3 sensitivity of two oak species (*Qs* and *Qm*), *Qs* would affect more at eO_3 for some certain elements due to its relatively higher sensitivity trait as compared with *Qm*.

(2) Under eO_3 accompanied with various soil types, eO_3 accelerates the leaf senescence. Soil fertility (or acidification) affects the retranslocation responses to eO_3. Moreover, foliar elements as well as their retranslocation rate can be highly related with both leaf and shoot growth patterns. Early-successional tree species may retranslocate less nutrients from foliage before senescence, and may become much less grown under infertile soils (Killingbeck, 2004; Koike, 2004). Consequently, birch *Bp* in my study is expected to have less retranslocation rate under eO_3, especially accompanied with serpentine soil. On the other hand, I also hypothesized that foliar elements in *Qm* may have the least responses to eO_3 when considering about the O_3 tolerance capacity of oak species. At the same time, more significant results of retranslocation rates are also expected in *Fc* due to the O_3 sensitive of beech species.

1.4.2 Defensive chemicals under environmental changes

I hypothesized that eO_3 accompanied with poor fertility soil can lead to a higher defense mechanism by changing C/N ration and several certain defensive chemicals. About the comparisons among the different species, I prefer re-considering again the O_3 sensitivity. I hypothesized that oak *Qm* can be a higher defense level species and become insusceptible to herbivores under environmental changes due to its higher tolerant capacity to eO_3.

1.5 Objectives of the study and approaches for the goal

In order to fulfill the achievements, I concentrated on the stoichiometry study on

foliar nutrients contents and their allocation dynamics of retranslocation as well as the aspects of defense chemicals under various environmental changes. Moreover, I also focused on the stoichiometry changes of representative foliar elements or chemical compositions to discuss their essential roles of physiological functions or defense mechanism in terms of specific species traits.

Firstly, I examined the concentrations of representative foliar nutrients (N, P, K, Mg, Ca, Mn) as well as accumulation of carbohydrates (starch and sugar) in two oaks (*Qs* and *Qm*) under eO_3 accompanied with eCO_2. I made a discussion in relation to the growth characteristics for each oak species and also investigated indices for understanding the effects of eO_3 and eCO_2 in these oaks under such future environmental changes

Furthermore, I quantified the both contents and retranslocation rates of various foliar elements (e.g. N, P, K, Mg, Ca, Mn, Fe, Ni, and Al, Cr) to determine the response to eO_3 accompanied with various soil conditions (B, V and S soil) for birch (*Bp*), beech (*Fc*) and oak (*Qm*) species over one or two growing seasons.

I also made comparisons among species on elemental retranslocation rate for a long-time O_3 exposure. Besides, I tested relationships among foliar elements within each species over long-time exposure to find any highly correlations for better explaining the alternations on elemental allocation dynamics and hence make clear about the physiological and biochemical functions in foliage.

Last but not least, I investigated the leaf defense traits by analyzing defensive indexes (LMA, N, C/N and secondary metabolites production such as lignin, total phenolics and condensed tannins) in response of eO_3 under two different soils (B and V soils) for three representative species (birch *Bp*, beech *Fc* and oak *Qm*) in northern Japan forests.

My study aims to provide further evidences on understanding of stoichiometry of foliar elements as well as defensive chemicals in response of high O_3 accompanied with various environmental factors, and hence to better understand the elements cycling and plant defense mechanism under elevated O_3 world. Eventually, achieve the final goal of ecosystem rehabilitation to make a sustainable production of broad-leaved trees under changing environment.

The frame work and flow chart of the research approach to obtain the research goals is presented in Figure 1.1.

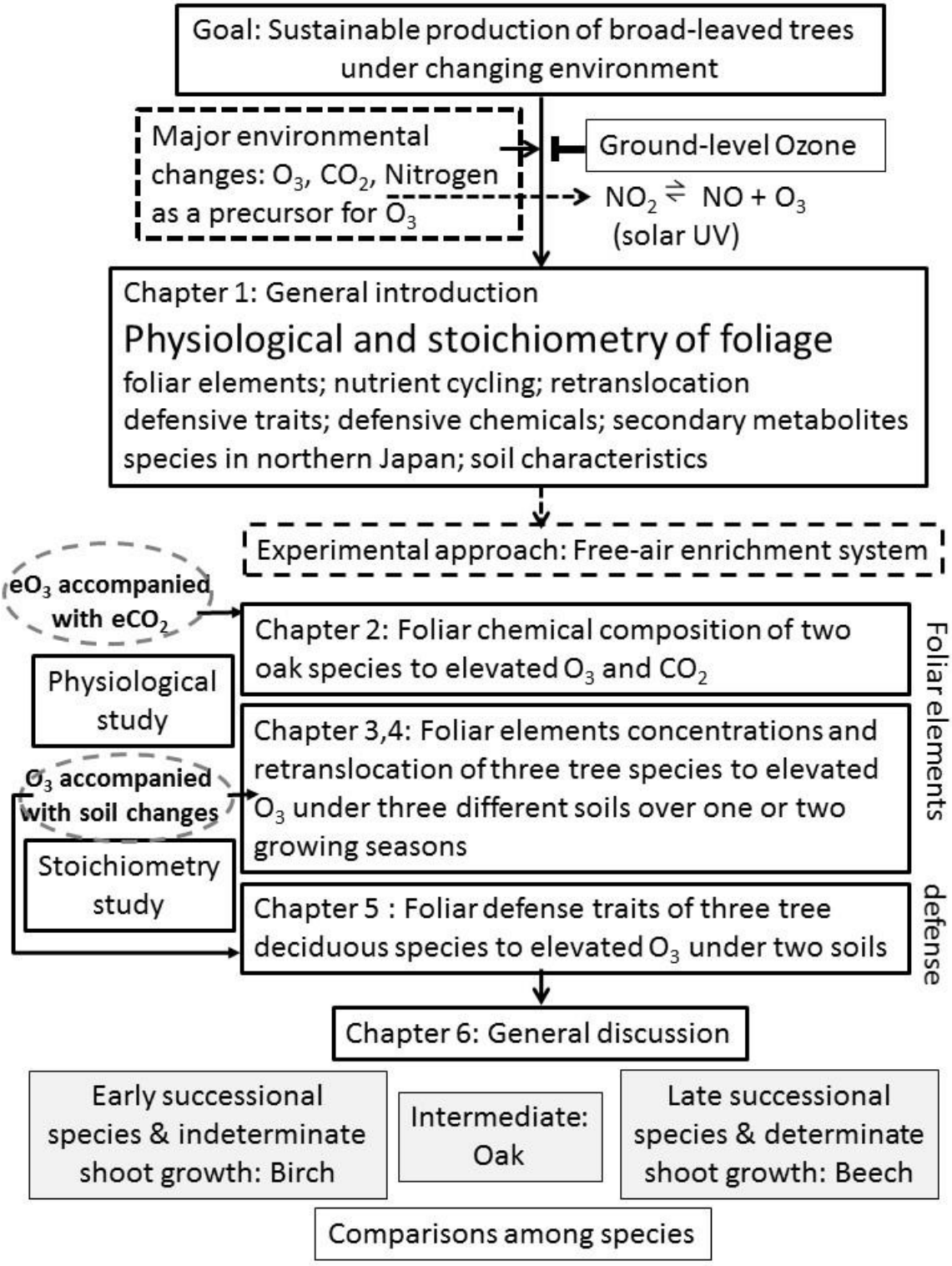


Figure 1.1 Flow chart of research approach to obtain the research goals

Table 1.1 General information about characteristics of soils that were applied in my study.

Soil types	Distribution	Elements composition	pH value	References
Brown forest (B) soil	Common soil in deciduous broad-leaved forest	Essentially contains necessary elements for plants growth	Big range; average around 5.3	e.g. Okitsu, 2003; Kim et al., 2011; Shi et al., 2017
Volcanic ash (V) soil	Distributed in regions where volcanoes are located. Mainly in eastern Hokkaido	Low: N, K, Mg, Ca, Mn High: Al, Fe, S	Lower pH	e.g. Takahashi and Shoji, 2002; Watanabe et al., 2010; Kayama et al., 2011;
Serpentine (S) soil	Widely distributed in northern Japan	Low: Ca and several essential plant nutrients High: Ni, Cr, Mg	Higher pH	e.g. Kayama et al., 2005; Okitsu, 2003

Table 1.2 Classification of Japanese forest trees to O_3 sensitivity groups. Relative species concerning my study are shown with bold

O_3 sensitivity	**Leaf habit**	**Leaf growth**	**Species**
High (sensitive to O_3)	Broad-leaved	Deciduous	*Populus maximowiczii,* *Populus nigra,* **Siebold's beech (*Fagus crenata*)** Japanese zelkova
		Evergreen	*Castanopsis sieboldii*
	Coniferous	Deciduous	Japanese larch
		Evergreen	Japanese red pine
Moderate	Broad-leaved	Deciduous	**Japanese white birch (*Betula plathpylla* var. *japonica)*** **Konara oak (*Quercus serrata)***
		Evergreen	*Quercus myrsinaefolia,* *Cinnamomum camphora*
	Coniferous	Evergreen	Nikko fir
Low (tolerant to O_3)	Broad-leaved	Deciduous	**Mizunara oak (*Quercus mongolica* var. *crispula*)**
		Evergreen	*Lithocarpus edulis,* *Machilus thunbergii*
	Coniferous	Evergreen	Japanese black pine Sugi cedar Hinoki cypress

(Edited by Koike T. on basis of Kohno et al., 2005; Yamaguchi et al., 2011)

Notes: List of common name and its corresponding Latin name of the species

Common name:	**Latin name**
Japanese zelkova	*Zelkova serrata*
Japanese larch	*Larix kaempferi*
Japanese red pine	*Pinus densiflora*
Nikko (Urajiro) fir	*Abies homolepis*
Japanese black pine	*Pinus thunbergii*
Sugi cedar	*Cryptomeria japonica*
Hinoki cypress	*Chamaecyparis obtusa*

Chapter 2: Physiological responses on foliar nutrients chemical composition of two oak species to elevated ozone and carbondioxide

2.1 Introduction

2.1.1 Background

Recently, tropospheric ozone or ground surface ozone (O_3) is continuously increasing in northeast Asia and reduces vigor and health of forests (e.g. Izuta, 2006; 2017; Takigawa et al., 2009; Yamaguchi et al., 2011; Koike et al., 2013). In Asian countries, the emission of precursors for O_3 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, O_3 in Asia, including China and Japan, will be also increasing for at least several decades (e.g. Koike et al., 2013). Thus, O_3 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, 2017; Watanabe et al., 2005). O_3 concentration usually shows the highest value from spring to summer in northeast Japan (Yamaji et al., 2006).

On the other hand, atmospheric carbon dioxide (CO_2) concentration has been yearly increasing and reached to nearly 400 μmol mol^{-1} from May 2013 (NOAA, 2014). Elevated CO_2 concentration (eCO_2) may have combined effects with elevated O_3 concentration (eO_3) on the growth and photosynthetic capabilities of representative forest species in Japan (e.g. Matsumura et al., 2005; Koike et al. 2015). It is said that eCO_2 can someway compensate the negative effect of high O_3 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 eO_3 or eCO_2; 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 eO_3 and/or eCO_2 affecting on woody species in terms of specific nutrient analysis even though many physiology 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 O_3 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 O_3 compared with *Qm* (Yamaguchi et al., 2011).

At eCO_2, stomatal conductance (Gs) is reduced accompanied with an enhanced photosynthetic rate because plants need not open stomata widely for getting CO_2 (Kopper and Lindroth, 2003; Paoletti and Grulke, 2005; Fares et al., 2014). A reduction in Gs would decrease xylem sap flow via a reduced transpiration. Conversely, eO_3 also induces a reduction in Gs but accompanied with a decline in photosynthetic function (Paoletti and Grulke, 2005; Hoshika et al., 2012b; 2013c). It has also been suggested that eO_3 can affect carbohydrate metabolism and hence to reduce their contents (Slid et al., 2002), yet is still not clear. Conversely, it is said that carbohydrates (e.g. starch and sugar) contents may be increased under eCO_2 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, eO_3 and/or eCO_2 would modify 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 eCO_2 (Fangmeier et al., 2002) whereas photosynthetic performances will be depressed under eO_3 exposure with decreasing of Mg and Mn (Saul, 1964; Paoletti et al., 2002).

2.1.2 Hypotheses and objectives

On basis of the above background, it is hypothesized that eO_3 and/or eCO_2 may alter the balance of water-soluble nutrients such as K, Mg, Mn, etc. through change in Gs. It was also expected that foliar nutrients balance in 2 oaks would be altered due to eO_3 effects. Especially, *Qs* may have big changes in some nutrient elements due to relatively higher O_3 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 fumigation of eO_3 and/or eCO_2. Based on findings of the changes in starch and sugar as well as 6 nutrient elements (P, N, K, Ca, Mg and Mn), we discuss on representative elements and starch

in relation to growth traits in the 2 oaks grown under eO_3 and/or eCO_2 and hence investigate indices for understanding the effects of eO_3 and eCO_2 in these oaks in such future environmental conditions.

2.2 Materials and methods

2.2.1 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).

2.2.2 Free-air enrichment system

The free-air fumigation 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℃.The system was installed with 4 factorial combination treatments of elevated O_3 (eO_3) and/or elevated CO_2 (eCO_2) as follow: ambient (control), eO_3, eCO_2, eO_3 and eCO_2 ($e(O_3+ CO_2)$) (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 O_3 (eO_3) concentration was 2 times higher than the ambient level and eCO_2 concentration was controlled at about 550 μmol mol^{-1} in order to simulate 2060 projections (IPCC, 2007). Mean 10 h daytime (from 7:00-17:00) O_3 concentration during the experimental period was 30.2 ± 0.4 and 52.2 ± 2.1 nmol mol^{-1} (mean ± SD) for ambient and eO_3, respectively. Meanwhile, mean 10h daytime (from 7:00-17:00) CO_2 concentration during the experiment period was 377 ± 2.9 and 546 ± 21.3 μmol mol^{-1} for ambient and eCO_2, respectively (Kitao et al.,

2015).

O_3 was generated from an ozone generator (Model PZ2A; Kofloc, Kyoto, Japan) and eO_3 (2 times of ambient) was released from a tubing system suspended throughout the plot. CO_2 was provided by AIR WATER INC. (Osaka, Japan) from liquid CO_2. An O_3 analyzer (Model EG-3000F; Ebara Jitsugyo Co. Ltd., Kanagawa, Japan) and an O_3 monitor (Model 205; 2B Technologies, Boulder, Colorado, U.S.A.) were used to control and monitor the O_3 concentration. In addition, CO_2 concentration was monitored with both a Carbon Dioxide Probe (Model GMP343; Vaisala, Helsinki, Finland) and an infrared CO_2 analyzer (Model LI-820; LI-COR Inc., Lincoln, Nebraska, USA) (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).

2.2.3 Measurements

After 2 to 4 months' fumigation of eO_3 and/or eCO_2, 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℃ 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 eCO_2 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 mill grounded into powder and made a digestion generally by following steps: 1) add 50 mg leaf samples into 100 ml beaker; 2) heat hotplate in the fume cupboard; 3) add concentrated nitric acid (HNO_3), hydrochloride acid (HCl), 30 % hydrogen peroxide (H_2O_2) in sequence with volume of 4 ml: 2 ml: 2 ml (or1 ml); 4) increase the temperature of hotplate until 200℃; 5) add diluted HNO_3 (2 %) until all the samples being decomposed; 6) add 20-25 ml distilled water to each beaker after it becomes almost dry; 7) increase the temperature of hotplate until 250℃; 8) continuously run the hotplate 30-45 min until no bubbles in the beaker; 9) rinse each beaker with 2% HNO_3 after it becomes almost dry; 10) make a filtration for the rinsed solution in each beaker; 11) fix each filtered solution to 50 ml flask with 2% HNO_3 and get the digested solution for each sample.

Afterwards, the concentrations of projected elements (K, P, Ca, Mg and Mn) of the treated leaf samples were determined with an Inductively Coupled Plasma Mass Spectrometry (ICP-MS; IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., Massachusetts, U.S.A.) analysis.

2.2.4 Statistical analysis

The defined threshold level of significance for rejection of the null hypothesis was set at α=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 randomized by blocks (GLM) (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 2.1. Since the responses to the gas treatments were not depended on the leaf types (except starch contents) and the combination of “leaf type” and “species”, the data are represented only for the interactions: Gas×Species and Leaf×Species (Tables 2.2 and 2.3, respectively), and 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 and sugar, N, P, K, Ca, Mg and Mn 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 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.

2.3 Results

Table 2.1 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 K, N, Ca, Mg and Mn contents were significantly affected by the gas treatments ("Gas") which included ambient air, eO_3, eCO_2, and $e(O_3+ CO_2)$. 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 interactions 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 2.1 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 eO_3 treatments as compared with control (Table 2.2). Meanwhile, starch was significantly increased by both eCO_2 and $e(O_3+CO_2)$. For *Qm*, although there was no significant difference on starch at eO_3, we observed that starch was 14 % lower at eO_3 but significantly recovered by 78 % from eO_3 to $e(O_3+CO_2)$. This recovery was smaller than that of *Qs* (which is 110 %). Apparently, with eCO_2 and $e(O_3+CO_2)$ 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 2.3). 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 Ca, Mg and Mn were significantly decreased by eO_3 treatments (Table 2.2).

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 2.3). 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 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 2.2), whereas SPAD values of *Qs* were significantly higher than those of *Qm* at eO_3. Given that they were not different at ambient air, it can be stated that eO_3 had higher effect on leaf greenness of *Qm* than that of *Qs*. Based on Table 2.3, 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 (Figure 2.1). 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 reflects 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, K, P and Mg. eO_3 and $e(O_3+ CO_2)$ contributes 58.9 and 39.1 % to PC1, respectively, while eCO_2 and $e(O_3+$

CO_2) contribute 64.7 and 27.6 %, respectively, to PC2.

2.4 Discussion

As Gs is usually reduced by eCO_2 (e.g. Norby and Zak, 2011), plants have higher water use efficiency and lower O_3 uptake (e.g. Koike et al., 2012), which may change the absorption of water-soluble elements with aging of leaves (Hoshika et al., 2013c). 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 eO_3, in both species, maybe because of declined photosynthetic function and low Gs which were induced by eO_3 (Yamaguchi et al., 2011; Kitao et al., 2015), as was also found in Siebolds' beech (*Fc*) sapling during young stage of leaf until late-July (Hoshika et al., 2012b; 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 eCO_2 has also been observed in our study in coincidence with many researches' results (e.g. Norby and Zak, 2011; Koike et al. 2015). 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 eO_3 but this reduction was slightly recovered by $e(O_3+ CO_2)$ at the 1st flush and by eCO_2 at the 2nd flush for *Qm*; while it was increased by $e(O_3+ CO_2)$ 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 O_3 damages and may be more tolerant to eO_3 than *Qm* under eCO_2 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 eO_3 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 eCO_2 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 eCO_2. This was also supported by Conroy's research (1992), indicating the view that eCO_2 increased the P demand of leaves. As one of the most actively sources for carbohydrates, leaves may prefer the

photosynthetic carbon reduction cycle at eCO_2 (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; Singh et al., 2013).

As one of the most important macro-nutrients, N is the major component of protein and various enzymes, importantly affects not only energy reserves but also the process of photosynthesis (Marschner, 2012). eO_3 did not significantly affect leaf N for both *Qm* and *Qs*. This result was also consistent with the 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 eCO_2 (e.g. Rogers et al., 1993). At eCO_2 and $e(O_3+ CO_2)$ treatments, oaks did not require to accumulate much N because of enriched CO_2 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 eCO_2 may be also attributed to the limitation on 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 eO_3 and eCO_2 fumigation decreased foliar K in potato and beech (e.g. Fangmeier et al., 2002; Thomas et al., 2006), what we found in this study was in consistence with Wang et al. (2015) that leaf K was not clearly different at eO_3 and eCO_2 from that grown under ambient condition as we showed in Table 2.2. It was possibly because that the decreased photosynthetic rate (Kitao et al., 2015) was somehow offset by the enhanced phyto-hormones induced by eO_3 or eCO_2 (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).

K, P 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 eO_3 may also be associated with eO_3 accelerated leaf senescence (e.g. Fuhrer et al., 1990; Fangmeier et al., 2002) and reduction of water use efficiency induced by stomatal sluggishness at eO_3 (Hoshika et al., 2015). For the eO_3 treatment and the 1st flush leaves in both May and August, SPAD readings of *Qs* showed 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 eO_3, which may result from the relatively higher susceptibility of *Qs* to eO_3 (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 Ca and Mn 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 Ca and Mn at the 1st flush in August were significantly higher than those in May, which is consistent with the low mobility of Ca and Mn (Kitao et al., 2001). Leaf Ca and Mn at the 2nd flush, however, had significantly lower values compared to 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 eO_3 by mid-August for the beech seedlings (Hoshika et al., 2012b; 2013c). Furthermore, the higher leaf Mn in *Qm* might be due to the higher Gs of *Qm*.

Due to the fact, N was reduced by eCO_2 and $e(CO_2 + O_3)$ but it was not affected by eO_3, while Mg was decreased by eO_3 but not affected by eCO_2 and $e(CO_2 + O_3)$ (Table 2.2), as well as the fact that one of the highest loading on PC1 (58.9 % contribution of eO_3) was that of N and that Mg had a high loading on PC2 (64.7 % contribution of eCO_2), 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. So the effect (or no effect) of the dominant pollutant will be observed. If N is reduced then there is a dominant effect of CO_2, whereas if Mg is decreased then there is a dominant effect of O_3. Consequently, investigation of both N and Mg would be of help in field studies to discriminate O_3 and CO_2 effects, especially under the projected high CO_2 levels. A positive association was indicated between eCO_2 and $e(O_3+ CO_2)$, which were negatively associated with eO_3 (PC1).

2.5 Summary

To sum up, in this study I investigated foliar starch and sugar contents and nutrients composition of leaves of 2-year-old oak seedlings grown under elevated O_3 and/or CO_2 in a free air enrichment system. From the data of effects of gas treatments, *Qs* has possibly a higher ability for being recovery from O_3 damages and likely become more tolerant to eO_3 than *Qm* under eCO_2. From the results of elements concentration, Mg and N may become the major indices in assessing the O_3 effects on these species and investigation of both of them would be of use in field studies to discriminate O_3 and CO_2 effects, especially under the projected elevated CO_2 levels. However, 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.

Table 2.1 Summary of the GLM results for the value of concentration of starch and sugar, N, P, K, Ca, Mg, Mn 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 O_3, elevated CO_2, elevated O_3 and CO_2. 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**	**39.45**	1.74	**3.25**	1.59	**3.36**	1.15
	(<0.001)	**(<0.001)**	(=0.193)	**(<0.010)**	(=0.215)	**(<0.050)**	(=0.351)
Sugar	**9.39**	0.13	2.89	0.61	0.49	0.69	0.30
	(<0.001)	(=0.940)	(=0.095)	(=0.721)	(=0.124)	(=0.560)	(=0.932)
P	**13.95**	1.79	3.89	0.59	0.91	0.50	0.62
	(<0.001)	(=0.161)	(=0.054)	(=0.734)	(=0.408)	(=0.687)	(=0.715)
N	**6.63**	**12.91**	**5.24**	0.62	**7.16**	1.28	0.65
	(<0.010)	**(<0.001)**	**(<0.050)**	(=0.716)	**(<0.010)**	(=0.293)	(=0.693)
K	**9.55**	**3.30**	3.18	0.68	3.13	**3.36**	0.54
	(<0.001)	**(<0.050)**	(=0.081)	(=0.664)	(=0.053)	**(<0.050)**	(=0.773)
Mg	**37.66**	**5.17**	**136.11**	1.20	**16.66**	2.20	1.20
	(<0.001)	**(<0.010)**	**(<0.001)**	(=0.319)	**(<0.001)**	(=0.101)	(=0.322)
Mn	**27.87**	**6.35**	**72.05**	1.45	**8.22**	**3.35**	1.23
	(<0.001)	**(<0.010)**	**(<0.001)**	(=0.216)	**(<0.001)**	**(<0.050)**	(=0.306)
Ca	**67.25**	**4.97**	0.88	2.57	3.14	0.95	0.39
	(<0.001)	**(<0.010)**	(=0.354)	(=0.051)	(=0.055)	(=0.424)	(=0.881)
SPAD	**20.56**	1.85	**18.93**	0.62	**9.10**	1.94	0.69
	(<0.001)	(=0.151)	**(<0.001)**	(=0.714)	**(<0.001)**	(=0.136)	(=0.657)

Table 2.2 Mean net values (± SD) of the concentrations (mg g^{-1} dry mass) of starch and sugar, N, P, K, Ca, Mg, Mn and the readings of SPAD (arbitrary units) under the 4 gas treatments (Gas×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 α=0.05.

	Ambient			Elevated O_3			Elevated CO_2			Elevated (O_3+CO_2)		
	Qm	***Qs***	**Pooled**	***Qm***	***Qs***	**Pooled**	***Qm***	***Qs***	**Pooled**	***Qm***	***Qs***	**Pooled**
Starch	42.87^{A}	56.9^{AB}	49.90^{a}	36.64^{A}	37.62^{A}	37.13^{b}	88.88^{D}	77.5^{BCD}	83.17^{c}	65.4^{BC}	79.3^{CD}	72.36^{c}
	(26.1)	(17.90)	(22.89)	(21.48)	(20.91)	(20.57)	(37.21)	(37.23)	(36.6)	(35.16)	(19.97)	(28.65)
Sugar	69.39^{A}	66.94^{A}	68.17^{a}	68.29^{A}	68.98^{A}	68.64^{a}	72.39^{A}	65.66^{A}	69.03^{a}	69.57^{A}	65.30^{A}	67.44^{a}
	(9.42)	(6.75)	(8.05)	(12.97)	(9.11)	(10.88)	(5.97)	(6.65)	(7.04)	(8.10)	(7.04)	(7.68)
P	1.17^{A}	1.07^{A}	1.12^{a}	1.41^{A}	1.22^{A}	1.32^{a}	1.30^{A}	1.20^{A}	1.25^{a}	1.21^{A}	0.81^{A}	1.01^{a}
	(0.53)	(0.47)	(0.49)	(0.43)	(0.38)	(0.41)	(0.42)	(0.54)	(0.47)	(0.68)	(0.43)	(0.59)
N	2.01^{A}	1.8^{ABC}	1.89^{a}	1.87^{AB}	1.80^{ABC}	1.84^{a}	1.61^{BC}	1.60^{C}	1.60^{b}	1.64^{BC}	1.57^{C}	1.60^{b}
	(0.28)	(0.19)	(0.26)	(0.20)	(0.21)	(0.20)	(0.23)	(0.19)	(0.21)	(0.17)	(0.15)	(0.16)
K	7.59^{AB}	7.20^{AB}	7.39^{ab}	8.05^{AB}	7.87^{AB}	7.96^{b}	6.63^{B}	7.12^{AB}	6.88^{a}	8.51^{A}	6.79^{B}	7.65^{ab}
	(1.22)	(1.19)	(1.19)	(1.46)	(1.24)	(1.32)	(0.96)	(1.13)	(1.05)	(1.06)	(1.28)	(1.44)
Mg	2.44^{AC}	4.03^{B}	3.24^{a}	2.03^{A}	3.12^{CD}	2.57^{b}	2.11^{A}	3.56^{BD}	2.83^{ab}	2.11^{A}	4.14^{B}	3.13^{a}
	(0.31)	(1.36)	(1.26)	(0.53)	(1.03)	(0.97)	(0.33)	(1.21)	(1.14)	(0.55)	(1.23)	(1.39)
Mn	0.32^{A}	0.17^{CD}	0.25^{a}	0.23^{BC}	0.14^{D}	0.18^{b}	0.26^{AB}	0.21^{BCD}	0.23^{a}	0.25^{B}	0.16^{CD}	0.21^{ab}
	(0.13)	(0.04)	(0.12)	(0.08)	(0.02)	(0.07)	(0.07)	(0.06)	(0.07)	(0.07)	(0.04)	(0.07)
Ca	21.33^{A}	18.7^{AB}	20.00^{a}	16.1^{AB}	14.99^{B}	15.55^{b}	19.55^{AB}	18.6^{AB}	19.09^{a}	18.0^{AB}	19.5^{AB}	18.75^{ab}
	(7.82)	(4.82)	(6.45)	(4.20)	(2.79)	(3.50)	(9.21)	(6.18)	(7.62)	(8.03)	(7.38)	(7.52)
SPAD	34.2^{AB}	34.90^{A}	34.53^{a}	29.72^{B}	34.89^{A}	32.31^{a}	32.06^{AB}	34.32^{A}	33.19^{a}	30.5^{AB}	34.81^{A}	32.68^{a}
	(4.27)	(2.75)	(3.51)	(4.80)	(4.38)	(5.19)	(5.24)	(2.43)	(4.13)	(5.04)	(2.40)	(4.41)

Note: Since foliar starch contents were significantly increased in elevated-CO_2-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).

Table 2.3 Mean net values (± SD) of the concentrations (mg g^{-1} dry mass) of starch and sugar, N, P, K, Ca, Mg, Mn and the readings of SPAD (arbitrary units) in the 3 types of leaves, independently of the gas treatment (Leaf×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 α=0.05.

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

Note: Since foliar starch contents were significantly increased in elevated-CO_2-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).

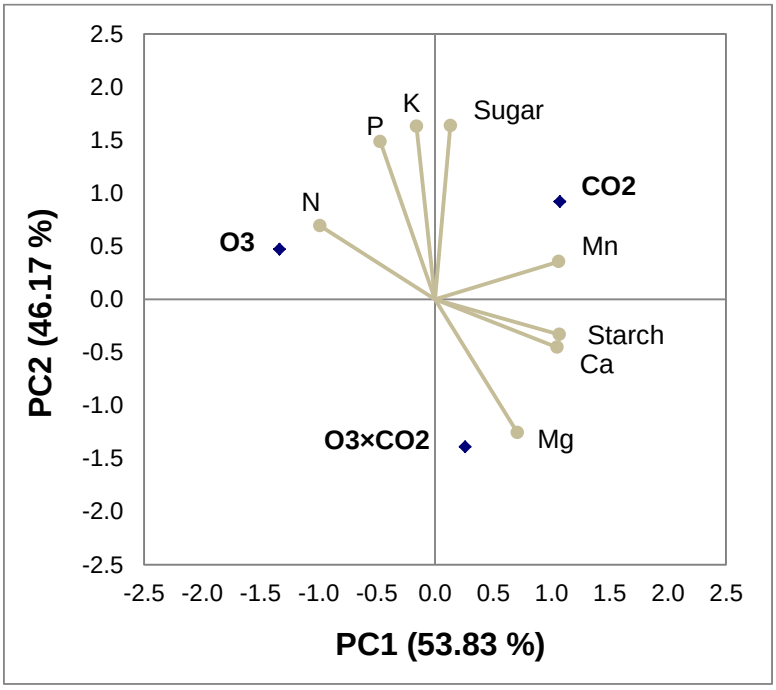


Figure 2.1 Biplot of the PCA ordination (with varimax rotation) of the 3 gas treatments, based on their Cohen's d of the gas-induced changes on the concentrations of starch and sugar, P, N, K, Mg, Mn, Ca. The circular dot points represent the scores of each response variable, while rhombus dot points represent the scores of the gas treatments, all after Cohen's d.

Chapter 3: Physiological responses on foliar elements retranslocation of three deciduous tree species to elevated ozone and three different soils

3.1 Introduction

3.1.1 Background

Retranslocation of foliar nutrients can be considered as the amount of certain elements depleted from aged plant organs and used for production in other parts of the plant (e.g. Salifu and Timmer, 2001). It is one of the most essential processes in nutrient dynamics and ecosystem function via decomposition processes, especially for broadleaf species (e.g. Killingbeck, 2004; Koike, 2004).

Retranslocation can be quantified by the parameter of element resorption efficiency (ERE), which is defined as the percentage of the foliar nutrient pool absorbed (Huang et al., 2007; Farahat and Linderholm, 2015). Positive ERE values indicate that plants are able to reuse rather than lose their internal nutrients with senescence, therefore plant growth, reproduction and competitive ability will be facilitated and vice versa for negative ERE values (e.g. Farahat and Linderholm, 2015).

Retranslocation has become characterized as an important strategy for trees to conserve nutrients, which eventually effects nutrient competition, absorption and productivity (e.g. Salehi et al., 2013). It is an essential mechanism for improving plant growth as well as nutrient competitiveness and species conservation (e.g. Salifu and Timmer, 2001; Uddlings et al., 2006).

These processes of nutrient retranslocation in plants are likely to be affected by plant genetic make-up or plant species, soil fertility, as well as other environmental conditions (e.g. Hagen-Thorn et al., 2004). Despite the fact that research has been conducted on the retranslocation response of foliar nutrients, most of them has mainly targeted one or various species (e.g. Helmisaari, 1992; Salehi et al., 2013) under different soil statuses, for instance, water irrigation and nitrogen fertilization (e.g. Salifu and Timmer, 2001; Zhao et al., 2013). Therefore, little information is available for understanding the effects of elevated ozone (hereafter: eO_3) and its relations and interactions with soil fertility (or acidification) on the retranslocation process of foliar nutrients (Uddlings et al., 2006).

Recently ground level O_3 concentration has been continuously increasing in northeast Asia and is negatively affecting the forest health (e.g. Serengil et al., 2011; Koike et al., 2013). Furthermore, elevation of O_3 is expected to continuously increase for at least several decades in the foreseeable future (e.g. Ohara et al., 2007). In addition, eO_3 can accelerate foliar senescence (Hoshika et al., 2012b) and reduce the retranslocation rate of nitrogen (N) (e.g. Kam et al., 2015), resulting in nutrient deficiencies in leaves caused by the decline of N acquisition (e.g. Karnosky et al., 2007).

Brown forest soil (mostly Cambisols) in Japan which is a common soil type for deciduous broad-leaved forests, contains the necessary elements for plant growth (e.g. Kayama et al., 2011; Kim et al., 2011). Serpentine soil is derived from weathered serpentine rock (e.g. Brady et al., 2005; Kayama and Koike, 2015) and is characterized by excessive Mg and heavy metals but having low contents of Ca and several essential nutrients (e.g. Brady et al., 2005). It has a high pH value and is also known to be widely distributed throughout Japan (e.g. Kayama and Koike, 2015). Volcanic ash soil is an acidic soil that contains high levels of aluminum (Al) and iron (Fe), but low contents in magnesium (Mg), calcium (Ca), manganese (Mn), nitrogen (N) and potassium (K) (e.g. Kayama et al., 2011; Watanabe et al., 2010). As the response of retranslocation seems to be influenced by soil fertility (Xu and Timmer, 1999) as well as soil acidification (Gjengedal, 1996; Adams et al., 2006), we have planted seedlings on brown forest soil, volcanic ash soil and serpentine soil. These soil conditions represent rich fertility with neutral soil, medium fertility with acidic soil and poor fertility with alkaline soil, respectively.

As the representative of deciduous species in the northern forests of Japan, Japanese white birch (*Bp*: *Betula platyphylla* var. *japonica*), Mizunara oak (*Qm*: *Quercus mongolica* var. *crispula*), and Siebold's beech (*Fc*: *Fagus crenata*) were chosen, since they all provide social, economic and environmental benefits that are important to the cool temperate forests of Japan (e.g. Koike, 1995; Koike et al., 1998). According to the Yamaguchi et al. (2011), O_3 tolerance capacity can be labeled from low to high on these 3 species and is as follows: beech (*Fc*), birch (*Bp*), oak (*Qm*).

3.1.2 Hypotheses and objectives

In general, most early successional tree species retranslocate less nutrients from their leaves before shedding them, compared to late successional trees, and this is especially so for plants grown in infertile soil condition (e.g. Killingbeck, 2004; Koike,

2004). In this study, I focus on the retranslocation and allocation dynamics of foliar nutrients (N, P, K, Ca, Mg, Mn and Fe, Al) to achieve the following major objectives: 1) Determine how O_3 concentration effects foliar nutrients retranslocation in *Bp*, *Fc* and *Qm*. 2) Quantify foliar nutrient retranslocation of element N, P, K, Ca, Mg, Mn and Fe, Al, and as well as to examine foliar nutrients retranslocation in different soil sites. 3) Evaluate the combined effects of both O_3 and soil on foliar nutrients retranslocation in species of *Bp*, *Fc* and *Qm*. In addition, I investigate the effects of O_3 on N contents in mature and senescing leaves, as well as the relation of N dynamics and leaf mass per area (LMA) to further clarify the effects of O_3 on foliar N dynamics in 3 representative woody plants seedlings. Based on these results, I also discuss the plausible understanding of the ecophysiological meaning of retranslocation of each element in leaves in relation to specific traits of O_3 effect.

3.2 Materials and methods

3.2.1 Plant materials

Two-year old seedlings of Japanese white birch (*Bp*: *Betula platyphylla* var. *japonica*), Siebold's beech (*Fc*: *Fagus crenata*) and Mizunara oak (*Qm*: *Quercus mongolica* var. *crispula*) were obtained near Sapporo, and were planted in mid-July 2014 at the nursery of Hokkaido University, Sapporo. These 3 deciduous tree species are the major components of cool temperate forests in Japan (e.g. Koike, 1988; 1995; Koike et al., 1998). All the seedlings were grown in the same soil-environment and growth media.

3.2.2 Experiment site description

The experimental site was located in Experimental Nursery of Hokkaido University Forests in center part of Sapporo in northern Japan (43°04′ N, 141°20′ E, 15 m a.s.l.) with about a population of two million people. Annual mean temperature and the total precipitation in 2014 were 8.2 ℃ and 1,129 mm respectively. A free-air O_3 enrichment system was utilized at the university experimental nursery with each system having three soil types (brown forest soil, serpentine soil, volcanic ash soil) and with each soil type being about 30 cm in depth. The enrichment lasted for one growing season. The

experimental site consists of 3 control and 3 treatment circular plots that are 6.5 m in diameter and with each system being surrounded by 5 m high dispersal pipes (Kim et al., 2011). Details of the O_3 exposure in field station as well as the plot design can be found in Watanabe et al. (2013) and Shi et al. (2017). O_3 concentration of 70 (±7) nmol mol^{-1} was applied during daylight hours (about 7 h everyday) from early-August to mid-November in treatment plots, while at the same time, the control plots were subjected to ambient daytime O_3 concentration of 26.5 nmol mol^{-1} (Kam et al., 2015)

Each of the free-air enrichment plot was unevenly divided into 3 parts, replaced with 3 different soil types: about 84 % of the plot area was divided into 2 parts, each equally consisting of brown forest (B) soil and volcanic ash (V) soil; about 16 % of the area was serpentine (S) soil. The detailed for the design of each free-air enrichment experimental circular plot as well as the distribution of those 3 soils in each plot is shown in Figure 3.1. All of these soil types are widely distributed across northern Japan (e.g. Kim et al., 2011). Brown forest soil is native to the Sapporo Experimental nursery, immature volcanic ash soil was brought from the Tomakomai Experimental Forest of Hokkaido University (Kim et al., 2011) and serpentine soil was brought from the eastern part of Teshio Experimental Forest (Watanabe et al., 2012), where serpentine soil is dominant (Kayama and Koike, 2015).

When taken together, the system was set up with 6 factorial combination treatments of O_3 exposure and soil types in the experimental nursery as follow: ambient O_3 × B soil (AB), eO_3 × B soil (EB), ambient O_3 ×S soil (AS), eO_3 × S soil (ES), ambient O_3 × V soil (AV), eO_3 × V soil (EV).

3.2.3 Collection of samples and measurements

Samples of mature and senescing leaves were taken from 3 tree species seedlings in each treatment (4 trees per replication) in two-stages: for mature leaves, samples were taken in mid-September during the peak nutritional activities of seedlings and for senescing leaves samples were taken at the mid-November (Shi et al. 2016b). The top crown or second leaf counted from the shoot-top was collected as mature leaves. The collected leaf samples were then measured the leaf area for calculation of the leaf mass per unit area (LMA). After rinsing their surface with distilled water, both collected mature and senescing leaves were dried at 70 ℃ for at least 5 days. Afterwards, all the leaves were mill ground into fine powder to be used for further analysis.

In order to analyze the projected elements (N, P, K, Ca, Mg, Mn and Fe, Al), the

powdered leaf samples were digested by nitric acid, hydrogen peroxide method and the concentration was measured with an Inductively Coupled Plasma Mass Spectrometry (ICP-MS; IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., Massachusetts, USA.) analysis. N concentration was determined by the combustion method using a NC analyzer (NC-900, Sumica, Osaka, Japan).

Moreover, for the purpose of evaluating the retranslocation rate and determining the proportion of the foliar nutrients resorbed before leaf abscission, the element resorption efficiency (ERE) from senescing leaves was calculated as follows:

$$ERE = (C_{mature} - C_{senescing})/C_{mature} ,$$

where ERE is the element (N, P, K, Ca, Mg, Mn and Fe, Al) resorption efficiency, for specific nutrient X, we will utilize XRE to stand for the resorption efficiency of nutrient X. C_{mature} is the nutrient concentration of mature leaves and $C_{senescing}$ is the nutrient concentration of senescing leaves (e.g. Helmisaari, 1992; Huang et al., 2007; Salehi et al., 2013).

3.2.4 Statistical analysis

The value of each species and for each treatment was averaged for at least 3-4 replications in order to provide the sample estimate for the actual replicate. Each ERE was subjected to linear mix model as follows;

$$ERE = GAS + Soil + GAS \times Soil + Plot,$$

where GAS is the treatment of ozone (ambient and eO_3), Soil is the treatment of soil (B, S and V), GAS × Soil is the interaction of ozone and soil, and Plot is the location of each circular plot. We used GAS, Soil and GAS × Soil as fixed factors, and Plot as a random factor. The effects of each fixed factors were analyzed by likelihood ratio test, and we applied Tukey's post-hoc test when the likelihood ratio test was significant ($P < 0.05$). These analyses were performed separately on each three species. These statistical analyses were performed using R (version 3.1.0, www.r-project.org) with the "lmer" function of lme4 package (Version 1.1-7, http://CRAN.R-project.org/p-ackage=lme4).

For mineral elements of Ca, K, Mg, Mn and Fe, Al, two-way analysis of variance (ANOVA) was used to test the effects of O_3 exposure and soil types as well as their interaction. Results were considered significant at $p < 0.05$. Duncan Multiple Range

tests were utilized to compare the ERE differences among the 6 treatments.

Significance of differences in O_3 treatments between mature and senescing leaves for a single species was tested by the Student's *t*-test. Correlation analysis between N contents in mature or senescing leaves and LMA were quantified and tested with Pearson's correlation coefficients. All the statistical works were performed by SPSS software (21.0, SPSS, Chicago, USA).

3.3 Results

Figure 3.2 and Table 3.1 indicate the results of ERE in birch species *Bp* under the factorial combination treatments of O_3 exposure and soil types. In different treatments, ERE of N, P and K were positive while that of Ca, Mg, Mn, Fe and Al were negative. NRE was found to be significant in interaction of eO_3 and soils (Figure 3.2a), as well as PRE was significantly influenced by eO_3 (Figure 3.2b). The median values of NRE were increased with poor sites (i.e. V and S: Figure 3.2 and Table 3.1) for the studied elements. While for NRE, values at eO_3 were decreased with the poor soil fertility (Figure 3.2a). The median values of NRE at ES and EV as well as the values of PRE at AS and AV were nearly identical (Figure 3.2b). Moreover, NRE and PRE values were clearly higher at eO_3. The only exception in Figure 3.2 occurred on NRE at EV treatment.

Table 3.1 shows that MgRE and KRE didn't show any significant differences. Whereas, according to ANOVA analysis, CaRE and AlRE were significantly affected by both O_3 and soil independently. Interaction influences were observed in ERE of Mn and Al. FeRE was observed to be highly affected by soil. At the same time, several differences among treatments were observed in the Table 3.1. MnRE under V soil as well as AlRE under BF were significantly influenced by eO_3. Furthermore, AlRE as well as CaRE and FeRE under both S and V were significantly different with B at ambient alone or at both gas treatments.

For beech species *Fc*, no significant influence on N and P was exerted by any individual and combined treatment (Figure 3.3). However, clearly differences on the metal elements were observed for effects of gas and soil (Table 3.2). In Figure 3.3a, the median value of NRE was decreased under B and V soil at eO_3 while it was conversely increased by eO_3 under S soil. In Figure 3.3b, the range of foliar PRE was enlarged by eO_3, especially when the seedlings planted under B and V. Meanwhile, the median

values of PRE under B and S were both decreased by eO_3 but it maintained the same in V.

On the other hand, based on Table 3.2, significantly differences were observed to gas and soil except Mg. On the other hand, merely the ERE (K, Mn and Al) had clearly responded to the interactive effects of O_3 exposure and soil types. Among the treatments, NRE under V soil was also affected by eO_3 except Mg. Similar effect as that under S soil was found except on Mn and Al. However, clearly influences of eO_3 on MgRE and MnRE were observed under B soil. Moreover, we also found that ERE of K, Ca, Mn and Al at eO_3 got significantly higher values under B than that under S or V soils.

The likelihood ratio test indicates that both NRE and PRE of *Qm* were significantly influenced by soil condition (Figure 3.4). Further analysis of soil pairwise test via Tukey's post-hoc test suggests that the significant effects were only found between V and S soils on NRE while no effect was found on PRE. Additionally, ERE (N and P) under S was both decreased at ambient. The NRE values under S as well as the PRE under V at eO_3 were exceeded the range to others. For NRE, the median values were enhanced by eO_3 in the 3 soils. For PRE, it was sharply declined by O_3 under B and even reduced more when planted under S soil. In V soil, PRE was also sharply increased at eO_3.

Similar to birch *Bp*, there was no significant effect observed on MgRE and KRE (Table 3.3). In contrary, based on the ANOVA, interaction effects of gas and soil were observed on rest of the mineral elements. FeRE was also significantly influenced by gas and soil independently. MnRE and AlRE were significantly affected by gas and soil respectively. As for treatment differences, MnRE and AlRE under both S soil, and V soil were significantly lower than that under B at ambient O_3. On the other hand, FeRE and AlRE under S and V soils were significantly higher than that under B soil at eO_3. Parallel results, CaRE at ambient O_3 was significantly decreased under S soil but was significantly increased under V soil. In contrast, ERE of Ca at eO_3 got completely opposite performance on that under S and V soils.

According to the comparisons results in Figure 3.5, foliar N in mature leaves typically stayed higher than N in senescing leaves for each species under the same O_3 condition. Especially for birch *Bp* at eO_3, nearly 60 % foliar N was retranslocated (Figure 3.5a), suggesting that *Bp* is likely relatively sensitive to eO_3 compared to the other two species. Besides, foliar N contents were affected by O_3 in senescing leaves for beech *Fc* (Figure 3.5b). As for oak *Qm*, O_3 significantly led to about 70 % lower

foliar N contents in mature leaves (Figure 3.5c), suggesting that eO_3 promoted oak to adopt the strategy to recycle N beforehand in leaf vigorous stage during the growing season.

Table 3.4 presented the Pearson's correlation efficient (r) between foliar N and corresponding LMA of the three species. With regard of foliar N contents at ambient air, there were significant negative correlations between foliar N and LMA of *Bp*, *Qm* in mature leaves as well as foliar N and LMA of *Bp* in senescing leaves. With regard of foliar N at eO_3, the only significant positive correlation was observed between foliar N and LMA in mature leaves of *Fc*. With regard of the main effects on gas treatments, the highly significant negative correlations were observed at ambient between LMA and foliar N, for both in mature and senescing leaves

3.4 Discussion

Generally speaking, foliar elements are transported between xylem and phloem and their contents are determined by the balance between influx via xylem and phloem, and efflux via phloem (e.g. Larcher, 2003). As for water soluble elements, N, P and K are known to be mobile in the phloem and readily retranslocated from older to younger leaves (Marschner, 2012). Consequently, with the process of leaf senescence, N, P and K are unstable nutrients and they are usually transported to other parts of plant which results in the decrease of concentrations in senescing leaves (Helmisaari, 1992).

On the other hand, the non-mobile mineral elements, Ca, Mn and Fe, Al are transported from roots to leaves through xylem but are poorly mobile in phloem (e.g. Schulze et al., 2005; Marschner 2012; Shi et al., 2016b). Therefore, deficiencies of these mineral element contents commonly occur in younger leaves.

Mg is the central atom of chlorophyll pigment, and is essential for ribosome aggregation. It is also often regarded as one of the indicators for leaf greenness and many physiological functions (e.g. Killingbeck, 2004). Because of its mobility and availability in plants (Gjengedal, 1996), Mg is usually considered to decrease with leaf senescence (Helmisaari, 1992). Therefore, positive values for N, P, K and Mg, along with negative values of ERE for Ca, Mn, Fe and Al may be the expected results.

3.4.1 Birch

Notwithstanding the above considerations, MgRE in birch species *Bp* was unexpectedly negative under every treatment according to Table 3.1, this result is in agreement with the study of Carr et al. (2003): foliar Mg concentration which increased with the age of leaves. It is possible that the dynamics of Mg in mature leaves were suppressed by the increased foliar K as there was a negative correlation between Mg and K (e.g. Kayama and Koike, 2015).

Previous research suggested that nutrient retranslocation in late successional species may be increased at nutrient poor sites (e.g. Killingbeck, 2004), and we also found similar results in Figure 3.2 and Table 3.1 at ambient O_3 condition for 5 elements (Ca, N, P and Fe, Al). In addition to the opposite result for NRE in Figure 3.2a at eO_3, it suggested that ERE may be independent of nutrients gradients in the soil (Nelson et al., 1995). Alternatively, the interaction of O_3 and soil would influence and completely change the trend of ERE with soil fertility as NRE was found to be significant in interaction of eO_3 and soils in Figure 3.2a. However, it was not always the case since the increased CaRE and FeRE at eO_3 in V and S soils (Table 3.1) were still consistent with the results increasing with poor soils. In any case, ERE was likely to be limited by the availability of nutrient gradients as ERE of N, Ca and Fe at eO_3 and ERE of Ca, P and Fe, Al at the ambient site all remained almost the same in V and S soils (Figure 3.2 and Table 3.1).

We expected that O_3 would be detrimental to plant photosynthetic performance (e.g. Uddling et al., 2005; Koike et al., 2013) and nutrient dynamics and thus may result in lower ERE at eO_3 due to O_3-induced reduction of translocation dynamics from aged to younger leaves. For example, the MnRE value at V soil (Table 3.1) was significantly decreased by eO_3. This depression may be attributed to the reduction of Mn in mature leaves caused by the declined of photosynthetic function and low Gs which was induced by eO_3 (Yamaguchi et al., 2011; Shi et al., 2016b). However, ERE of N and P (Figure 3.2a and b) as well as the value of Al (Table 3.1) were markedly increased by eO_3. This result can be explained by the study of Hoshika et al. (2013b): mature leaves may be less susceptible to O_3 stress than senescing leaves in birch since mature leaves should have a higher capacity of avoidance for O_3-induced low Gs (Hoshika et al., 2012b; 2013b). Therefore, the decreased concentration in senescing leaves at eO_3 may be a result of increased ERE.

What is more, Mn was significantly accumulated more in senescing leaves when planted in V soil at eO_3 compared to other soils at eO_3 (Table 3.1). As previous literature has pointed out, Mn appears to have low mobility when it is limiting to the plant (Kitao et al., 1998), the deficiency of Mn in V soil may explain the inducement of higher accumulation.

Furthermore, I found a highly significant difference between ambient air and eO_3 in senescing leaves in *Bp* (Figure 3.5a). Birch species *Bp*, as one of the key species in boreal forests, has a high photosynthetic and responds rapidly to the environment (Koike, 1995). Consequently, O_3-induced early defoliation and shorten growing season make birch has to recycle the N more quickly before senescence as a self-protection strategy, especially when eO_3 accelerate the process of senescence. Alternatively, O_3-induced early leaf defoliation may easily take place in senescing leaves since they are more susceptible to eO_3 (Hoshika et al., 2013b).

3.4.2 Beech

For beech species *Fc*, NRE and PRE were decreased at eO_3 except the increase on NRE from AS and ES. Since no significant influence on N and P was exerted by any individual and combined treatment (Figure 3.3), the decrease of ERE was possible because concentration of N and P is rather susceptible to leaching at eO_3 during senescence (Helmisaari and Mälkönen 1989). In contrast, NRE at ES got the highest value compared to other treatments examined (Figure 3.3a), suggesting that more N was needed in mature leaves of beech for new plant growth and leaf growth was in critical need of N derived from retranslocation. This was especially so, when planted under S soil as it is poor in fertility.

Yamaguchi et al. (2007) concluded that the sensitivity of growth and photosynthetic parameters of beech to O_3 became high with increasing amounts of N in soil. Combined with the research conducted by Hoshika et al. (2013b) that late leaves in *Bp* were more susceptible to O_3 stress, therefore ERE may result in higher values at eO_3 in high fertility soil as a consequence of lower retention in senescing leaves. Therefore, ERE (K, Ca, Mn and Al) at eO_3 was markedly higher in B soil than in other soils in Table 3.2.

Moreover, mineral elements that are shown in Table 3.2 such as Fe and Al that ERE was significantly enhanced by eO_3 under both V and S soils and under V soil alone, respectively. Fe and Al were indicative of reduced accumulation of the elements in

senescing leaves at the specific treatment. It has recently been shown that N deficiency-induced leaf senescence (V and S soils in this study) enhances Fe mobilization in old leaves and therefore favored the decline of foliar Fe accumulation with senescence (Barunawati et al., 2013; Shi et al., 2012). As for Al, from the discussion in *Bp*, we believe that the explanation of enhancement on AlRE by eO_3 for birch species could also work on beech species *Fc*. Alternatively, the O_3-induced reduction in aged leaves for beech species (Hoshika et al., 2013b, Yamaguchi et al., 2007) may not be adequately compensated by enhancement of foliar Al during leaf senescence at acidic soil (Gjengedal, 1996). As a result, increase of AlRE at EV was observed in Table 3.2.

On the other hand, from Table 3.2, MgRE in B and S soils, ERE of K and Ca in S and V soils, MnRE in B and V soils were decreased by eO_3. Other than the O_3-induced reduction of translocation dynamics as we explained in birch species, those nutrients that were lost in the mature leaves are likely to be due to leaching (Helmisaari, 1992; Gjengedal, 1996), as leaching removes various compounds during senescence (e.g. Vergutz et al., 2012). Although previous research demonstrates that foliar leaching of Ca, K and Mg can be observed on acidified soil and is believed to occur via cation exchange (e.g. Gjengedal, 1996). These results may suggest that foliar metal leaching is independent with the soil acidification at least for beech species. In addition to leaching, the Al toxicity may also be the cause of inhibition of some mineral nutrients resorption during senescence (e.g. Schulze et al., 2005).

Beech species usually have a longer growing season compared to the other two species. As shown in Figure 3.5b, the effect of O_3 to senescing leaves in *Fc* (* p=0.018*)* was not as much as that in *Bp* shown in Figure 3.5a (** p = 0.006). This is probably because beech is much easier than birch to obtain N from the N cycling at eO_3 since its litter decomposition may become faster with the O_3-induced accelerant process of defoliation (Kavvadias et al., 2001). Beech species, hence, is unnecessarily for mature leaves to keep such quantities of N during the process of senescence. As a result, considerable N is remained in senescing leaves of beech *Fc*. Besides, either birch or beech species adopted the N recycling in the late stage of a growing season.

3.4.3 Oak

According to results of oak in Figure 3.4, although we found eO_3 gave rise to the increase of NRE and PRE in both S and V soils, there were no significant differences between ERE and O_3 in N and P. However, NRE and PRE in V soil were clearly higher

than that of the results from S soil. In the study of Helmisaari (1992), it is emphasized that foliar ERE did not vary in response to soil fertilization, we thus hold the view that higher NRE and PRE in V soil may refer to the soil depletion and acidification.

Consequently, depletion-driven losses of N and P in soil were associated with a decreased retention of N and P in senescent leaves and strongly increased EREs (Hayes et al., 2014). Moreover, higher ERE of N and P in V soil (Figure 3.4) may result from the high depletion-driven loss in V soil (acidic) as output-N had a high negative correlation with soil pH (Dise and Wright, 1995).

Similarly, higher CaRE in V soil than that of S soil were also markedly observed at ambient in Table 3.3, suggesting that higher depletion-driven loss of soil in V soil may also be related to Ca. In contrary, significantly lower CaRE in V than in S soil was observed with eO_3. This opposite result may be caused by the interaction influence of O_3 and soil. Namely, the enhanced ERE caused by soil was not adequately compensated by O_3-induced detrimental effects on ERE. Such combined influences may be broadly applicable on other elements for oak species.

For oak species *Qm*, AlRE in high fertility soil (B soil) was significantly the highest at ambient but clearly the lowest at eO_3 (Table 3.3). Retranslocated nutrients are generally essential for the production of new tissues and organs throughout all stages of development for plants. This suggests that soil and environmental factors that facilitate plant growth development also promote nutrient retranslocation (Nambiar and Fife, 1991) and vice versa. As a result, ERE is expected to be increased by high soil fertility but decreased by eO_3. Therefore, the markedly highest MnRE and AlRE in AB (Table 3.3) may be the result of being grown on a relatively high fertility soil condition as in B soil. Whereas the significant low FeRE and AlRE in EB (Table 3.3) may be due to the interaction effect of O_3 and soil. The declined ERE caused by eO_3, nevertheless, may be aggravated by relative high N in soil and finally results in the low values (Yamaguchi et al., 2007).

Moreover, in Figure 3.5c, although oak was labeled as the least sensitive to O_3 among the three species in Yamaguchi et al. (2011), the present result in our study shows that mature leaves of oak were not less responsive to eO_3. As we indicated in our previous study that retranslocation of foliar N of oak was also affected by soil conditions (Shi et al., 2016b). These was clarified that leaf N was concern with the enhancement of roots, the sensitivity of nutrient dynamics in one species to O_3 exposure may be controlled by several parameters, environmentally and bio-physiologically.

3.4.4 Comparisons among birch, beech and oak species

Consider overall impacts of the eO_3 exposure on those foliar mineral nutrients (Ca, K, Mg, Mn and Fe, Al in this study), *Fc* was observed to be most affected (Table 3.2), followed by *Qm* (Table 3.3) and *Bp* (Table 3.1), sequentially. This was consistent with the previous study that the highest and the lowest sensitivity to eO_3 among those 3 species should be beech and oak, respectively (Yamaguchi et al., 2011). These results may be due to a difference in growth patterns (Hoshika et al., 2013a). Roughly classifying leaf development (Kikuzawa and Lechowicz 2011), birch has an indeterminate growth pattern (Koike 1995, Hoshika et al., 2013b), while beech and oak typically shows determinate (e.g. Kitao et al., 2009) and semi-determinate growth patterns (Holbrook and Zwieniecki, 2011), respectively. Since the indeterminate growth pattern may have ability to produce new leaves to compensate the eO_3 damage (e.g. Hoshika et al., 2013a; 2013b), this may be the reason why *Bp* which has an indeterminate growth pattern had least effects on mineral nutrients by eO_3 among the 3 species (Table 3.1).

With a similar explanation, *Fc* with determinate shoot growth (Koike et al., 1998; Kitao et al., 2009) was most crucially affected by soil conditions on those mineral nutrients (Table 3.2). Although *Qm* is usually considered as a semi-determinate growth species, these classifications of shoots growth patterns are not clear-cut and may still involve considerable overlaps. Therefore, the soil condition impact on *Qm* is not necessarily in between *Fc* (Table 3.2) and *Bp* (Table 3.1) but was the most tolerant among the 3 species (Table 3.3). Although beech is a typical late-successional tree species (Koike, 1988; 2004; Kitao et al., 2009), it often retranslocates more foliar nutrients before leaf shedding in infertile soil condition, compared to early or mid-successional species such as birch and oak species (Killingbeck, 2004; Eguchi et al., 2008), which likely lead to the obtained results of the essential impacts of soil conditions on the beech species as well.

Moreover, LMA is considered as the essential indicator of leaf thickness for leaf trait analysis in the leaf morphology of those species (Hayashi et al., 2005). Generally, LMA is decreased with the natural process of leaf senescence due to the reduction of dry mass and the decline of the nutrient in senescing leaves, but O_3-induced modification in leaf traits may reduce the leaf size and thus enhance the LMA values (Paoletti, 2007). Based on the Table 3.4, N_{mature} at ambient in *Bp* and *Qm* was higher

compared to that at eO_3 which was regulated by their relatively lower LMA values at ambient air. And those results are consistent with the Figure 3.5a and c. Similarly, LMA of senescing leaves in *Bp* at ambient was a lower value than that in either mature leaves or at eO_3. Based on the negative correlations in *Bp* in Table 3.4, I expected a higher $N_{senescing}$ at ambient compared to eO_3 which also in agreement with the result in Figure 3.5a.

Despite the fact that LMA in mature leaves was expected a higher value compared to senescing leaves as well as a lower value compared to eO_3 at the same time. Higher N_{mature} at ambient in Figure 1C indicated that eO_3 did not affect much on the correlations between LMA and foliar N in this study. Alternatively, eO_3 did not simply increase the LMA, the changes on LMA may also be controlled by other factors that may need to be further tested. As a consequence, LMA can be utilized as an index parameter in speculation of the foliar N dynamics at ambient.

3.5 Summary

To sum up the results, I indicated that eO_3 as well as soil had a great influence on ERE and revealed that there are considerable differences in nutrient acquisition and loss in leaves among Japanese representative tree saplings (Koike, 2004; Hayes et al., 2014). O_3 and soil may have different effects on the ERE of various nutrients with different species with different growth patterns. Even though it is hard to find a specific pattern between the species and the impacts of combined gas and soil treatments, retranslocation was more efficient concerning mineral nutrients for beech *Fc* leaves.

Therefore, beech: *Fc* being the representative of determinate shoot growth species is likely to have a relatively high sensitivity of foliar ERE to either eO_3 or soils, which may be a good index species when investigating the mineral nutrient retranslocation system in field surveys. It is necessary to take into account the indirect effects of physiological environments that determine the dominant Japanese deciduous forest species. Studying about retranslocation of foliar nutrients will be of help to develop appropriate strategies of nutrient management in plantations (Salehi et al., 2013) and finally achieve the goal of forest conservation.

Moreover, effects of foliar N dynamics in response to exposure varied upon different species. As O_3 accelerated the process of senescence, foliar N dynamics of birch species is also accelerated by eO_3 due to the sensitivity to O_3, especially for the

senescing leaves. Both *Bp* and *Fc* adopt the recycling of N at late stage of the growing season, specifically for N recycling in *Bp* is more sensitive than beech when exposure to eO_3. Moreover, although the correlations between LMA and the effect of O_3 on foliar N dynamics are under unclear, LMA is capable to be utilized as one of the indicators in speculation of the foliar N dynamics at ambient condition.

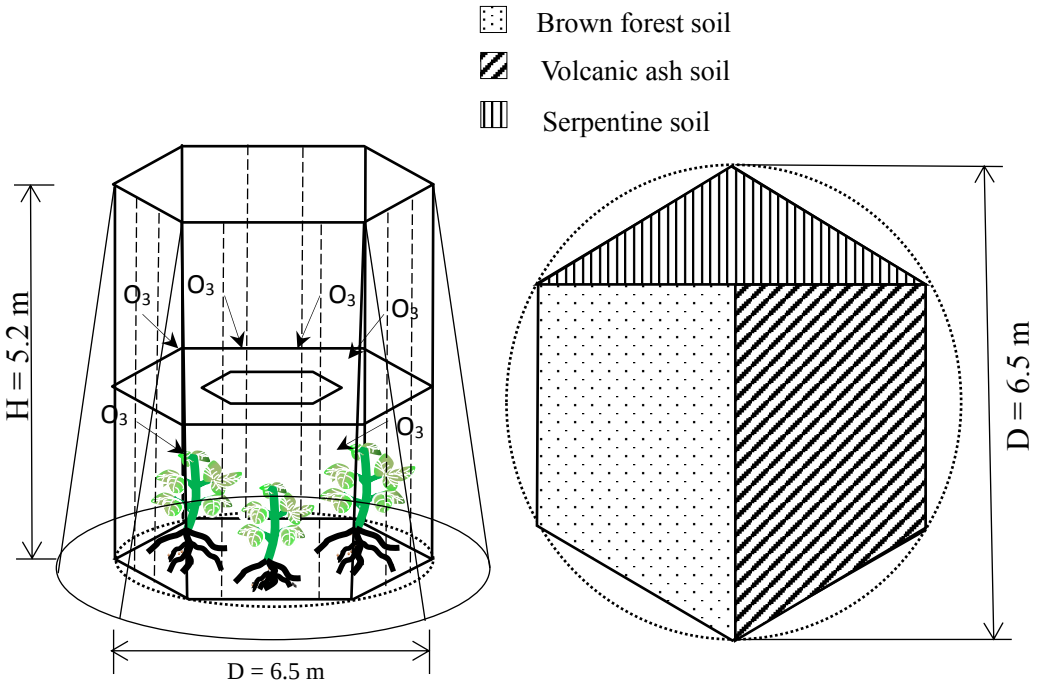


Figure 3.1 Design of a free-air enrichment experimental circular plot and a bird-view of soil distribution for each plot at Hokkaido University experimental nursery.

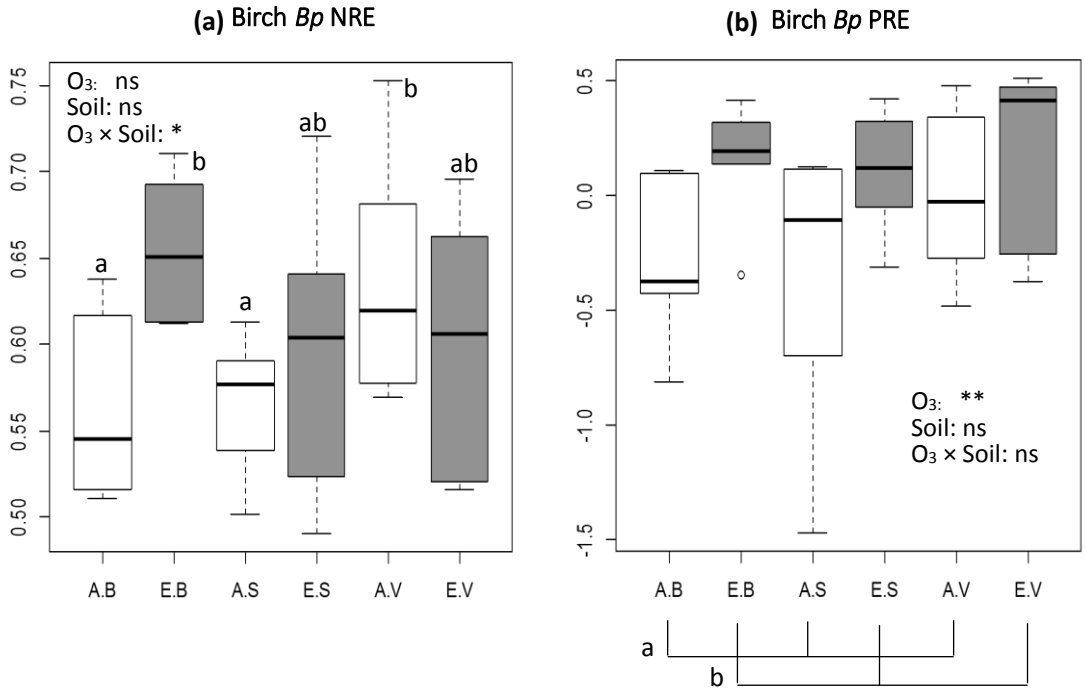


Figure 3.2 Boxplot of element resorption efficiency (ERE) values of a). Nitrogen: NRE and b). Phosphorus: PRE for birch *Bp*, comprising 6 treatments with 3 replications. Three types of soil (B=brown forest soil, S=serpentine soil, V=volcanic ash soil) and 2 gas treatments (A=ambient O_3, E=elevated O_3). The effects of each fixed factors were analyzed by likelihood ratio test, and Tukey's post-hoc test was used when the likelihood ratio test was significant. Each variable marked with different letter differ statistically significantly at a level of significance α=0.05. (Box: interquartile range; whiskers: lower and upper quartiles; median horizontal line of boxes: median; circles: extremes. ns: treatment effect not significant. * P<0.05, ** P<0.01).

Table 3.1 Mean element resorption efficiency (ERE) (±SD) of K, Mg, Ca, Mn and Fe, Al under 3 types of soil (B=brown forest soil, S=serpentine soil, V=volcanic ash soil) and 2 gas treatments (A=ambient O_3, E=elevated O_3) in birch species *Bp*. Two-way analysis of variance (ANOVA) followed by Duncan Multiple Range tests were used to compare the ERE differences among 6 treatments. Means within each variable marked with different letter differ statistically significantly at a level of significance α=0.05.

Birch *Bp*	**AB**	**EB**	**AS**	**ES**	**AV**	**EV**	**Gas**	**Soil**	**Gas × Soil**
MgRE	-0.472a	-0.010a	-0.177a	-0.510a	-0.227a	-0.472a	ns	ns	ns
	(0.086)	(0.314)	(0.249)	(0.173)	(0.098)	(0.066)			
KRE	0.261a	0.257a	0.239a	0.242a	0.334a	0.157a	ns	ns	ns
	(0.009)	(0.031)	(0.046)	(0.015)	(0.026)	(0.084)			
CaRE	-0.62 cd	-0.744 d	-0.242 a	-0.36 abc	-0.26 ab	-0.541 bc	*	**	ns
	(0.160)	(0.078)	(0.075)	(0.021)	(0.077)	(0.064)			
MnRE	-0.650 a	-0.605 a	-0.611 a	-0.508 a	-0.328 a	-1.050 b	ns	ns	*
	(0.269)	(0.113)	(0.043)	(0.057)	(0.049)	(0.065)			
FeRE	-3.180 b	-3.224 b	-1.262 a	-1.579 a	-1.513 a	-1.854 a	ns	***	ns
	(0.504)	(0.517)	(0.039)	(0.073)	(0.275)	(0.224)			
AlRE	-4.575 b	-2.503 a	-2.250 a	-2.649 a	-2.597 a	-2.186 a	*	**	**
	(0.371)	(0.268)	(0.312)	(0.139)	(0.363)	(0.058)			

ns: treatment effect not significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (ANOVA)

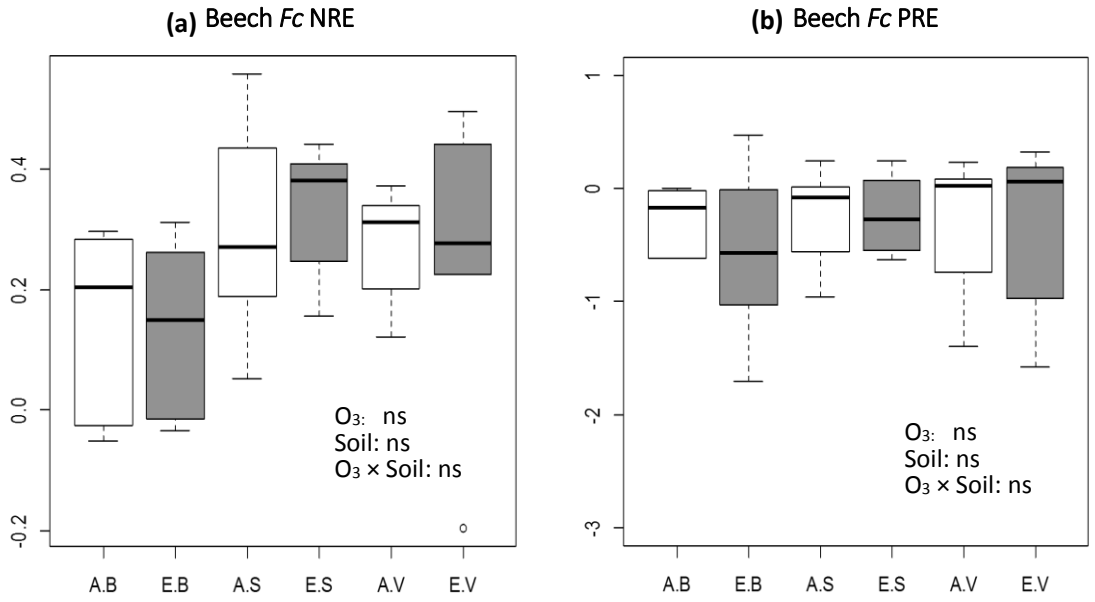


Figure 3.3 Boxplot of element resorption efficiency (ERE) values of a). Nitrogen: NRE and b). Phosphorus: PRE for beech *Fc*, comprising 6 treatments with 3 replications. The effects of each fixed factors were analyzed by likelihood ratio test, and Tukey's post-hoc test was used when the likelihood ratio test was significant. (Box: interquartile range; whiskers: lower and upper quartiles; median horizontal line of boxes: median; circles: extremes. ns: treatment effect not significant).

Table 3.2 Mean element resorption efficiency (ERE) (±SD) of K, Mg, Ca, Mn and Fe, Al under 3 types of soil (B=brown forest soil, S=serpentine soil, V=volcanic ash soil) and 2 gas treatments (A=ambient O_3, E=elevated O_3) in beech species *Fc*. Two-way analysis of variance (ANOVA) followed by Duncan Multiple Range tests were used to compare the ERE differences among 6 treatments. Means within each variable marked with different letter differ statistically significantly at a level of significance α=0.05.

Beech *Fc*	**AB**	**EB**	**AS**	**ES**	**AV**	**EV**	**Gas**	**Soil**	**Gas × Soil**
MgRE	-0.02 ab	-0.849 c	0.436 a	-0.45 bc	0.202 ab	-0.14 abc	**	ns	ns
	(0.174)	(0.863)	(0.006)	(0.207)	(0.055)	(0.049)			
KRE	0.216 a	0.238 a	0.310 a	-0.063 b	0.144 a	-0.020 b	**	*	**
	(0.053)	(0.083)	(0.069)	(0.070)	(0.017)	(0.012)			
CaRE	0.043 a	-0.09 ab	-0.10 ab	-0.345 c	0.068 a	-0.150 b	**	**	ns
	(0.028)	(0.041)	(0.073)	(0.085)	(0.021)	(0.049)			
MnRE	0.487 a	-0.039 b	-0.630 c	-0.352 c	0.135 b	-0.455 c	**	***	**
	(0.035)	(0.043)	(0.066)	(0.097)	(0.072)	(0.171)			
FeRE	-0.148 a	0.095 a	-1.082 b	0.005 a	-1.420 b	-0.416 a	***	**	ns
	(0.017)	(0.125)	(0.343)	(0.058)	(0.246)	(0.105)			
AlRE	-0.28 ab	0.013 a	-0.561 b	-0.353 b	-1.265 c	-0.467 b	***	***	*
	(0.058)	(0.030)	(0.179)	(0.053)	(0.239)	(0.088)			

ns: treatment effect not significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (ANOVA)

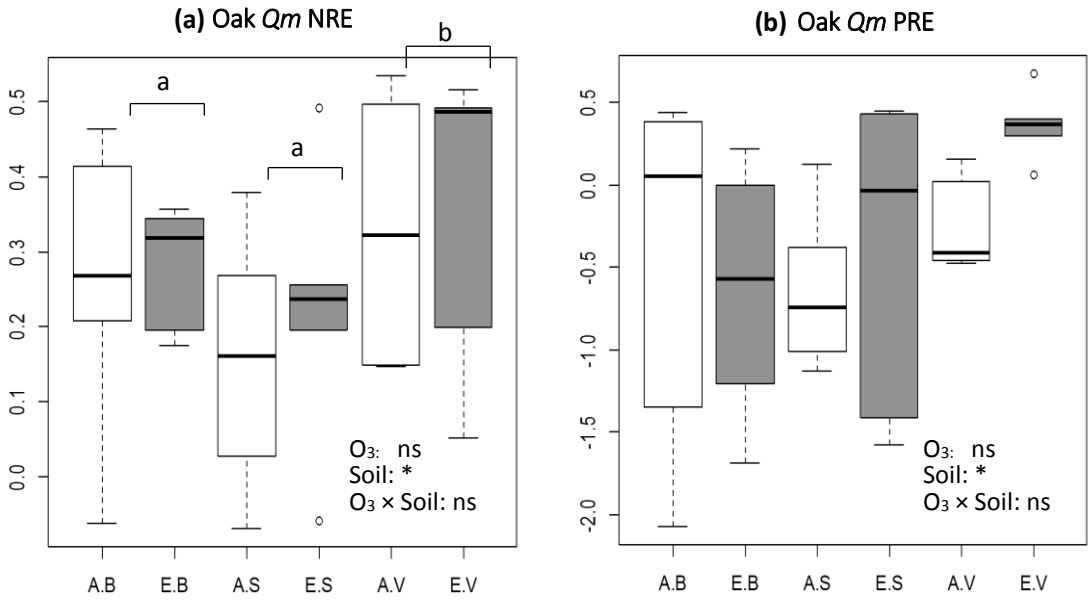


Figure 3.4 Boxplot of element resorption efficiency (ERE) values of a). Nitrogen: NRE and b). Phosphorus: PRE for oak *Qm*, comprising 6 treatments with 3 replications. The effects of each fixed factors were analyzed by likelihood ratio test, and Tukey's post-hoc test was used when the likelihood ratio test was significant. Each variable marked with different letter differ statistically significantly at a level of significance α=0.05. (Box: interquartile range; whiskers: lower and upper quartiles; median horizontal line of boxes: median; circles: extremes. ns: treatment effect not significant, * P<0.05).

Table 3.3 Mean element resorption efficiency (ERE) (±SD) of K, Mg, Ca, Mn and Fe, Al under 3 types of soil (B=brown forest soil, S=serpentine soil, V=volcanic ash soil) and 2 gas treatments (A=ambient O_3, E=elevated O_3) in oak species *Qm*. Two-way analysis of variance (ANOVA) followed by Duncan Multiple Range tests were used to compare the ERE differences among 6 treatments. Means within each variable marked with different letter differ statistically significantly at a level of significance α=0.05.

Oak *Qm*	AB	EB	AS	ES	AV	EV	Gas	Soil	Gas × Soil
MgRE	-0.166a	0.269a	-0.317a	0.075a	0.172a	-0.167a	ns	ns	ns
	(0.046)	(0.222)	(0.252)	(0.058)	(0.122)	(0.179)			
KRE	-0.189a	-0.007a	0.068a	0.053a	0.073a	-0.308a	ns	ns	ns
	(0.022)	(0.062)	(0.051)	(0.183)	(0.062)	(0.230)			
CaRE	-0.055 bc	-0.109 bc	-0.280 d	0.148 a	0.049 ab	-0.157 cd	ns	ns	***
	(0.064)	(0.026)	(0.020)	(0.160)	(0.033)	(0.021)			
MnRE	-0.753 b	-0.444 ab	-1.759 c	0.100 a	-2.121 c	0.255 a	***	ns	**
	(0.330)	(0.203)	(0.444)	(0.010)	(0.033)	(0.034)			
FeRE	-0.208 a	-1.730 b	-0.722 a	-0.312 a	-0.327 a	-0.085 a	**	*	**
	(0.059)	(0.490)	(0.181)	(0.343)	(0.040)	(0.048)			
AlRE	-0.241 a	-1.481 d	-0.593 b	-0.164 a	-0.984 c	-0.513 b	ns	***	***
	(0.092)	(0.085)	(0.037)	(0.090)	(0.069)	(0.115)			

ns: treatment effect not significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (ANOVA)

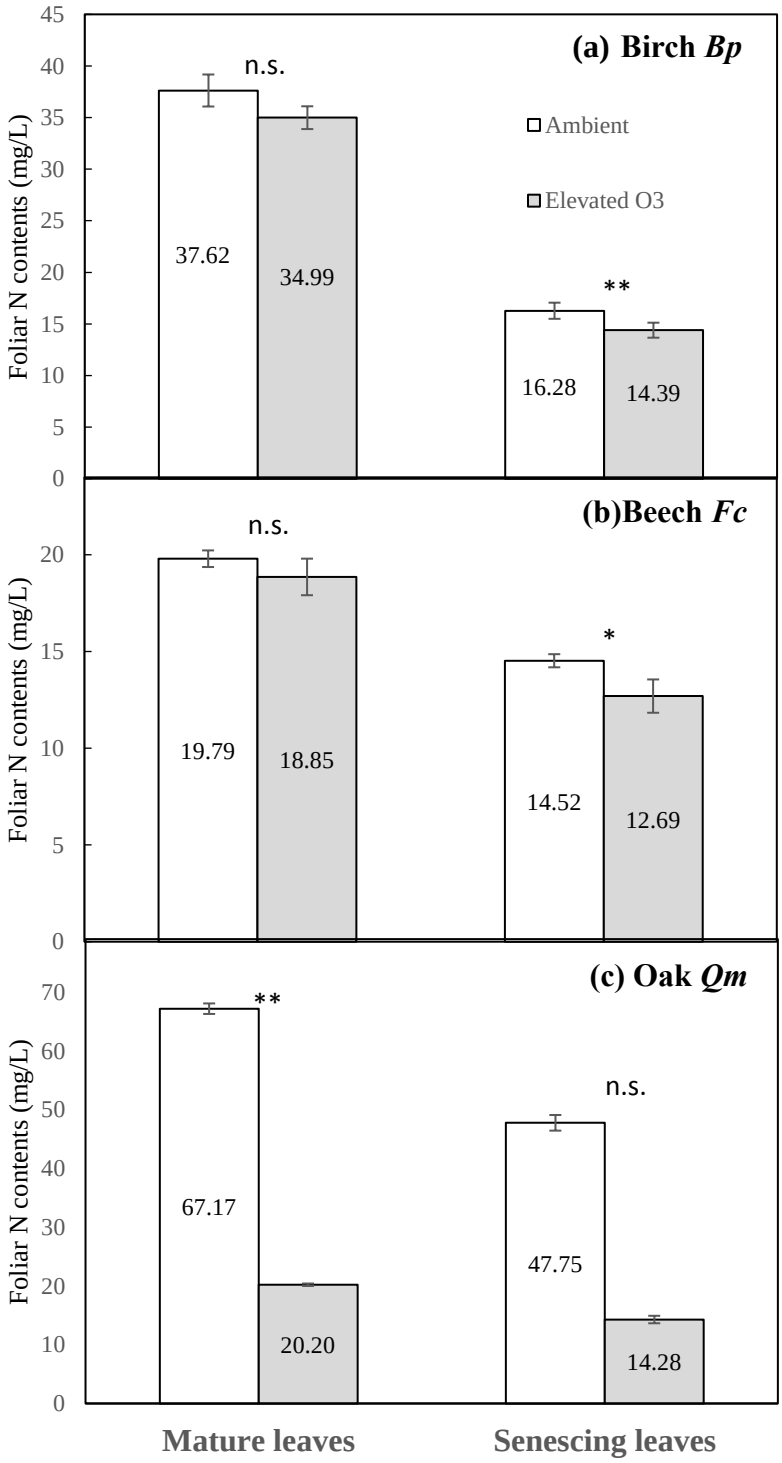


Figure 3.5 Nitrogen contents (mg/l) in live and senescing leaves of (a) birch *Bp* (b) beech *Fc* and (c) oak *Qm* seedlings grown under ambient and elevated O_3 as measured within the growing season. Data are represented as mean ± SD (n=4-6). *t*-test: n.s. no significant, **P*<0.05, ***P*<0.01.

Table 3.4 Pearson's correlation coefficients (r) between N concentrations in leaves (dependent variable) and the corresponding LMAs (independent variable) of three species (n=35; Pooled = the main effects of the gas treatments).

Foliar N	Ambient				Elevated O_3			
	Birch *Bp*	Beech *Fc*	Oak *Qm*	Pooled	Birch *Bp*	Beech *Fc*	Oak *Qm*	Pooled
N_{mature}	-0.29*	0.02	-0.14**	-0.46**	-0.07	0.009	0.23*	0.27
$N_{senescing}$	-10.51*	2.03	5.47	-0.56**	-0.34	-3.63	2.34	0.41

*P<0.05, **P<0.01

Chapter 4: Stoichiometry study on various foliar elements of three deciduous tree species exposed to elevated ozone under three different soils over two growing seasons

4.1 Introduction

4.1.1 Background

Tropospheric ozone, namely ground-level ozone (O_3) as the characteristic of strongly oxidizing, is recognized as one of the important widespread atmospheric pollutants, inducing morphological and chemical changes in leaves that adversely affect the vigor and health of forests (e.g. Bussotti et al., 2005; Yamaguchi et al., 2011; Feng et al., 2015). O_3 concentrations have been increased in East Asia and it is expected to be continuously enhanced for decades in foreseeable future because of dramatic increases in emissions of precursor gases (e.g. Akimoto 2003; Ohara et al., 2007; Paoletti, 2007; Watanabe et al., 2010). Recently, relatively high O_3 concentration has been detected frequently over wide areas of Japan (Wakamatsu et al., 1998; Takeda and Aihara, 2007; Watanabe et al., 2010; Koike et al., 2013).

As the major deciduous broad-leaved tree species native to northern Japan (Koike 1988), Japanese white birch (hereafter *Bp*) is a typical pioneer tree species with heterophyllous shoot development, continuously unfold leaves one by one (Koike, 1995; Wang et al., 2000; Hoshika et al., 2013b); Siebold's beech (*Fc*) is typical determinant shoot growth and high re-translocation ability of foliar nutrients (Shi et al. 2016a). This species can be of help in conservation of soil and maintain the biodiversity in forests (Watanabe et al., 2010); and both have diffuse porous wood. Mizunara oak (*Qm*) has ring porous wood and succeeding type shoot development (Kitao et al. 2015; Koike 1988; Shi et al. 2016a;b).

These 3 species play an essential role in cool temperate forests and provide benefits in social, economic and environment (Koike, 1988; Koike et al., 1998). However, forest decline and dieback of beech species as well as oak species wilt disease symptoms have been markedly observed in Japan that was believed to be likely due to the high O_3 concentrations (Kume et al., 2009; Watanabe et al., 2010; Izuta, 2017). Although there was no reduction effect on short-time O_3 exposure in *Bp*, long-time O_3 exposure may have a negative impact on the growth (Matsumura et al., 2005; Hoshika et al., 2012a).

There are big variations in sensitivity to O_3 between various species grown in Japan. Among the 3 species, *Fc* with determinate shoot growth pattern is more sensitive to O_3 and its nutrient retranslocation is more efficient (Kohno et al., 2005; Shi et al. 2016a). *Qm* is reported as a tolerant species with a highly capacity of O_3 stress according to screening experiments of Kohno et al. (2005) and Yamaguchi et al. (2011).

The effect of elevated O_3 concentration (hereafter, eO_3) on tree species is also greatly affected by soil conditions due to plants receive most elements from soils and fertilizers (e.g. Agathokleous et al., 2016). For example, beech species may become more sensitive to O_3 with increasing of nitrogen (N) load in soil (Yamaguchi et al., 2007); the adverse effects of O_3 on beech growth become less under chronic mild water-stressed condition (Watanabe et al., 2005). Therefore, I selected 3 different common soil properties that widely distributed in northern Japan as 3 different soil conditions in this study. Brown forest soil (B) which is mostly Cambisols, is a fertile soil type containing sufficient necessary nutrients for plant growth (Kayama et al., 2011; Kim et al., 2011); volcanic ash soil (V) which is deemed strongly acidic soil, contains high contents in aluminum (Al) and iron (Fe) but low in major and secondary nutrient elements (Kayama et al., 2011; Watanabe et al., 2010); serpentine (S) soil is lacking in several necessary nutrients with high ratio of magnesium (Mg)/calcium (Ca) and excessive heavy metals such as nickel (Ni) and chrome (Cr) that is widely known to be an infertile soil type in Japan (Brady et al., 2005; Kayama and Koike, 2015).

Leaf stoichiometry plays an essential role in the evaluation of plants composition, ecosystem function and nutrient limitation, etc. (Elser et al., 2000; Allen and Gillooly, 2009; Wu et al., 2012). Ecological stoichiometry deals with the balance of multiple elements in ecology interactions (Elser et al., 2000; Rong et al., 2015). Several studies have revealed that eO_3 may have great impacts on foliar nutrient contents and can accelerate leaf senescence (Ribas et al., 2005; Hoshika et al., 2012b), resulting in an elemental imbalance in plants (Kam et al., 2015; Shi et al., 2016a). On one hand, a frequently observed effect of eO_3 is a decline of N acquisition in plants, causes the nutrient deficiencies in leaves and thereby affects foliar physiological and biochemical functions (Karnosky et al., 2007; Yamaguchi et al., 2010). Moreover, there were observations on the reduction of N, phosphorus (P) contents (Cao et al., 2016) and alternation on their allocations dynamics under eO_3 treatment (Shi et al., 2016a). On the other hand, there were considerations suggested that the compounds for the biosynthesis of protein were increased by eO_3 and the metabolism of N was enhanced

through the increased activities of foliar enzymes (Yamaguchi et al., 2007).

Until recently, although many studies of foliar stoichiometry in response to various environmental conditions have been carried out, most of which focus on the elevated CO_2 (e.g. Huang et al., 2012) or soil fertilization (e.g. Dordas and Sioulas, 2009; Ostertag, 2010) as an independent treatment and discuss only primary nutrients such as N, P for one specific tree species (e.g. Elser et al., 2000; Zhang et al., 2010; Huang et al., 2012; Rong et al., 2015). A handful of studies were about the effect of eO_3 on foliar stoichiometry with secondary nutrients or trace elements: fewer studies were conducted with free-air enrichment system for field-grown trees; much-limited studies were concerned with combined effects of eO_3 and soil conditions on various foliar elements stoichiometry among different species.

4.1.2 Hypotheses

In a previous work, I studied the re-translocation of foliar nutrients compared with only mid-summer and leaf senescence period in deciduous broad-leaved trees with typical shoot development in responses to eO_3 and soils (Shi et al. 2016a). From previous results, it is expected that re-translocation of the studied elements under poor nutrition soil without toxic substances are likely more effective in *Fc* due to its determinate shoot growth pattern and the foliar stoichiometry in *Fc.* This is supposed to be more sensitive to eO_3. In this study, I further present a biochemical and physiological analysis of the stoichiometry of 10 foliar elements (Ca, K, Mg, Mn, N, Ni, P and Al, Cr, Fe) in 3 tree species for longer-time free-air O_3 exposure to examine the effects of eO_3 alone and together with different soil conditions.

4.1.3 Objectives

In order to fulfill the achievements, I investigated the stoichiometry issues from following aspects: 1) Effects of O_3 or/and soil on foliar element contents in different species, and I expected that elements in *Qm* leaves may have the least effects of O_3 since *Qm* is considered to be O_3 tolerant in many previous studies (e.g. Kohno et al., 2005; Paoletti et al., 2007; Yamaguchi et al., 2011); 2) Effects of O_3 or/and soil on foliar retranslocation rates, and I expected that mineral element retranslocation may be sensitive to the treatment in *Fc* (Shi et al., 2016a); 3) Comparisons among species on elemental retranslocation rate during two growing seasons, and I expected that long-

term exposure of eO_3 has more adversely influence on foliar elements dynamics, especially for *Fc*; 4) Foliar elements contents and their relationships, and I expected that various highly correlations among foliar elements and also different contents of elements in leaves for each species may change the elements allocation dynamics and thereby affect the physiological and biochemical function in foliage; 5) Profiling the elements in different species by principal component analysis and I expected to observe index elements that are able to assess the O_3 and soil effects on these species.

Based on the findings, I also discussed on the essential roles of physiological functions for each element in terms of specific traits of each tree species, and establish interactions between the environmental factors and the foliar elements among different species. The understanding of foliar elements stoichiometry in response of high O_3 in this study can be of help to understand elements cycling under elevated O_3 condition.

4.2 Materials and Methods

4.2.1 Plant materials and experimental site

On mid-July 2014, two-year old seedlings of Japanese white birch (*Bp*: *Betula platyphylla* var. *japonica*), Siebold's beech (*Fc*: *Fagus crenata)*, and Mizunara oak (*Qm*: *Quercus mongolica* var. *crispula*) which were obtained from Hokkaido Horti-Green Co. Ltd. near Sapporo were planted in field at nursery of Sapporo Experimental Forest, Hokkaido University, in northern Japan (43°04' N, 141°20' E, 15 m a.s.l.), and were exposed to an experimentally enhanced O_3 regime with a free-air O_3 enrichment system (Shi et al, 2016a) for two growing seasons.

All the plants were grown naturally without intentionally irrigation and fertilization. The enrichment system was temporarily closed during the snow period of Sapporo (from mid-December to early-May). Therefore, the O_3 exposure system was employed from early-August to mid-November in 2014 and from mid-June to early-December in 2015. The annual mean temperature and total precipitation of Sapporo in 2014 and 2015 were 8.2℃, 1129 mm and 10.3℃, 1331mm respectively.

The experimental sites consist of 6 circular plots, 3 for ambient O_3 and another 3 for elevated O_3. Each circular plot is 6.5 m in diameter with 5.2 m high dispersal pipes surrounded by (Kim et al., 2011). The distance between the ambient plots and elevated O_3 plots was about 20 m. The method of O_3 exposure applied in this study is based on

the system used at Kranzberg Forest in Germany (Nunn et al, 2002; Werner and Fabian, 2002). Watanabe et al. (2013) as well as Shi et al. (2017) described more details about the O_3 exposure as well as the plot design in the field station. The target O_3 concentration of the elevated O_3 plots was 70 ± 7 nmol mol^{-1} during daylight hours (about 7 h d^{-1}), while the ambient plots were subjected to daytime O_3 concentration of 30 ± 4 nmol mol^{-1}.

Each of the experimental system plot was divided into 3 parts and replaced with 3 different typical soils distributed in northern Japan. Brown forest (B) soil which was regarded as control in this study, is native to the nursery of Sapporo Experimental Forest; Volcanic ash (V) soil was taken from the Tomakomai Experimental Forest in southern Hokkaido (Kim et al, 2011); Serpentine (S) soil was taken from the eastern area of Teshio Experimental Forest in northern Hokkaido (Watanabe et al, 2012). The detailed for the design of each free-air enrichment experimental circular plot as well as the distribution of those 3 soils in each plot was shown in Figure 3.1 in the previous chapter.

4.2.2 Samplings and measurements

Leaf sampling taken from 3 seedlings per species at each treatment (6 treatments in total) with 3-4 replications was conducted every other month during the snow-free period when the free-air O_3 enrichment system was running. Leaf samples were collected totally 5 times within two growing seasons as the different development stages of leaf growth: mid-September and mid-November for first growing season in 2014; mid-July, early-September and mid-November for second growing season in 2015. Samples I collected in September and November are considered respectively as mature leaves with peak and senescing leaves with low nutritional activities in this study. Mature leaves were collected from the first or the second leaf counted from top of a shoot and senescing leaves were collected from the fallen leaves after those shoots gently being shaken. Every time I collected 1-2 sample leaves for the same seedling at each treatment. All the collected leaf samples were dried at 70 ℃ for 5-7 days after rinsing the surface with distilled water and were mill grounded into powder.

Soil cores at the depth of 0-10 cm (100 ml) were collected from each plot (3 cores per the treatment, totally 18 cores) in April 2015 and were air-dried for over one month to evaluate the chemical properties of the soil. After passed through a 2 mm mesh of sieve for a pretreatment, soil samples were prepared for next measurements. In order to determine the soil pH values, 10 g of soil sample was shaken with 25 ml de-ion water

for 1 h (Watanabe et al., 2012) and was afterwards measured with a pH meter (HM30V, TOA Electronics Ltd., Tokyo, Japan). After the measurements of pH values, soil samples were dried at 105 ℃ for at least 24 h. In order to extract the exchangeable cations from soil samples for further elemental concentration analysis, 2 g of soil sample was shaken with 40 ml of 1 M ammonium acetate solution for 1 h (Thomas, 1982; Watanabe et al., 2012) and was well filtered to get an extract soil solution.

For the purpose of measurements of the elemental concentrations of Mg, K, Ca, Mn, Fe, Ni, Al, Cr and foliar P, 50 mg leaf samples as well as the extract solutions of soil that obtained in the above step were digested by nitric acid and hydrogen peroxide. Analyses of the elemental concentrations of leaf and soil samples were then conducted with an Inductive Coupled Plasma Mass Spectrometer (ICP-MS, IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., MA, U.S.A.). For the purpose of measurements of available P in soils, P was extracted with the Bray-2 method as follow: 1g soil sample was shaken with 20 ml extracting solution containing 0.03M ammonium fluoride and 0.1 M hydrochloric acid (Bray and Kurtz, 1945). The extracted solution of P was then determined by molybdenium blue method with ascorbic acid (Murphy and Riley, 1962) using with a spectrophotometer (Gene Spec III, Hitachi, Tokyo, Japan) at the wavelength of 710 nm. The combustion method with a NC analyzer (NC-900, Sumica, Osaka, Japan) was utilized to measure the N concentration in both foliar and soil samples.

As described in chapter 3, retranslocation rate can be expressed by element resorption efficiency with the formula:

$$\mathrm{ERE} = \frac{\mathrm{Cmature} - \mathrm{Csenescing}}{\mathrm{Cmature}},$$

where ERE is the elements (Ca, K, Mg, Mn, N, Ni and Al, Cr, Fe) resorption efficiency (RE). For each specific element X, the element X resorption efficiency is expressed by "XRE" for being better distinguished in this test; C_{mature} is the element concentration of mature leaves which collected in September and $C_{senescing}$ is the element concentration of senescing leaves which collected in November (Huang et al., 2007; Shi et al., 2016a). From this equation, the percentage of elements resorbed prior to leaf abscission as well as the retranslocation rate can be estimated.

4.2.3 Soil chemical properties

According to the soil chemical properties shown in Table 4.1, there was no significant difference in soil pH values as well as the chemical elements contents between the two O_3 treatments. As a non-essential element, Cr content in brown forest (B) soil was significantly lower ($p<0.5$) among the 3 soils. As expected, Fe and Al contents in volcanic ash (V) soil were significantly higher ($p<0.5$) than that in brown forest and serpentine soils. Serpentine (S) soil with pH about 6.1, had a significantly higher ($p<0.001$) value than that of B soil and V soil. Besides, Mg, Ni and Cr contents in S soil were about 68.6, 0.33 and 0.11 mg $100g^{-1}$, respectively, significantly higher (Mg, Ni $p<0.001$, Cr $p<0.05$) than that in B soil and V soil. Contrary, S soil contents of K, Ca, Mn, Al and N were significantly lower (N $p<0.01$, others $p<0.05$) compared to the other two soil types. Mg/Ca ratio in S soil was about 0.64 which was the highest among 0.10 in B soil and 0.09 in V soil. Soil content of P did not have significantly differences among the soil types, which was consistent with the results of northern Japan by Kayama and Koike (2015).

4.2.4 Statistical analysis

The distribution of the data was tested for normality with the Kolmogorov-Smirnov test. Two-way ANOVA followed by post hoc Tukey's hsd test was conducted to compare the difference in soil chemical properties among the soil types under different O_3 enrichment. Since I collected the same seedlings each time over two growing seasons, a two-way (O_3 and Soil) repeated-measures analysis of variance (ANOVA) with collection time or growing seasons as within-subjects variable was utilized to compare the effects of O_3 (ambient O_3 and elevated O_3 concentration) and Soil (brown forest soil, volcanic ash soil and serpentine soil) on foliar contents or retranslocation rate in different time over two growing seasons for each species. Comparison results among the 6 treatments for both foliar contents and retranslocation rates were obtained by general liner model (GLM) analysis or post hoc Tukey's hsd test, after significant result of the GLM based on the standardized data. In order to assess the retranslocation differences between two growing seasons as well as the species differences within one growing season, one-way repeated-measures ANOVA followed by post hoc Tukey's hsd was applied. For correlation analysis, Pearson correlation coefficients (r) with Student's t test with a conservative p value cut off of 0.001 were applied to identify the

significant correlations among foliar elements in three species. Descriptive statistics and the coefficient of variation (CV) were applied to evaluate the elements foliar contents among different species.

The overall foliar contents of 10 elements of 3 species with O_3 and soil treatments were profiled by principal component analysis (PCA) in order to reduce the number of variables and screen the data for different treatments by clustering elements with close interactions into novel principal component. The rotation method was the varimax with the Kaiser Normalization based on the Cohen's d. The treatments of O_3 and soil were applied as the classification criteria in assessing the relations among them and the elements.

All the statistics analyses were performed with SPSS 21.0 (IBM, NY, USA) and MS EXCEL 2010 to visualize the differences of 10 elements in 3 species under 6 combined factorial treatments of O_3 and soils over two growing seasons.

4.3 Results

4.3.1 Variations of leaf-element contents and the element retranslocation with environmental variable

Table 4.2 provides statistics summary for the levels of two-way repeated-measures ANOVA on foliar elements contents and their retranslocation rates (ERE) with collection time ("Time") and growing season ("GS") as within-subjects variable, respectively, for the effects of O_3 exposure ("O_3") and soil types ("Soil") in each species. Foliar contents (K, Ca, Mg, Mn and Al, P) and their corresponding retranslocation rates as well as NiRE and CrRE in these 3 species were detected to have a notable effect in response to either O_3 or soil. Leaf Mg, Mn, P contents in birch species *Bp* (Table 4.2a) were significantly affected by O_3 exposure. The O_3 exposure affected also leaf NiRE and PRE in *Bp* though no significant differences were observed among the 6 combined treatments of O_3 and soil on NiRE as shown in Table 4.4a.

In birch species *Bp*, only leaf K and its KRE were detected to be significantly affected by soil and interaction of O_3 x Soil, respectively. In beech species *Fc*, eO_3 effects were detected on foliar contents of K, Al as well as retranslocation rate of Mg, Ca and P (Table 4.2b). Moreover, leaf Mg, K, Al and KRE, CaRE, PRE were significantly affect by soil types. Foliar Mn and Al, ERE of K and P were detected to

be influenced by interactions of O_3 x Soil in *Fc*. As for *Qm* shown in Table 2.2c, O_3 exposure effects were significantly detected on leaf Al, P and their retranslocation rates in addition to MnRE. Although no significant differences were observed between soil and foliar contents, KRE, CaRE, CrRE and PRE were found to be notable affected by soil types. Besides, interaction of O_3 and Soil was observed to significantly affect leaf Ca content and KRE, PRE in oak species. In addition, time factors ("Time" or "GS") had markedly affected foliar contents but not retranslocation rates in three species (Table 4.2). There were no significant effects on interactions of time factors x O_3 x Soil for both foliar element contents and retranslocation rates. In this study, I presented the main effects on the parameters and factors that were determined and only the significant results were addressed to be discussed.

4.3.2 Effects of O_3 and soil treatments on foliar element content

Table 4.3 illustrates the mean foliar element concentrations in *Bp*, *Fc* and *Qm* species and the comparison results among the 6 treatments that combined with O_3 (Ambient: amb, Elevated: e) and soils (B, V, S). For *Bp*, it is apparently from the Table 4.3a that leaf Mg under serpentine soil as well as Mn under all soil types were dramatically decreased by eO_3. Foliar P concentration was dramatically lower at eO_3-S than that at amb-V. Table 4.3b reported significantly more foliar Mg but less K were observed at S soil condition in *Fc*. Foliar Al content under S soil was dramatically decreased at ambient but it was significantly enhanced at eO_3. Whereas in *Qm* (Table 4.3c), leaf Ca content at eO_3 was significantly reduced by grown under S soil. I also found that foliar P in *Qm* was significantly affected by eO_3 when leaf P was markedly reduced at eO_3-V compared with that at amb-S. In addition, foliar element concentration (Table 4.3) was higher than that in soil (Table 4.1).

4.3.3 Effects of O_3 and soil treatments on foliar element resorption efficiency (retranslocation rate)

Table 4.4 presents the treatment effects on foliar retranslocation rate of various elements in the 3 tree seedlings. In *Bp*, KRE and PRE were found to be significantly increased under S soil at eO_3 (Table 4.4a). In *Fc*, MgRE as well as CaRE was decreased with eO_3, especially CaRE was highly significantly decreased at eO_3. Moreover, KRE was significantly higher at eO_3-B soil than that at amb-V soil in *Fc*. PRE in *Fc*, similar

as it was in *Bp*, was identified to be significantly increased under S soil at eO_3 (Table 4.4b). In *Qm* (Table 4.4c), similar to the results in birch *Bp*, KRE and PRE were both significantly enhanced at eO_3-S soil. Contractively, CaRE was detected to be considerable decreased under S soil at amb. MnRE and PRE were exposed notably higher by eO_3 under V soil in my results of *Qm*. Reduction of AlRE and CrRE were also identified at eO_3-S and amb-S, respectively in *Qm*.

4.3.4 Differences of foliar element resorption efficiency (retranslocation rate) during two growing seasons among species

The results of ERE differences between two growing seasons among the three species are set out in Table 4.5. Depending on comparison of the effects within one growing season as well as across two growing seasons, the consequences could be divided into 6 groups in terms of the exact same effects on ERE. 1) MgRE and PRE were found to have distinct differences in *Bp* within 2nd growing season, meanwhile had considerable differences in *Fc* across two growing seasons. 2) KRE and MnRE in *Qm* were detected to be significantly different not only within 1st growing season in 2014 but also between the two growing season in 2014 and 2015. 3) CaRE, FeRE and together with AlRE were assessed to have notable differences in *Bp* within both 1st and 2nd growing seasons, respectively. 4) NiRE in *Qm* was similar to group (2) that had significant differences of across growing seasons but the within one growing season differences were found in 2nd growing season. 5) NRE in *Bp* was comparable to group (3) that was found to be significantly different within both 2014 and 2015, while the major differences across two growing seasons were found in *Qm*. 6) CrRE in none of the species was observed to have differences either within one growing season or across two growing seasons.

4.3.5 Foliar element contents

Foliar contents of 10 elements in this study varied by 3 orders of magnitude from about 0.02 mg/g for Cr to about 20 mg/g for N, with the general order of Cr < Ni < Al < Fe (Mn) < P < Mg < K < Ca < N for these 3 tree species (Figure 4.1). There were a bit differences on foliar contents of Mn and Fe depending upon species. Descriptive statistics including some main statistical parameters such as mean, standard deviation

(SD), maximum and minimum values of foliar elements contents were analyzed, and the results are shown in Table 4.6. *Bp* contains more foliar Fe than Mn (Figure 4.1a) while *Fc* and *Qm* contain more foliar Mn than Fe as shown in the Figure 4.2b and 4.2c respectively, which were consistent with the results presented in Table 4.6. This is probably related to *Bp* with indeterminate shoot growth pattern.

In order to visualize the species variance influencing each element, coefficient of variation (CV) was presented in Figure 4.2. The CV of macronutrients in the studied 3 species including Ca, K, Mg, N and P varied <50 %, whereas trace elements including Ni and Cr had CVs >100 %. The pattern of CV was found to be concordant with the stability of limiting elements: Ca, K, P in *Bp*, N and Ca in *Fc*, as well as K and Ca in *Qm* were < 30%, whereas Al and Fe in *Bp*, Mn and Al in *Fc* and *Qm* were > 50 %, and Ni and Cr were approximated to 120 % in all species.

4.3.6 Relationships among foliar elements

Leaf elements which are controlled by the homeostatic mechanism are reported to have interaction effects not only within foliar nutrients but also across the species (Baxter et al., 2012; Chu et al., 2015). Therefore, in order to further understand the foliar stoichiometry and better explain the foliar elements contents affected by either O_3 or soil type as well as their allocations and dynamics, we investigated the correlations among 10 foliar elements within each species to identify the correlated foliar elements in *Bp*, *Fc* and *Qm* as shown in the whole plots in Figure 4.3. I also detected a large number of positively correlated foliar elements in all species as shown in Figure 4.3, however, negatively correlated ones were very limited. In datasets of *Bp*, there were 3 pairs of foliar elements negatively correlated (N-Fe, N-Mn and N-Ca) while in other two species, only N-Al and N-Fe were detected negatively correlated in *Fc* and *Qm*, respectively (Figure 4.3b and 4.3c).

4.3.7 Principle component analysis

In order to identify the foliar elements associated with the effects of O_3 and soil treatments and also screen variables to summarize the data for different treatments across different species, principle component analysis (PCA) was conducted and the results are shown in Figure 4.4. Ambient O_3 with B soil were regarded as control during the analyses. There were 10 foliar elements converted to 2 linearly uncorrelated PCs

that in total contributed to 63.2 % of total variance of elements among species. PC1 which can be regarded as the major axis; explained 42.5 % of the total variance and it was dominantly incorporated by beech species *Fc* and O_3 treatment with contribution of 91 % and 25.2 %, respectively. Meanwhile, PC2 explained 20.8 % of total variance and was mainly affected by birch species and soil treatment with contribution of 73.4 % and 14.2 %, respectively.

From PCA result shown in Figure 4.4, PC1 was clearly separated *Bp* from *Fc* and *Qm* as well as separated combined O_3 and soil effects from independent treatment of O_3. Therefore, *Bp* and the combined treatments were characterized by their higher foliar N with 75 % contribution loading on PC1, whereas *Qm* and control were characterized by their higher metal leaf contents (Al, Fe, Mn, Ni and Cr with contribution of 78.5 %, 76.3 %, 75.5 %, 72.8 % and 72.2 %, respectively). PC2 was determined by foliar K, P and Mg with the contribution of 78.9 %, 67.4 % and 63.3 %, respectively which were the top 3 highest for soil treatment and *Bp*.

4.4 Discussion

4.4.1 General analysis of effects of O_3 and soil on foliar contents and retranslocation rates

Foliar mineral content depends on availability and extent of translocation of an element between xylem flow and phloem loading (Gjengedal, 1996); correspondingly, retranslocation is a process of a nutrient element removal from aged plant tissue (e.g. foliage) into other plant organs (e.g. fine roots), providing nutrients for the production prior to senescence (Killingbeck, 2004; Shi et al., 2016a). Foliar contents and their retranslocation rate are closely linked with each other. It is anticipated that those environmental factors or treatments which promote the plant growth also facilitate the retranslocation rate of elements and vice versa (Nambiar and Fife, 1991). As a result, an agreement result was found in my study that the effects of O_3 and soil treatments on the retranslocation rate can be detected in those elements basically detected to have significantly treatment effects on foliar contents (Ca, K, Mg, Mn, P and Al) among 3 species (Table 4.3, 4.4). Therefore, understanding the mechanism of both foliar nutrient contents and their corresponding retranslocation rate are essential to be of help understand the dynamics of nutrient cycling as well as the maintenance of soil fertility

in forest ecosystems (Onyekwelu et al., 2006).

Generally speaking, the nutrients re-translocated more on nutrient poor soil sites, manifesting the essential function of retranslocation in the nutrient conservation mechanism (Koike, 2004; Uddling et al., 2006). The amount to retranslocation as well as retranslocation rate can be varied depending on the elements mobility, plant species, and environmental factors such as O_3 effect and soil nutrient status (Hagen-Thorn et al., 2004). Among the studied 10 elements, K, Mg, N Ni and P are known as water soluble elements and are also able to mobile in phloem translocated from old to younger leaves. Contrarily, Ca, Fe, Mn, Al and Cr as the non-mobile elements are move upward via xylem from roots to foliage but poorly mobile in phloem (Zayed and Terry, 2003; Marschner, 2012). Therefore, during leaf senescence, mobile elements are unstably transported out from the senescing leaves and anticipate positive values on retranslocation rates; while non-mobile elements are basically remained in older leaves and result in the deficiencies in younger leaves, expecting negative values on retranslocation rates (Helmisaari, 1992; Marschner, 2012; Shi et al., 2016a).

Within two growing seasons, different treatments of combined O_3 and soils had different effects on foliar elements contents in each species. Significant effects were detected in different collection time for all (or almost) foliar elements contents in *Bp*, *Fc* and *Qm*. This is because I collected foliage samples in different development stages of leaf growth and the significant differences detected for foliar nutrients at different leaf stages may due to the different shoot growth pattern of different species (Shi et al., 2016). Soil elements: Ca, K, Mg, Mn, N, Ni and Al, Cr, Fe (projected nutrient elements except P) exhibited significant differences among soil types. As the same aged seedlings were planted and developed under the same site conditions, the nutrient differences of surface soil (depth 0–10 cm) are likely due to the elemental function of each species (e.g. Salehi et al., 2013).

4.4.2 Birch

As Mg is the central atom of chlorophyll pigment and an essential element for the aggregation of ribosomes (Marschner, 2012) as well as Mn promotes the water oxidation occurrence in photosynthetic oxygen evolution (e.g. Kitao et al., 1998). Both Mg and Mn are the key elements playing important roles in photosynthesis. As leaf Mg and Mn contents were influenced by eO_3 exposure in a similar manner (Table 4.2a) in birch species *Bp*, lower value of foliar Mg and Mn at eO_3 (Table 4.3a) may associate

with the eO_3-induced accelerants of foliar senescence and the lower stomata conductance at eO_3 (Hoshika et al., 2015). The similar effects of eO_3 on Mg and Mn are possibly also attributed to the positive correlations as I identified in *Bp* (Figure 4.3a). As one of the primary essential elements for plants growth, K must be dissolved into soil water and enters the plant as roots uptake from water (Marschner, 2012). Therefore, foliar K as a highly mobile element in plant was observed significantly affected by soil conditions as shown in Table 4.2a. Leaf K in *Bp* was decreased with nutrient infertility soil independent of the O_3 condition (Table 4.3b).

Contrastively, accumulation of toxic elements (Mg, Ni and Cr) performed higher effects in soils (Table 4.1). I consider that the major factor for leaf K inhibition of *Bp* grown in S soil was the toxic elements accumulation in the infertility soil. Previous research reported that a sufficient N habitat (e.g. B soil) induced *Bp* greater resistance to O_3 (Pääkkönen and Holopainen, 1995), which may result to increased nutrient concentration in leaf. Considering the significant effect of O_3 was found in foliar P (Table 4.2a), a possible explanation for the reduced results in foliar P might because of the higher sensitivity effects to O_3 for *Bp*. Moreover, the enhanced KRE and PRE at eO_3-S (Table 4.4a) were in agreement with the study of Koike (2004), reported that the nutrient retranslocation in late successional seedlings can be increased at nutrient deficiency soil. Consider about the effects (or combined effects) of eO_3, there is a possibility that both KRE and PRE were increased under nutrient limit soil (Table 4.4a) to enhance the competitive at plant-level so as to overcome the stressed environment (e.g. eO_3) (Aerts and Chapin, 2000). Similar explanation can be also applied on enhanced result of PRE in *Fc* (Table 4.4b) as well as PRE and KRE in *Qm* (Table 4.4c).

4.4.3 Beech

According to the research reported by Conti and Geiger (1982), K is able to regulate the photosynthates translocation in sap of plants and higher K may accelerate the functions in photosynthesis process. Therefore, the decreased foliar K at amb-S and eO_3-S (Table 4.3b) were likely because of the reduced photosynthesis process caused by K-limited S soil (Table 4.1) and eO_3, respectively since both soil and eO_3 were found to significantly affect the foliar K content in *Fc* species (Table 4.2b). Another possible explanation for this that was supported by Kayama and Koike (2015) was that the uptake K in *Fc* leaves might be suppressed by accumulation of Mg in leaves under infertility soil as it decreases the leaf water potential and photosynthetic rate. Serpentine

soil contained higher Cr and Ni (Table 4.1) but I did not detect any effects on Cr or Ni in *Fc* species (Table 4.2b).

As Al is a highly rhizo-toxic and a major stress element to plants on acid soils like V soil (Table 4.1) as found by Brunner and Sperisen (2013), I considered that the significant decreased content of foliar Al in *Fc* for both infertile soil and eO_3 (Table 4.2b, 4.3b) likely suggested that *Fc* has a strong detoxification mechanism (Kayama et al., 2011; Kayama and Koike, 2015) to protect itself from adverse environment condition (e.g. S soil, eO_3) and make the leaves suppress the Al uptake since low content of Al has beneficial effects on growth (Marschner, 2012).

With the respect of retranslocation in *Fc* species (Table 4.2b, 4.3b), I considered that eO_3 may have a detrimental effect on Mg dynamics and make Mg accumulated in aged leaves to get a lower MgRE at eO_3. What's more, the decreased results of MgRE and CaRE in *Fc* were likely due to the hormone changed regulated by K dynamics as KRE was found to be enhanced at eO_3-B soil. On the other hand, the reduction of Mg and Ca in foliar mature leaves of *Fc* species that likely induced the lower values of MgRE and CaRE maybe due to the foliar leaching via cation exchange (Helmisaari, 1992).

Leaching of Ca in mature leaves was probably weaken at S soil due to its limited content of soil Ca (Table 4.1) but was likely strengthen at lower pH soil (V soil, Table 4.1) as reported by Gjengedal (1996) or at eO_3. Similarly, PRE was significantly decreased by eO_3 and the interaction O_3 x Soil (Table 4.4b) which was likely due to the leaching of P in senescing leaves under eO_3 since late leaves of *Bp* (senescing leaves in this study) were more susceptible to eO_3 (Helmisaari and Mälkönen, 1989; Hoshika et al., 2013b).

4.4.4 Oak

With regard to *Qm* species combined with the results in Table 4.2c and 4.3c, *Qm* is perhaps more tolerant than other 2 species due to fewer elements were observed to be affected by O_3 and/or Soil. Apparently, foliar Ca content was affected by interaction O_3 x Soil due to the declined Ca was identified at eO_3-S soil. As serpentine soil contains higher toxic elements including high ratio of Mg/Ca and excessive Ni and Cr (Table 4.1), in addition to the detrimental effects of eO_3 on the detoxification mechanism for *Qm* species, the reduced Ca possibly suggested that the accumulation of toxic elements (Mg, Ni and Cr) in soil decreased the photosynthetic rate at eO_3 and thereby decrease

the foliar uptake of Ca.

Alternatively, there was no adequately Ca available in S soil (Table 4.1) to be absorbed from roots and eventually lead a lower leaf Ca in *Qm*. Moreover, Ca as the relatively immobile elements, dramatically reduction of CaRE may be attributed to the higher accumulation in senescing leaves that induced by the effect of infertile soil. Similar explanation is able to apply on AlRE and CrRE as well in *Qm* (Table 4.4c) for their reductions at eO_3 and S soil, respectively. As one of the essential components of key molecules such as nucleic acid and ATP, P is involved in regulation of enzyme reactions, promotes metabolic process and helps strengthen plants (Lawniczak, 2011).

According to the study of Thomas et al. (2006), the reduction of leaf P in *Qm* (Table 4.3c) likely depends on the soil types and on the impact degree of metabolism of O_3 since eO_3 also alters the mycorrhization as reported by Edwards and Kelly in 1992. Furthermore, PRE was found to be affected by interaction O_3 x Soil and it was significantly higher in both S soil and at eO_3 (Table 4.4c), suggesting that alive leaves may be able to avoid the detrimental effects of eO_3 and P as a mobile element is retranslocated more in living leaves (Hoshika et al., 2013).

Furthermore, the lower retention of Mn and P in senescing leaves may result in the significant effects of eO_3 on MnRE and PRE since nutrient contents in late leaves were susceptible to be decreased by O_3 stress (Hoshika et al., 2013b). Although KRE was found to be affected by interaction O_3 x Soil as well (Table 4.2c), there was no difference between ambient and eO_3, suggesting that the effects of O_3 on foliar K dynamics is considering too small due to more effects of eO_3 might be detected on roots.

4.4.5 Comparisons among different species

Regarding with the compositive results of nutrient re-translocations in Table 4.2c and 4.4c, although oak is considered as the most tolerant species to eO_3 among the three species on the foliar contents (Kohno et al., 2005; Yamaguchi et al., 2011), it is possibly susceptible to eO_3 on dynamics of immobile elements (e.g. Al, Mn) and it is possible also the most sensitive to soil conditions on foliar retranslocation rates compared to other 2 species. Taking account with growing seasons for different species, I divided the discussion of results in Table 4.5 into two aspects. As re-translocated nutrients are available for reusing by the plant in the next year's growth, for long term monitoring on tree growth, Mg and P are retranslocated from old to younger leaves the most in *Fc*

species, meanwhile N are translocated the most and Ni is remained considerably in *Qm* leaves; for short-term monitoring (one growing season or less) on *Qm* growth, foliar retranslocation of K and Mn are observed to be the most sensitive pair of elements; retranslocation of Ca, Fe, N and Al is able to be considered as the indices in monitoring the growth of *Bp* species for either short- or long-term.

4.4.6 Foliar elements contents and the elemental relationships in different species

Generally, foliar elements contents and their retranslocation rates are varied across the species with different shoot growth patterns (Shi et al., 2016a) and mineral accumulation pattern for metal elements (Watanabe et al., 2007). Therefore, the difference foliar contents for Fe and Mn between *Bp* and other two species (*Fc, Qm*) is probably related to the shoot growth and also accumulation patterns. Based on the results of varietal difference contributed to variation of foliar elements and the descriptive statistics among species shown in Table 4.6, considerable differences among species were also detected in all essential elements excluding Ni in leaves. Ni is generally accumulated in roots but not leaves (Jones and Hutchinson, 1988). This trend was agreed with this result as shown in Figure 4.1. The differences for the projected elements corresponding to variations in addition to the species were likely due to the environmental factors such as soil and climate, or the heterogeneous distribution of non-essential elements in the field (Chu et al., 2015). Although Ni is known as the metal component required for numbers of enzyme activity in biological process (Marschner, 2012), while the metabolic activity involving Cr in plants remains unclear, similar results of Cr and Ni with the previous study (Chu et al., 2015) were observed. Cr and Ni had the largest two CV values among the given elements for all the species (Figure 4.2) which also opposite to their lowest values on foliar concentration (Figure 4.1), suggesting that Cr and Ni may become the most two sensitive of the projected species grown in the field.

With regard to relationships of foliar elements, N was detected to be one of the elements pair that had negative correlations independent with the species (Figure 4.3). Previous evidence shows that uptake of N was suppressed by the accumulation of the several mineral toxic elements such as Mg, Ni and Cr (Kayama et al., 2005; Singh et al., 2013). I can suggest the similar trends for present species with the negative correlations of N-Fe, N-Mn and N-Ca in *Bp*, N-Al in *Fc* and N-Fe in *Qm*. This trend

could be attributed to the fact that accumulation of these mineral elements induces the decrease of leaf water potential, resulting in closed stomata and reduced photosynthetic rate (Rao et al., 1987; Kayama and Koike, 2015). On the other hand, Figure 4.3 indicated the opposite results with the positive correlations of N-K and N-Al in *Bp* as well as N-Al in *Qm*. Thus, interrelations between foliar N and non-essential toxic element such as Al varied with different species as summarized by Watanabe et al. (2007). For those species that positive interrelations with N-Al, the negative correlations of N-Fe were accompanied with (Figure 4.3a, 4.3b). An explanation could be that the toxic elements such as Al may have a trait that makes N uptake suppressed by those mineral elements including at least Fe (Schmitt et al., 2016). Alternatively, N uptake was suppressed by the interaction effects between Fe and other mineral elements.

Besides, *Bp* and *Fc* are relatively more sensitive to O_3 compared with *Qm* may concern with the plenty of interactions among foliar nutrients. Especially, the uptake of N was much easier suppressed by various mineral elements when it correlated with foliar K, which closely related with the regulation of hormone control in a plant (Conti and Geiger, 1982). Nevertheless, foliar N was independent with Mg, Ni, P and Cr in studied species, which was consistent with the results of Kayama and Koike (2015). The independence between N and Mg supports my previous research into N-Mg as a pair of major indices in assessing the O_3 effects in a free air system (Shi et al., 2016b).

Other correlations among mineral elements detected in this study were all positive. I found the most positive correlations in Cr and Ni, which were comparable to my previous results in Figure 4.1 and 4.2. Therefore, the sensitive of Cr and Ni to the projected species are like to be related to their highly positive interrelations with other mineral elements. Moreover, Mg, Ni, Cr as well as Mn, Fe, Ni were positively correlated with each other in all 3 datasets. As Mg, Fe and Mn assist in the chlorophyll manufacture and other biochemical process (Marschner, 2012) as well as the essential roles of Cr and Ni binding the cell wall components (Seregin and Kozhevnikova, 2006), Fe, Mg, Mn, Ni and Cr are highly involved in the basic structures and cells function, thus have similar biochemical pathways along with positive correlations (Wu et al., 2012). Furthermore, pairs of foliar elements Fe-Ni, Fe-Al and Fe-Cr have been found to highly significant correlated with $r > 0.8$ in all species, suggesting a possible link of those metal elements on their transport pathway (Schmitt et al., 2016) and the uptake of toxic mineral elements like Fe in leaves was more readily affected by other toxic mineral elements like Al and Cr (excess Ni).

4.4.7 Profiling the foliar elements in birch, beech and oak

Combined with the results of effects of O_3 and soil on foliar element content in Table 4.3 as well as the PCA in Figure 4.4, the following 4 facts were observed in this study: 1) foliar Al was reduced by combined effects of O_3 and soil in *Fc* as well as was notably affected by O_3 in *Qm* species; 2) foliar Mn was reduced by eO_3 in birch *Bp* as well as was markedly affected by combined effects in *Fc* species; 3) foliar K was reduced by soil condition in *Bp* as well as was affected by both independent treatments of O_3 and soil in *Fc*; 4) Mn and Al had highest loading on PC1 as well as K had highest loading on PC2.

Consequently, K combined with either Mn or Al will be a pair nutrients that simultaneously respond to the treatments of eO_3 and soils. But in this study, I will not take Al into account for the index elements as Al is a non-essential element and high content is toxic to plants, except some tropical species (Watanabe et al., 2007; Schmitt et al., 2016). Accordingly, when leaf Mn is reduced, eO_3 effect is taken into consideration, whereas when K is reduced, soil conditions are possibly the dominant effect. Therefore, Mn and K may be considered as the indices in assessing the O_3 and soil effects for long-term growth monitoring in seedlings of *Bp*, *Fc* and *Qm*.

4.5 Summary

In conclusion, my research has been focused on interpreting the environmental impact factors by studying the stoichiometry of foliar nutrients in 3 broad-leaved forest species that are representative of cool temperate region of Japan. My results suggest that both O_3 and soil properties are the essential impact factors on foliar stoichiometry on *Bp, Qm,* and *Fc* seedlings.

With regard to the 3 species, *Qm* can be considered as the most susceptible species to eO_3 upon leaf dynamics of immobile elements even though it was regarded as relative more tolerant species to eO_3 in many previous researches (e.g. Kohno et al., 2005; Yamaguchi et al., 2011). *Fc* with determinate shoot growth pattern is more sensitive than birch and oak species to eO_3 effects on foliar contents. *Bp* species has negative correlations between foliar N and those metal elements (Ca, Fe and Mg).

Regardless the tree species, soil nutrients (soil types) have distinct impacts on retranslocation rate of K, P and Fe Although Cr and Ni may be the most two sensitive

elements for the projected species grown on the field, the sensitivity may concern with their considerable positive interrelations in leaf, thus they will not be the case after effects of elevated O_3 or/and soil conditions are taken into account. From my results, Mn and K can become indices in assessing the O_3 and soil effects in both short and long term monitoring on growth of these tree species.

These findings are essential in identifying the pattern of variation in leaf nutrient and its regulation variables as well as further understanding the mechanism of responses of O_3 tolerance in 3 species in terms of nutrient dynamics.

Table 4.1 Chemical properties of brown forest soil, volcanic ash soil and serpentine soil, sampled at depth 0–10 cm (mean ± SD, n = 3–6). Pool is the main effects of the soil type, which indicates the pooled values for both ambient and elevated O_3 treatments.

	Brown forest			Volcanic ash			Serpentine			Statistics		
	Amb	O_3	Pool	Amb	O_3	Pool	Amb	O_3	Pool	Soil	O_3	Soil x O_3
pH	5.24 (0.05)	5.30 (0.03)	5.27^{B} (0.03)	4.28 (0.15)	4.57 (0.08)	4.43^{B} (0.10)	5.90 (0.14)	6.36 (0.05)	6.13^{A} (0.12)	***	ns	ns
Mg mg $100g^{-1}$	16.79 (1.78)	21.97 (1.12)	19.38^{B} (1.49)	13.66 (0.98)	18.82 (3.32)	16.24^{B} (1.93)	61.76 (10.42)	75.44 (7.42)	68.60^{A} (6.50)	***	ns	ns
K mg $100g^{-1}$	21.25 (3.66)	26.85 (3.21)	24.05^{A} (2.51)	21.55 (1.11)	26.27 (4.12)	23.91^{A} (2.18)	12.87 (3.61)	18.98 (2.43)	15.93^{B} (2.38)	*	ns	ns
Ca mg $100g^{-1}$	143.7 (13.7)	260.3 (32.1)	202.0^{A} (30.4)	116.9 (10.2)	230.4 (45.5)	173.7^{A} (32.8)	69.61 (8.93)	143.6 (27.3)	106.6^{B} (20.9)	*	ns	ns
Mn mg $100g^{-1}$	1.043 (0.02)	1.081 (0.16)	1.062^{A} (0.07)	1.061 (0.02)	1.004 (0.02)	1.032^{A} (0.02)	0.736 (0.11)	0.809 (0.09)	0.773^{B} (0.06)	*	ns	ns
Fe mg $100g^{-1}$	0.654 (0.15)	0.445 (0.07)	0.549^{AB} (0.09)	0.597 (0.12)	1.221 (0.40)	0.909^{A} (0.23)	0.266 (0.05)	0.357 (0.12)	0.311^{B} (0.06)	*	ns	ns
Ni mg $100g^{-1}$	0.182 (0.01)	0.172 (0.01)	0.177^{B} (0.006)	0.172 (0.01)	0.183 (0.00)	0.178^{B} (0.00)	0.328 (0.04)	0.322 (0.03)	0.325^{A} (0.02)	***	ns	ns
Cr mg $100g^{-1}$	0.098 (0.00)	0.096 (0.00)	0.097^{B} (0.002)	0.097 (0.00)	0.109 (0.00)	0.10^{AB} (0.00)	0.115 (0.01)	0.110 (0.00)	0.113^{A} (0.00)	*	ns	ns
Al mg $100g^{-1}$	1.303 (0.25)	0.596 (0.27)	0.950^{AB} (0.23)	1.108 (0.19)	1.375 (0.50)	1.242^{A} (0.25)	0.606 (0.19)	0.376 (0.11)	0.491^{B} (0.11)	*	ns	ns
P mg $100g^{-1}$	2.070 (0.10)	2.317 (0.23)	2.194^{A} (0.12)	1.95 (0.14)	1.702 (0.05)	1.828^{A} (0.09)	1.924 (0.23)	2.395 (0.11)	2.160^{A} (0.15)	ns	ns	ns
N mg/g	2.308 (0.21)	2.602 (0.27)	2.455^{A} (0.17)	2.285 (0.06)	2.349 (0.12)	2.317^{A} (0.06)	1.717 (0.31)	1.332 (0.20)	1.524^{B} (0.18)	**	ns	ns

ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, no significant.

Table 4.2 Levels of a two-way repeated-measures ANOVA, with collection time (Time) or growing season (GS) as within-subjects variable, for the effects of O_3 (ambient and elevated O_3 concentration) and Soil (brown forest soil, volcanic ash soil and serpentine soil) on foliar concentration of 10 elements (N, P, K, Ca, Mg, Mn, Fe, Ni and Al, Cr) and their retranslocation rates (RE) in (a) birch *Bp*, (b) beech *Fc* and (c) oak *Qm* seedlings for 5 collections over two growing seasons.

(a) Birch *Bp*	O_3	Soil	O_3 x Soil	Time	Time x O_3	Time x Soil	Time x O_3 x Soil
df	2	3	6	5	10	15	30
Mg	**0.016**	0.195	0.400	**0.002**	**0.031**	0.408	0.405
K	0.450	**0.006**	0.094	**<0.001**	0.341	0.078	0.219
Ca	0.494	0.183	0.445	**<0.001**	0.237	0.539	0.975
Mn	**0.021**	0.244	0.29	**<0.001**	0.628	0.625	0.146
Fe	0.184	0.077	0.296	**<0.001**	**0.023**	**0.019**	0.183
Ni	0.063	0.174	0.170	**<0.001**	0.847	0.325	0.08
Al	0.101	0.239	0.221	**<0.001**	0.157	**0.017**	0.196
Cr	0.284	0.696	0.444	**<0.001**	0.221	0.932	0.355
P	**0.042**	0.208	0.653	**0.039**	0.206	**0.043**	0.528
N	0.201	0.911	0.945	**<0.001**	0.057	0.122	0.421
	O_3	Soil	O_3 x Soil	GS	GS x O_3	GS x Soil	GS x O_3 x Soil
df	2	3	6	2	4	6	12
MgRE	0.350	0.805	0.247	0.889	**0.037**	0.973	0.367
KRE	0.260	0.276	**0.03**	0.056	0.494	0.257	0.495
CaRE	0.623	0.700	0.773	0.251	0.240	0.400	0.709
MnRE	0.838	0.978	0.422	0.18	0.372	0.776	0.124
FeRE	0.618	0.162	0.979	0.069	**0.016**	0.055	0.763
NiRE	0.201	0.535	0.487	0.174	0.449	0.940	0.869
AlRE	0.527	0.772	0.404	0.134	0.606	0.609	0.424
CrRE	0.470	0.122	0.194	0.403	0.502	0.816	0.278
PRE	**0.004**	0.068	0.991	0.194	0.244	**0.036**	0.49
NRE	0.454	0.758	0.086	0.281	0.084	0.144	0.058

Table 4.2 (continued)

(b) Beech *Fc*	O_3	Soil	O_3 x Soil	Time	Time x O_3	Time x Soil	Time x O_3 x Soil
df	2	3	6	5	10	15	30
Mg	0.344	**0.038**	0.609	**<0.001**	**0.032**	0.047	0.29
K	**0.042**	**0.037**	0.261	**0.007**	0.14	0.13	0.297
Ca	0.940	0.051	0.653	**0.001**	0.068	**0.009**	0.59
Mn	0.462	0.053	**0.012**	**0.002**	**0.021**	0.177	0.207
Fe	0.752	0.056	0.132	**<0.001**	**0.008**	**0.01**	0.277
Ni	0.570	0.614	0.868	**<0.001**	0.232	0.803	0.435
Al	**0.045**	**0.047**	**0.005**	**0.004**	0.197	0.352	0.115
Cr	0.074	0.393	0.980	**<0.001**	**0.016**	0.278	0.525
P	0.255	0.148	0.379	**<0.001**	0.218	0.497	0.26
N	0.679	0.526	0.186	**<0.001**	0.224	0.054	0.249
	O_3	Soil	O_3 x Soil	GS	GS x O_3	GS x Soil	GS x O_3 x Soil
df	2	3	6	2	4	6	12
MgRE	**0.030**	0.340	0.574	0.105	0.056	0.198	0.068
KRE	0.503	**0.030**	**0.049**	0.037	0.128	0.229	0.306
CaRE	**0.018**	**0.027**	0.591	0.145	0.492	0.587	0.065
MnRE	0.169	0.079	0.225	0.203	0.175	0.05	0.057
FeRE	0.057	0.052	0.235	0.134	**0.029**	**0.011**	0.073
NiRE	0.091	0.700	0.857	0.267	0.084	0.558	0.274
AlRE	0.989	0.672	0.997	0.252	0.166	0.507	0.797
CrRE	0.136	0.791	0.370	0.206	0.318	0.432	0.933
PRE	**0.012**	**0.023**	**0.018**	0.12	0.921	0.76	0.133
NRE	0.203	0.338	0.571	0.052	0.078	0.31	0.058

Table 4.2 (continued)

(c) Oak *Qm*	O_3	Soil	O_3 x Soil	Time	Time x O_3	Time x Soil	Time x O_3 x Soil
df	2	3	6	5	10	15	30
Mg	0.495	0.349	0.358	**0.016**	0.338	0.065	0.305
K	0.339	0.854	0.84	**0.001**	**0.025**	0.216	0.787
Ca	0.194	0.361	**0.01**	0.128	0.158	0.359	0.147
Mn	0.087	0.528	0.273	**<0.001**	**0.002**	**0.001**	0.092
Fe	0.234	0.062	0.867	**0.001**	**0.025**	0.176	0.051
Ni	0.341	0.771	0.094	**<0.001**	0.081	0.218	0.232
Al	**0.014**	0.534	0.933	**<0.001**	0.171	0.606	0.241
Cr	0.951	0.389	0.897	**<0.001**	0.974	**0.001**	0.91
P	**0.031**	0.419	0.501	0.329	0.709	0.158	0.661
N	0.802	0.165	0.356	**<0.001**	**0.006**	0.185	0.969
	O_3	Soil	O_3 x Soil	GS	GS x O_3	GS x Soil	GS x O_3 x Soil
df	2	3	6	2	4	6	12
MgRE	0.225	0.055	0.559	0.797	0.819	0.72	0.117
KRE	0.674	**0.049**	**0.031**	0.072	0.989	0.827	0.970
CaRE	0.728	**0.014**	0.283	0.112	0.329	0.073	0.455
MnRE	**0.031**	0.533	0.094	0.098	0.087	0.212	0.533
FeRE	0.199	0.465	0.297	0.052	0.416	0.232	0.165
NiRE	0.376	0.431	0.822	0.35	0.445	0.347	0.626
AlRE	**0.022**	0.198	0.201	0.173	0.232	0.139	0.078
CrRE	0.363	**0.048**	0.836	0.168	0.23	0.579	0.325
PRE	**0.026**	**0.048**	**0.03**	0.148	0.167	0.415	0.093
NRE	0.836	0.422	0.342	0.308	0.846	0.267	0.453

Table 4.3 Mean (±SD) foliar element concentration (mg g^{-1}) of N, P, K, Ca, Mg, Mn, Fe, Ni and Al, Cr in (a) birch *Bp*, (b) beech *Fc* and (c) oak *Qm* seedlings grown on three soil types under two gas treatments. Gas treatments include ambient O_3 (A) and elevated O_3 (E); Soil types includes brown forest (B) soil, volcanic ash (V) soil and serpentine (S) soil. Comparisons were made within each line among the 6 treatments. The results of each variable obtained by general linear model (GLM) analysis or a post hoc Tukey's hsd test, after significant results of the GLM analysis based on standardized data. Different letters within each row indicate statistical differences at $P < 0.05$.

(a) Birch	**AB**	**AV**	**AS**	**EB**	**EV**	**ES**
Mg	3.976 **b** (0.615)	4.082 **ab** (0.585)	5.143 **a** (1.205)	3.643 **b** (1.186)	3.715 **b** (0.659)	3.742 **b** (0.788)
K	8.801 **ab** (1.064)	10.000 **ab** (2.258)	7.962 **b** (1.541)	10.110 **a** (1.074)	9.294 **ab** (1.296)	8.289 **ab** (1.360)
Ca	11.630 a (1.655)	10.440 a (1.601)	9.999 a (1.802)	11.507 a (1.964)	11.632 a (1.502)	10.890 a (1.360)
Mn	0.393 **a** (0.074)	0.373 **a** (0.068)	0.284 **a** (0.069)	0.219 **b** (0.044)	0.217 **b** (0.064)	0.215 **b** (0.051)
Fe	0.419 a (0.245)	0.328 a (0.126)	0.318 a (0.110)	0.383 a (0.213)	0.343 a (0.168)	0.292 a (0.128)
Ni	0.050 a (0.036)	0.045 a (0.030)	0.056 a (0.035)	0.045 a (0.032)	0.051 a (0.037)	0.047 a (0.032)
Al	0.139 a (0.099)	0.148 a (0.078)	0.095 a (0.050)	0.089 a (0.062)	0.072 a (0.050)	0.078 a (0.062)
Cr	0.021 a (0.015)	0.021 a (0.015)	0.022 a (0.016)	0.022 a (0.016)	0.024 a (0.017)	0.022 a (0.015)
P	3.853 **ab** (0.716)	4.356 **a** (1.331)	3.455 **ab** (0.721)	3.666 **ab** (0.757)	3.437 **ab** (0.485)	2.989 **b** (0.482)
N	27.091 a (5.825)	26.544 a (6.362)	27.502 a (5.838)	29.531 a (7.387)	29.223 a (6.720)	29.196 a (7.377)

Table 4.3 (continued)

(b) Beech	AB	AV	AS	EB	EV	ES
Mg	1.907 **b** (0.718)	1.803 **b** (0.577)	3.406 **a** (0.864)	2.128 **b** (1.032)	1.970 **b** (0.504)	3.069 **ab** (0.680)
K	5.663 **ab** (0.817)	6.657 **a** (1.313)	4.460 **b** (1.080)	5.120 **ab** (0.982)	5.200 **ab** (0.715)	4.261 **b** (1.116)
Ca	11.688 a (1.575)	11.498 a (1.474)	11.694 a (1.194)	10.865 a (1.427)	12.162 a (1.423)	12.318 a (1.319)
Mn	0.615 a (0.236)	0.416 a (0.093)	0.465 a (0.128)	0.421 a (0.140)	0.602 a (0.267)	0.382 a (0.133)
Fe	0.314 a (0.079)	0.272 a (0.097)	0.249 a (0.077)	0.260 a (0.051)	0.288 a (0.076)	0.308 a (0.071)
Ni	0.044 a (0.032)	0.040 a (0.035)	0.046 a (0.033)	0.038 a (0.035)	0.047 a (0.033)	0.047 a (0.033)
Al	0.215 **a** (0.062)	0.157 **ab** (0.052)	0.108 **b** (0.034)	0.105 **b** (0.025)	0.146 **ab** (0.040)	0.119 **b** (0.032)
Cr	0.021 a (0.015)	0.017 a (0.014)	0.020 a (0.015)	0.020 a (0.018)	0.025 a (0.018)	0.023 a (0.017)
P	1.647 a (0.326)	1.700 a (0.367)	2.398 a (2.466)	1.210 a (0.345)	1.463 a (0.405)	1.423 a (0.386)
N	20.090 a (2.881)	18.220 a (3.772)	20.008 a (3.496)	20.194 a (4.155)	19.897 a (4.106)	18.468 a (4.922)

Table 4.3 (continued)

(c) Oak	AB	AV	AS	EB	EV	ES
Mg	2.412 a (0.425)	2.204 a (0.442)	3.011 a (0.760)	2.887 a (1.258)	2.028 a (0.508)	2.920 a (0.516)
K	7.709 a (1.712)	6.866 a (1.564)	6.828 a (1.182)	9.041 a (1.232)	8.565 a (2.113)	8.430 a (2.233)
Ca	13.456 **ab** (2.020)	15.315 **a** (2.208)	13.817 **ab** (1.858)	14.943 **a** (1.713)	12.716 **ab** (1.821)	11.505 **b** (2.017)
Mn	0.471 a (0.101)	0.439 a (0.126)	0.507 a (0.231)	0.320 a (0.106)	0.322 a (0.103)	0.351 a (0.120)
Fe	0.249 a (0.040)	0.236 a (0.055)	0.236 a (0.063)	0.327 a (0.140)	0.253 a (0.060)	0.242 a (0.074)
Ni	0.046 a (0.032)	0.052 a (0.039)	0.059 a (0.043)	0.067 a (0.049)	0.039 a (0.031)	0.040 a (0.028)
Al	0.144 a (0.039)	0.130 a (0.030)	0.112 a (0.023)	0.128 a (0.064)	0.124 a (0.068)	0.100 a (0.055)
Cr	0.021 a (0.015)	0.021 a (0.015)	0.022 a (0.017)	0.023 a (0.015)	0.019 a (0.015)	0.019 a (0.015)
P	2.513 **ab** (0.637)	2.865 **ab** (0.612)	3.944 **a** (2.504)	2.681 **ab** (0.512)	1.885 **b** (0.513)	2.377 **ab** (0.540)
N	23.212 a (3.529)	21.246 a (3.882)	24.229 a (4.136)	23.601 a (5.015)	20.949 a (6.125)	24.521 a (6.415)

Table 4.4 Mean (±SD) elements resorption efficiency (RE) of N, P, K, Ca, Mg, Mn, Fe, Ni and Al, Cr in (a) birch *Bp*, (b) beech *Fc* and (c) oak *Qm* seedlings grown on three soil types under two gas treatments. Gas treatments include ambient O_3 (A) and elevated O_3 (E); Soil types include brown forest (B) soil, volcanic ash (V) soil and serpentine (S) soil. Comparisons were made within each line among the 6 treatments. The results of each variable obtained by general linear model (GLM) analysis or a post hoc Tukey's hsd test, after significant results of the GLM analysis based on standardized data. Different letters within each row indicate statistical differences at $P < 0.05$.

(a) Birch	**AB**	**AV**	**AS**	**EB**	**EV**	**ES**
MgRE	-0.413 a	-0.441 a	-0.353 a	-0.131 a	-0.347 a	-0.314 a
	(0.071)	(0.081)	(0.078)	(0.103)	(0.045)	(0.048)
KRE	0.249 **ab**	0.417 **a**	0.294 **ab**	0.134 **b**	0.209 **ab**	0.364 **a**
	(0.008)	(0.032)	(0.044)	(0.025)	(0.022)	(0.030)
CaRE	-0.510 a	-0.480 a	-0.478 a	-0.673 a	-0.472 a	-0.383 a
	(0.057)	(0.060)	(0.085)	(0.055)	(0.055)	(0.047)
MnRE	-0.638 a	-0.450 a	-0.472 a	-0.434 a	-0.657 a	-0.561 a
	(0.069)	(0.039)	(0.088)	(0.044)	(0.079)	(0.041)
FeRE	-2.274 a	-1.540 a	-1.186 a	-2.179 a	-1.464 a	-1.095 a
	(0.200)	(0.145)	(0.085)	(0.226)	(0.091)	(0.103)
NiRE	-0.543 a	-0.136 a	-0.137 a	-0.225 a	-0.113 a	-0.002 a
	(0.120)	(0.041)	(0.078)	(0.081)	(0.050)	(0.056)
AlRE	-4.997 a	-12.271 a	-4.499 a	-2.488 a	-1.948 a	-7.120 a
	(0.123)	(3.539)	(0.822)	(0.221)	(0.079)	(2.455)
CrRE	-0.627 a	-0.680 a	-0.249 a	-0.475 a	-0.329 a	-0.205 a
	(0.100)	(0.080)	(0.039)	(0.138)	(0.063)	(0.078)
PRE	-0.538 **b**	-0.275 **ab**	-0.256 **ab**	-0.144 **ab**	0.105 **a**	0.162 **a**
	(0.086)	(0.075)	(0.064)	(0.064)	(0.077)	(0.061)
NRE	0.547 a	0.589 a	0.517 a	0.574 a	0.469 a	0.540 a
	(0.008)	(0.016)	(0.009)	(0.018)	(0.027)	(0.015)

Table 4.4 (continued)

(b) Beech	AB	AV	AS	EB	EV	ES
MgRE	0.179 **ab** (0.162)	0.383 **a** (0.071)	0.296 **ab** (0.080)	-0.267 **b** (0.339)	0.085 **ab** (0.105)	-0.118 **ab** (0.179)
KRE	0.248 **ab** (0.093)	0.026 **b** (0.062)	0.358 **ab** (0.045)	0.411 **a** (0.100)	0.192 **ab** (0.126)	0.271 **ab** (0.178)
CaRE	0.168 **a** (0.073)	0.152**ab** (0.045)	-0.012 **abc** (0.063)	-0.145 **b** (0.054)	-0.045 **bc** (0.065)	-0.219 **b** (0.080)
MnRE	0.162 a (0.179)	0.068 a (0.077)	-0.292 a (0.178)	-0.060 a (0.022)	-0.122 a (0.168)	-0.235 a (0.072)
FeRE	-0.157 a (0.101)	-0.729 a (0.347)	-0.467 a (0.318)	-0.193 a (0.148)	-0.404 a (0.093)	-0.160 a (0.097)
NiRE	-0.454 a (0.391)	-0.663 a (0.328)	-0.320 a (0.085)	-0.197 a (0.076)	-0.111 a (0.063)	-0.025 a (0.137)
AlRE	-0.540 a (0.340)	-0.839 a (0.191)	-0.770 a (0.437)	-0.700 a (0.486)	-0.853 a (0.293)	-0.759 a (0.298)
CrRE	-0.212 a (0.156)	-0.269 a (0.292)	-0.323 a (0.100)	-0.211 a (0.085)	0.090 a (0.101)	-0.126 a (0.086)
PRE	-0.002 **ab** (0.128)	-0.458 **b** (0.233)	0.401 **a** (0.172)	0.387 **b** (0.153)	0.205 **ab** (0.109)	0.158 **ab** (0.173)
NRE	0.324 a (0.035)	0.326 a (0.047)	0.401 a (0.046)	0.279 a (0.060)	0.321 a (0.054)	0.368 a (0.064)

Table 4.4 (continued)

(c) Oak	AB	AV	AS	EB	EV	ES
MgRE	-0.025 a	0.095 a	-0.133 a	0.270 a	0.178 a	-0.151 a
	(0.110)	(0.087)	(0.179)	(0.117)	(0.198)	(0.125)
KRE	0.031 **ab**	0.146 **ab**	0.224 **ab**	0.128 **ab**	-0.206 **b**	0.270 **a**
	(0.119)	(0.093)	(0.075)	(0.090)	(0.217)	(0.157)
CaRE	0.151 **ab**	0.224 **a**	-0.171 **b**	0.022 **ab**	0.011 **ab**	-0.028 **ab**
	(0.107)	(0.080)	(0.172)	(0.076)	(0.108)	(0.105)
MnRE	-0.444 **ab**	-1.075 **b**	-1.033 **b**	-0.709 **ab**	0.155 **a**	-0.498 **ab**
	(0.205)	(0.472)	(0.429)	(0.362)	(0.146)	(0.374)
FeRE	-0.194 a	-0.264 a	-0.429 a	-1.086 a	-0.362 a	-0.849 a
	(0.068)	(0.054)	(0.156)	(0.440)	(0.202)	(0.540)
NiRE	-0.132 a	-1.522 a	-0.380 a	-1.146 a	-3.618 a	-1.031 a
	(0.122)	(0.740)	(0.136v	(0.426)	(3.465)	(0.822)
AlRE	-0.505 **a**	-0.636 **a**	-1.341 **a**	-1.827 **a**	-3.781 **ab**	-8.071 **b**
	(0.292)	(0.176)	(0.824)	(0.403)	(2.145)	(4.452)
CrRE	-0.304 **a**	-0.315 **a**	-1.194 **b**	-0.137 **a**	-0.098 **a**	-0.759 **ab**
	(0.178)	(0.196)	(0.522)	(0.095v	(0.065)	(0.175)
PRE	-0.325 **c**	-0.277 **c**	-0.194 **bc**	-0.479 **c**	0.414 **a**	0.191 **ab**
	(0.248)	(0.070)	(0.087)	(0.189)	(0.065)	(0.082)
NRE	0.354 a	0.426 a	0.336 a	0.363 a	0.358 a	0.432 a
	(0.052)	(0.033)	(0.062)	(0.063)	(0.080)	(0.072)

Table 4.5 Mean (±SD) foliar element resorption efficiency (RE) of N, P, K, Ca, Mg, Mn, Fe, Ni and Al, Cr in 3 species during the growing seasons of 2014 and 2015 (GS X Species). One-way repeated-measures ANOVA followed by Tukey's hsd test, were applied within each row of the table. Different letters indicate statistical differences at $P < 0.05$

	1st growing season (2014)			2nd growing season (2015)		
	Birch *Bp*	**Beech *Fc***	**Oak *Qm***	**Birch *Bp***	**Beech *Fc***	**Oak *Qm***
MgRE	-0.311 **c** (0.193)	-0.112 **bc** (0.316)	-0.0280 **abc** (0.191)	-0.355 **c** (0.291)	0.280 **a** (0.139)	0.103 **ab** (0.219)
KRE	0.248 **a** (0.047)	0.130 **ab** (0.096)	-0.0579 **c** (0.131)	0.307 **a** (0.146)	0.362 **a** (0.182)	0.244 **a** (0.218)
CaRE	-0.462 **b** (0.134)	-0.106 **a** (0.095)	-0.0802 **a** (0.090)	-0.536 **b** (0.255)	0.0606 **a** (0.122)	0.154 **a** (0.199)
MnRE	-0.625 **b** (0.168)	-0.157 **ab** (0.252)	-0.839 **c** (0.567)	-0.445 **abc** (0.236)	-0.0206 **a** (0.136)	-0.362 **ab** (0.443)
FeRE	-2.102 **b** (0.549)	-0.501 **a** (0.377)	-0.579 **ab** (0.393)	-1.144 **b** (0.413)	-0.207 **a** (0.175)	-0.431 **a** (0.446)
NiRE	-0.272 **ab** (0.194)	-0.174 **a** (0.126)	-0.425 **ab** (0.253)	-0.113 **a** (0.315)	-0.388 **ab** (0.411)	-2.210 **c** (2.938)
AlRE	-2.793 **b** (0.537)	-0.467 **a** (0.226)	-0.692 **a** (0.276)	-8.314 **b** (8.418)	-0.986 **a** (0.574)	-4.430 **a** (3.924)
CrRE	-0.241 **ab** (0.189)	-0.0654 **a** (0.093)	-0.287 **ab** (0.232)	-0.614 **b** (0.359)	-0.266 **ab** (0.269)	-0.651 **b** (0.518)
PRE	0.0291 **ab** (0.134)	-0.110 **b** (0.186)	-0.235 **b** (0.247)	-0.345 **b** (0.325)	0.332 **a** (0.277)	0.0157 **ab** (0.257)
NRE	0.576 **a** (0.023)	0.300 **d** (0.064)	0.291 **d** (0.045)	0.502 **ab** (0.073)	0.373 **bcd** (0.073)	0.464 **c** (0.083)

Table 4.5 (continued)

	df	MgRE	KRE	CaRE	MnRE	FeRE	NiRE	AlRE	CrRE	PRE	NRE
GS	2	0.155	**0.009**	**0.002**	**0.013**	**0.006**	0.131	**0.023**	**0.002**	0.152	**0.035**
Species	3	**0.001**	**0.004**	**<0.001**	**0.001**	**<0.001**	0.093	**0.011**	0.071	**0.026**	**<0.001**
GSxSpecies	6	0.065	0.208	**0.027**	0.299	0.075	**0.034**	0.193	0.653	**<0.001**	**<0.001**

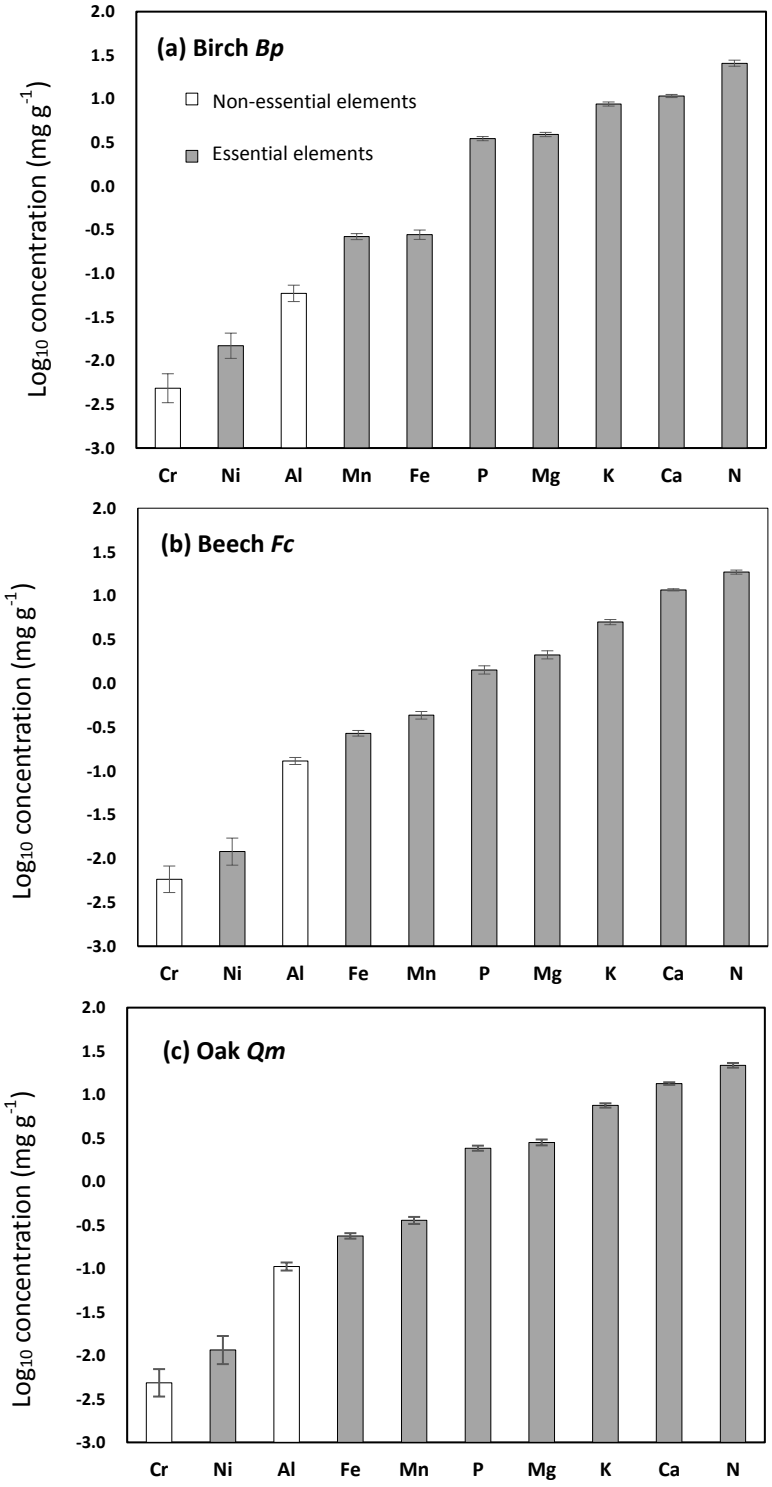


Figure 4.1 Average foliar contents of 10 elements in (a) birch *Bp* (b) beech *Fc* and (c) oak *Qm* species. Black columns indicate the essential elements, while white columns indicate the non-essential elements.

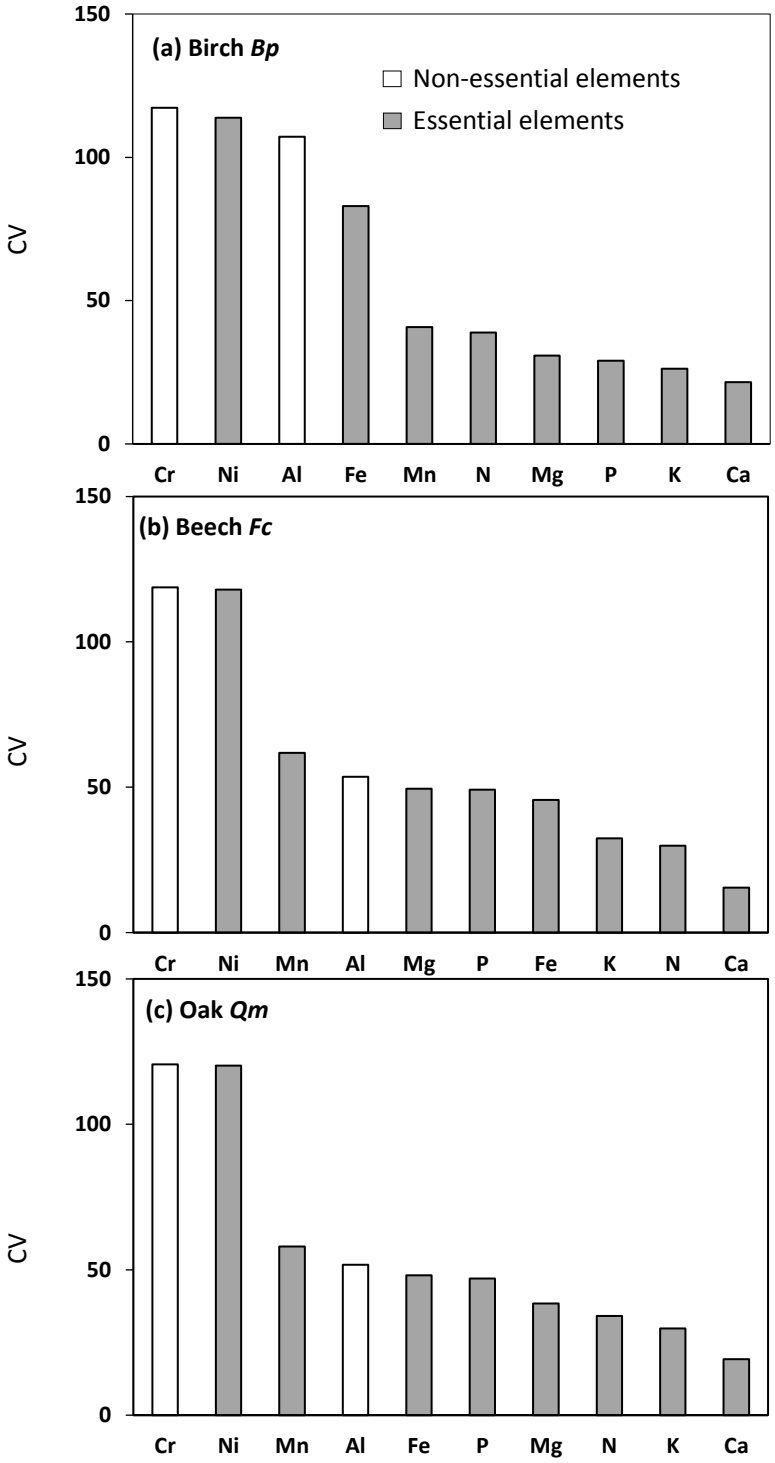


Figure 4.2 Relationship between average foliar contents and their coefficients of variation (CV) in (a) birch *Bp* (b) beech *Fc* and (c) oak *Qm* species. CV is the ratio of standard deviation values to average values of all seedlings for each species. Black columns indicate the essential elements, while white columns indicate the non-essential elements.

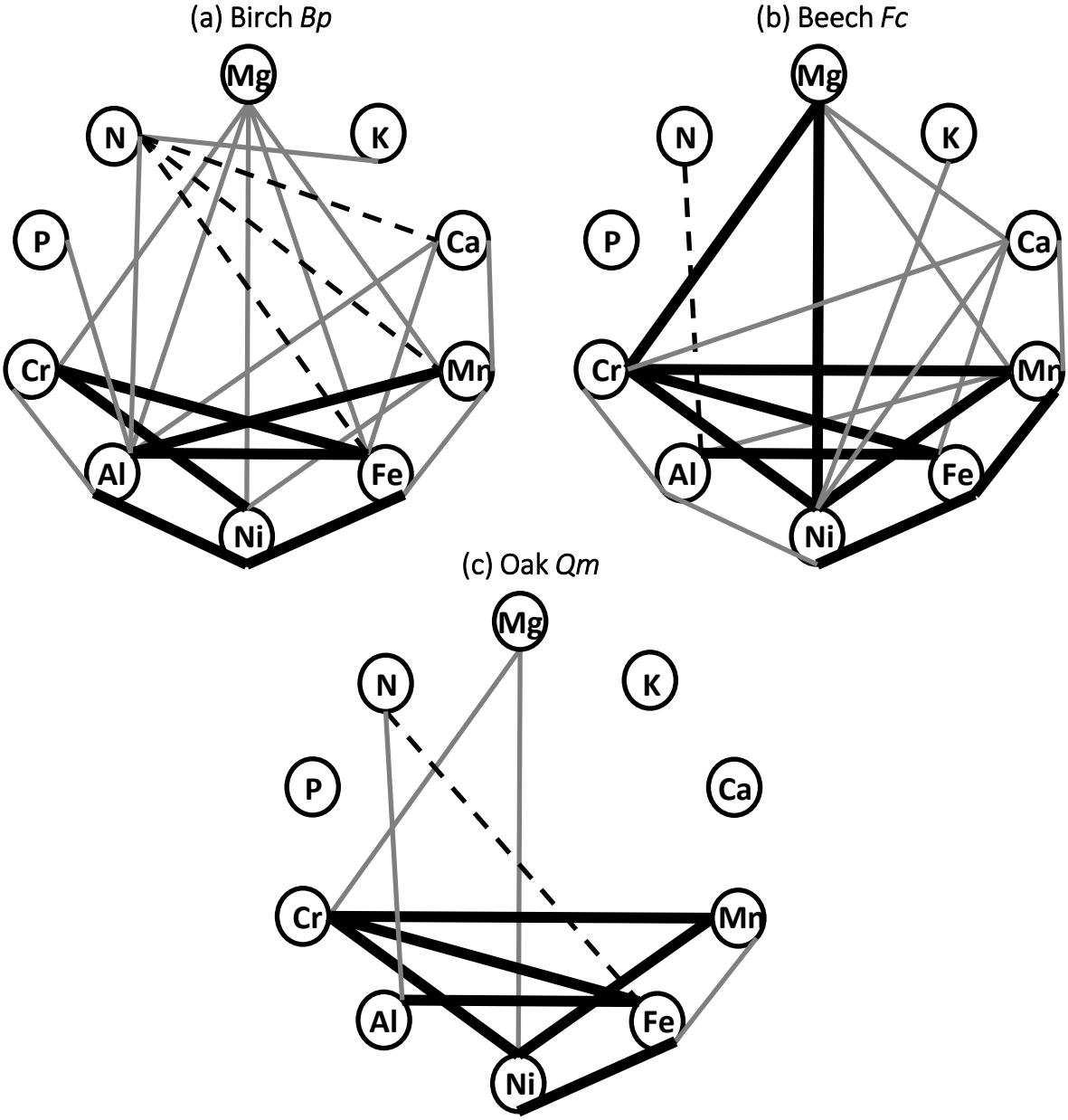


Figure 4.3 Correlations wheels showing significant correlations between foliar elements of (a) birch *Bp* (b) beech *Fc* and (c) oak *Qm* seedlings measured during two growing seasons. Correlations were derived from Pearson r correlation analysis and only significant correlation with $p < 0.001$ and $r > 0.5$ are illustrated on each wheel. Thin gray lines denote when $0.5 < r < 0.7$, and thick black lines denote $r \geq 0.7$. Solid lines indicate positive correlations, and dashed lines indicate negative correlations.

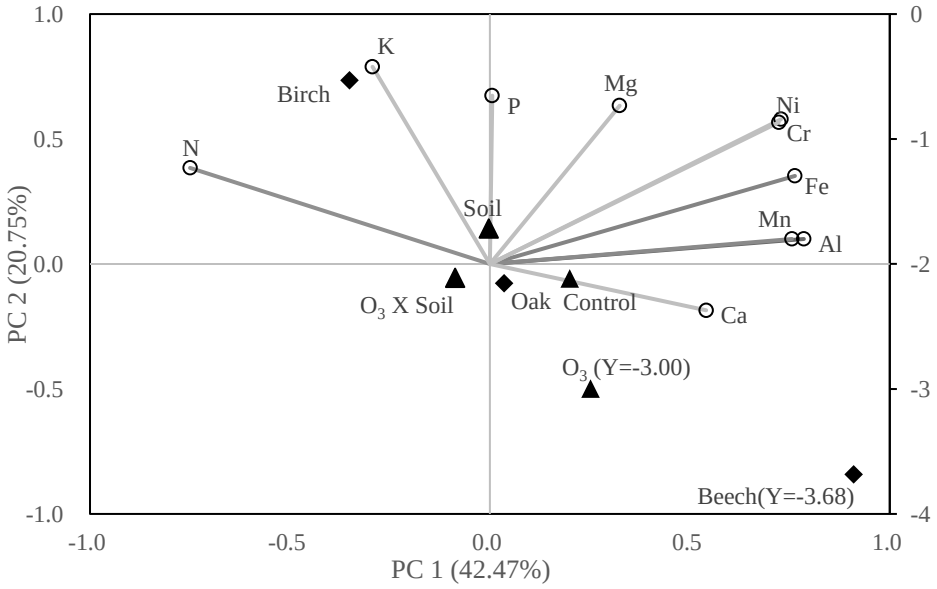


Figure 4.4 Biplot of the PCA results for foliar elements. "Control" indicates the Ambient O_3 with brown forest soil condition. Circular dot points stand for the scores of each response variable; triangle dot points stand for the scores of the treatments of O_3 and/or soil; rhombus dot points stand for the scores of species. (Y indicates the scores loading on axis of PC2, based on the scale in right side)

Chapter 5: Leaf defense characteristics of deciduous tree species seedlings in different soils exposed to a free-air ozne enrichment system

5.1 Introduction

5.1.1 Background

Unlike living organisms, plants are incapable to escape from the unfavorable surrounded environment so they have several defense mechanisms against biotic or abiotic stresses and would like to make appropriate bio-physiological response positively or negatively, to achieve the purpose of survival in hostile environment. Therefore, plant defense is a vital mechanism for biological conservation and improvement of which is essential on forest maintenance and biological diversification (Ohgushi et al., 2007; Kagata et al., 2005; Nakamura et al., 2005; Aoyama et al., 2010).

Plant growth is closely related to an integration of leaf gas exchange and leaf areal dynamics through growing season. Then how can plants keep leaf area through growing season in temperate regions within limited growth period (e.g. Koike 1990)? Leaf area is usually reduced with grazing insect herbivores, which is strongly influenced by environmental conditions, such as elevated ozone (O_3) and carbon dioxide (e.g. Lindroth 2010; Goodger and Woodrow 2014). In general, most plant defenses are originated from photosynthates, therefore they are usually strongly influenced by atmospheric condition as well as soil fertility (e.g. Oksanen 2003; Koike et al. 2006b; 2015；Lindroth 2010).

The defense mechanism of plants against herbivores is composed of physical, chemical, phonological, and other defensive strategies (Karban and Baldwin, 1997; Novriyanti et al., 2010). For most of the woody species, plants defense is complex and consists many characteristics (e.g. Haukioja, 2003). Foliage parts of plants defend themselves against pests or herbivores by employing mainly chemical and physical defense mechanisms (Stamp, 2003; Matsuki et al., 2004). Physical defense is usually on basis of morphological defense, which is possibly in category of both constitutive resistance and inducible defense (Rautio et al., 2002). Leaf physical defense which involves leaf toughness, leaf mass per area (LMA) and some other traits, making leaves become tougher, harder and better equipped with hair or sticky substances (e.g.

Haukioja, 2003; Koike et al., 2006b). Matsuki et al. (2004) indicated that leaf toughness tended to be greater in younger leaves which the optimal defense hypothesis (ODH) is in operation (Riipi et al., 2002; Stamp, 2003), suggesting that defense is favored allocated to more valuable parts. Whereas, leaf chemical defense of woody plants primarily relates the production of carbon-based chemicals to make leaves become harsh flavor, bad taste and even poisonous (Schoonhoven et al. 1998; Koike et al. 2003; Aoyama et al., 2010).

According to the growth differentiation balance hypothesis (GDBH), plants with higher growth rates have less defense chemicals (Herms and Mattson, 1992). The trade-off between growth processes (e.g. lignin synthesis) and production of secondary metabolites (e.g. total phenolics and condensed tannin) has ecological consequences that affected resources allocation since plants have to set up defenses effectively when they are grazed by herbivores to obtain sufficient growth and reproduction for survival (Schoonhoven et al. 1998; Herms and Mattson, 1992; Novriyanti et al., 2012).

There are several plant defense theories in addition to ODH and GDBH that can explain the certain behavior of plants with certain nutrients and resources (Novriyanti et al., 2012). The carbon-nutrient balance hypothesis (CNBH), namely the environmental constraint hypothesis, which proposed by Bryant et al. (1983) explains the effects of soil nutrients on plant defensive chemicals via the allocation of carbon-nutrient in plants. It is confirmed that the plants grown under unfertilized soil condition have higher level of carbon-based defense mechanism (Stamp, 2003) since sufficient nutrient soil condition can stimulate the plant growth and make it develop more leaves to compensate the loss of leaves grazed by herbivores. The individual defensive ability of the plant will be hence decreased (e.g. Aoyama et al., 2010). Gleadow et al. (1998) and Koike et al. (2006b) pointed out that nitrogen (N) deposition in soils are able to alter the foliar defense characteristics. Because the nutrient availability in soil is altered by N deposition and it may also change the defensive strategy of plants (Galloway et al., 2004).

Therefore, brown forest soil and volcanic ash soil were utilized as different soil fertility conditions in this study. Brown forest soil known as mostly Dystric Cambisols, is a common soil type and can be regarded as a fertile soil containing necessary soil nutrients for growth of deciduous broad-leaved forest trees (Kayama et al., 2011; Kim et al., 2011; Shi et al., 2017). Meanwhile, volcanic ash soil (Vitric Andosols) widely distributed in northern Japan, has been known to highly accumulation in organic carbon

due to the stabilization by aluminum (Al), iron (Fe) and sulfur (S) but low contents in several essential elements such as nitrogen (N), potassium (K), magnesium (Mg), calcium (Ca) and manganese (Mn) in the soil (Watanabe et al., 2010; Kayama et al., 2011; Hashimoto et al., 2012).

Additionally, changes in atmospheric environment should also be considered. The tropospheric ozone (O_3) concentration is continuously to increase in northeastern Asia and is expected to affect forest vigor and the ecosystems negatively (e.g. Di Baccio et al., 2008; Koike et al., 2013; Shi et al., 2016a). Several studies have reported that elevated O_3 (hereafter: eO_3) likely changes the physical and chemical defense characteristics of leaves (Kangasjärvi et al., 1994; Watanabe et al., 2005; Goodger and Woodrow, 2014) and consequently effects on productivity of tree species. For instance, foliar secondary metabolite (e.g. phenolic compounds) concentration was increased by eO_3 (Watanabe et al., 2005; Di Baccio et al., 2008); stomatal sluggishness acts to exclude O_3 (Hoshika et al., 2012b) from leaves may be more effective on defense functions within a leaf in reducing damage by O_3 (Padu et al., 2005; Peltonen et al., 2005; Kawaguchi et al., 2012); O_3-induced depression of photosynthesis and growth rate in Europe (e.g. Matyssek et al., 1993, 2012) and in Asia (Koike et al., 2012) may hence result in the changes of defense function based on the growth rate hypothesis (GRH) (Stamp, 2003).

Species-specific and genetic variation has been found in leaf defense to protect against various herbivores (Coley et al., 1985; Matsuki and Koike, 2006; Rousi et al., 2006; Novriyanti et al., 2010). Matsuki and Koike (2006) indicated that trees have shorter leaf lifespan may have higher photosynthetic rate but have less defense capacity and consequence of more susceptible to herbivore attack. Given the two leaf types (early and late) in birch as it is a typical heterophyllous species (e.g. Clausen and Kozlowski, 1965; Matsuki et al. 2004), late leaf (new leaf) in birch should be considered as resistant to insect herbivores.

In this study, as the representative deciduous tree species in northern Japan, Japanese white birch (hereafter *Bp*: *Betula platyphylla* var. *japonica* Hara), Siebold's beech (hereafter *Fc*: *Fagus crenata* Blume), and Mizunara oak (hereafter *Qm*: *Quercus mongolica* var. *crispula* Ohashi) were selected due to the essential roles they played in cool temperate forests, providing social, economic and environmental benefits (e.g. Koike, 1995; 2013; Shi et al., 2016a, 2017).

Plant defense characteristics are various with different successional traits. Given

Bp, Fc and *Qm* as early, mid and late successional species, respectively (Koike 1988), birch as an early-successional heterophyllous species that has a shorter leaf lifespan (Coley, 1988; Matsuki and Koike, 2006) with the indeterminate shoot growth, is expected as susceptible to herbivores in this study. According to the previous study reported by Kohno et al. (2005) and Yamaguchi et al. (2011), *Qm* has relatively higher capacity on O_3 stress, and followed by *Bp*, *Fc* (the most susceptible to O_3) in sequence.

Thus the variations in sensitivity to O_3 for different species should also be taken into account, because defensive availability depends on the strong influence of both species genotype and environmental conditions (e.g. Novriyanti et al., 2010).

Currently, although there are plenty of researches and studies about plant defense traits, lots of which focus on CO_2 as it is an important greenhouse gas in the world, and discuss only within one species or one family species (e.g. Nabeshima et al., 2001; Zvereva and Kozlov, 2006; Stiling and Cornelissen, 2007; Aoyama et al., 2010; Novriyanti et al., 2012). As predicted by Sitch et al. (2007), gross photosynthesis will be suppressed about 30 % in East Asia. What about the leaf defense characteristics of *Bp*, *Fc* and *Qm* in different soils under eO_3 exposure? At eO_3, photosynthetic capacity is usually suppressed and consequently defense level of leaves would be lower because most of defense chemicals are secondary metabolites originated from photosynthates (Matyssek et al., 2012). The inter-relation of genetic basis for defensive chemistry and environmental conditions (such as elevated O_3 under volcanic ash soil) is still unclear.

5.1.2 Hypothesis and objectives

In this study, I investigated the leaf defense traits by analyzing defensive indexes (LMA, N, C/N and secondary metabolites production such as total phenolics, condensed tannin, and lignin) in response of eO_3 under different soils (brown forest and volcanic ash soil) for *Bp*, *Fc* and *Qm* species. I expected that low soil nutrients availability and eO_3 that may give rise to a higher level of defense by altering the C/N ratio and amount of defensive compounds.

Besides, I also made comparisons among the three species and expected that *Qm* may have higher level of defense due to the genetic basis for tolerance (Shi et al. 2016b). My results will not only be of help to keep active on health of mixed broad-leaved forests, but also will provide a plausible understanding on the role of soil nutrients accompanied by eO_3, inducing the changes of defensive chemicals in *Bp*, *Fc* and *Qm* species.

5.2 Materials and methods

5.2.1 Plant material and study site

Two-year-old seedlings (as of 2014) of Japanese white birch (*Bp*: *Betula platyphylla* var. *japonica*), Siebold's beech (*Fc*: *Fagus crenata*), and Mizunara oak (*Qm*: *Quercus mongolica* var. *crispula*) were obtained from Hokkaido Holti-Green Co. Ltd. near Sapporo and were planted directly in the field at the nursery of Sapporo Experimental Forest, Hokkaido University, Japan (43°04' N, 141°20'E, 15m a.s.l.) under full sun.

Seedlings of each species were exposed to an experimental enhanced O_3 circular plot (6.5 m in diameter and 5.2 m in height) with a free-air enrichment system and in each plot planted with two soil types: brown forest soil and volcanic ash soil. Brown forest soil (hereafter: B soil) is a native soil type to the nursery, while volcanic ash soil (hereafter: V soil) was taken from the Tomakomai Experimental Forest located in the vicinity of Mt. Tarumae in Hokkaido (Kim et al., 2011). Seedlings were planted randomly over each treatment area and were grown in field naturally without artificial irrigation or fertilization. The experiment conducted in this study was started in the 3rd year (in 2016) from the seedlings being planted (in 2014).

This study was processed within one growing season in 2016 and the free-air O_3 enrichment system was running during the snow-free period from mid-May until early-December. The annual average temperature and total precipitation in 2016 in Sapporo were 7.4 ℃ and 1161 mm, respectively.

The experimental site consists of three plots each for ambient (A) and eO_3 (E). The distance between ambient and eO_3 was over 20m. The details about O_3 exposure as well as design of those circular plots for free-air enrichment system in the field are available in Watanabe et al. (2013) and Shi et al. (2017). Ambient plots were proposed to contain about 26-34 nm mol^{-1} of O_3 concentration during about 7 hr daylight time, meanwhile the elevated ones were subjected to 73-77 nm mol^{-1}.

When taken together, there were 4 combination treatments in the experimental site for each species (*Bp*, *Fc* and *Qm*): ambient x B soil (AB), eO_3 x B soil (EB), ambient x V soil (AV), and eO_3 x V soil (EV).

5.2.2 Samplings and measurements

As a typical heterophyllous species, birch *Bp* has two types of leaves: early leaves that flushed at end of April, and late leaves that flushed at early June and July (Koike 1995; Hoshika et al., 2013b). Whereas, *Fc* and *Qm* have almost the same leaf age due to simultaneous unfold leaves. In total 6 times of leaf samples collection were conducted as the different development stages of leaf growth in order to assess the leaf defense characteristics at different stages. After almost two-year O_3 exposure (from early-August in 2014, except the snow period), the 1st sampling targeted for the chemical defense research was carried out in late-May 2016 when the female adults of leaf beetles were grazing and *Bp* at this time only had early leaves with developing late leaves.

The 2nd sampling was carried out in early-July for *Bp* when larvae stared to eat on leaf surface, because both early leaves and late (mature) leaves were necessarily analyzed due to the heterophyllous of *Bp* (Coley, 1988; Koike, 2004). Until October, leaf sampling was continuously conducted every month in *Bp*, while samplings of both *Fc* and *Qm* were carried out every other month. In other words, *Bp* leaves were collected from late-May, early-July, early-August, late-August, late-September until late-October. Leaves sampled in May were classified as early leaves flushed in April, whereas leaves sampled after July were classified as late leaves. *Fc* and *Qm* leaves were collected at the same time every other month from late-May, early-August until late-September. All the leaves were taken from 3 seedlings with similar appearances (including size, shaper, color and so on) for each species at each treatment with 9-12 replications. Afterwards, I divided all the collected leaf samples into 3 sections for different measurements with 3-4 replications for each measurement.

Leaf samples in the 1st section were for measurements of physical defenses index (e.g. LMA, mg/m^2; Koike et al. 2006b). Leaf samples were dried at 70℃ for 7-10 days after rising the surface with distilled water. After measurements of the leaf dry mass and leaf area, they were milled grounded into powder in order to further analysis of foliar N, C contents via combustion method with a NC analyzer (NC-900, Sumica, Osaka, Japan).

For the rest measurements, the collected leaf samples were immediately freeze-dried (FLEX-DRY, FTS Systems, Stony Ridge NY, USA) for 24 h and then were milled grounded into powder for preparation of further analyses. Leaf samples in the 2nd

section were utilized for determination of defense chemicals (condensed tannins and total phenolics) with the Folin-Ciocalten method and proanthocyanidin method (Bate-Smith, 1977; Julkunen-Tiito, 1985). The detailed of analysis method of condensed tannins and total phenolics is summarized step by step in the Appendix 1. Moreover, leaf samples in the 3rd section were applied for lignin determination by the Acetyl Bromide Method (Dence, 1992; Moreira-Vilar et al., 2014). The detailed procedures of analysis of lignin I utilized are summarized step by step in the Appendix 2.

5.2.3 Statistical analysis

Kolmogorov-Smirnov test was applied to examine the data normality distribution in this study. The effects of O_3 and soil types on foliar traits and defensive chemicals (including LMA, N content, C/N ratio, condensed tannins, total phenolics, and lignin) were tested by two-way analysis of variance (ANOVA) and two-way repeated-measures ANOVA was applied when variable of harvest time (or leaf type) was involved. To examine the comparisons of parameters among the 4 different treatments within one species as well as among the 3 different species, general linear model (GLM) analysis or post hoc Tukey's hsd test was conducted. The different letters marked in the tables and figures are based on the statistical differences at $P < 0.05$. In order to test the seasonal changes of leaf traits and defensive chemical contents, one-way repeated-measures ANOVA was utilized for each species. All the statistical analyses were conducted by MS EXCEL 2010 and SPSS 21.0 (IBM, NY, USA).

5.3 Results

5.3.1 Effects of O_3 and soil on leaf traits and defensive chemicals within each species

The effects of O_3 and soil in 3 collection times of *Bp* species were presented in Table 5.1, foliar LMA, N, C/N, and condensed tannins (hereafter: ConTan) in *Bp* were observed to be significantly affected by the interaction of leaf type (Leaf) x O_3. Among them, LMA and C/N were considerably affected by leaf types. LMA was markedly enhanced in late leaves that harvest after August (late-2 leaves) at ambient O_3 under both soils, while C/N was significantly increased in both late leaves. Whereas foliar N content was significantly reduced in late leaves that harvest in early July (late-1 leaves).

Physical defensive parameters LMA and C/N as well as a defensive chemical total phenolics (hereafter: ToPhe) were highly influenced by leaf types of *Bp*. Significant differences of interaction of Leaf x O_3 x Soil were found in ConTan and ToPhe. No significant difference was observed in leaf lignin for *Bp*.

Based on the results of *Fc* in Table 5.2, all the projected leaf traits parameters were considerably affected by collection times (Time). Physical defense parameter LMA was strongly influenced by Time in every treatment. In addition, C/N was also found to be easily affected by both O_3 and soil condition. N was influenced by soil condition as well as the interaction of O_3 x Soil. The defensive chemicals including ConTan, ToPhe and lignin were susceptible to O_3 since we found significantly higher values of those at eO_3 under both soils.

It can be seen from the data in Table 5.3 that almost all leaf traits factors in *Qm* except lignin had significant differences with Time. Both LMA and ConTan were significantly influenced by soil conditions as significantly higher LMA and lower ConTan by late-Sept. were observed under V soil for eO_3 and ambient, respectively. Besides，ConTan was apparent affected by interaction of Time x O_3.

5.3.2 Comparisons of leaf traits and defensive chemicals among the different species

Figure 5.1 illustrates the main characteristics of leaf traits and defensive chemicals among the *Bp*, *Fc* and *Qm*. The results shown in Figure 5.1 was quite revealing in three general trends. No significant differences in LMA and foliar lignin contents were detected among the three different species (Figure 5.1A and 5.1F). Foliar N contents in beech *Fc* species (Figure 5.1B) and ConTan concentration in *Qm* leaves (Figure 5.1D) were identified to be significantly lower. The contrastive results that foliar C/N ratio in beech *Fc* species (Figure 5.1C) as well as ToPhe contents in *Qm* leaves (Figure 5.1E) were detected. There were considerably higher than that in rest two species.

Closer inspection of the characteristics of leaf defensive indexes influenced by O_3 and soil among species, as shown in Figure 5.2. This indicated that foliar N (Figure 5.2B) seemed to be affected by O_3 and soil in *Bp* and soil independently in *Fc*. Moreover, foliar C/N was detected to be significantly affected by O_3 in *Bp* and by soil in *Fc.* Contents of ConTan (Figure 5.2C) in *Bp* leaves as well as ToPhe and lignin contents in *Fc* leaves were found significantly influenced by O_3 as shown in Figure 5.2D-F. No

differences were statistically significant in LMA (Figure 5.2A) as well as for the effects of O_3 x Soil on the projected defensive characteristics among these three species.

5.3.3 Seasonal changes of leaf traits and defensive chemicals in birch, beech and oak

Seasonal changes of those leaf traits and defensive chemicals in each species were compared using a repeated-measure ANOVA and results were presented in Figure 5.3. An overview of the figure, most of the defensive characteristics had significantly differences in all the studies species except the foliar contents of lignin in *Qm* species. For *Bp* leaves, it seems to need about 3-4 months to fully expand after early flushing in late-April as the maximum peak values appeared in August (either early or late) for LMA, C/N, ConTan, and phenols in *Bp* species. Apparently, N contents are negatively correlated with C/N so they have opposite tendency with each other. Furthermore, C/N and ToPhe in *Qm* leaves as well as ConTan in *Fc* leaves were found to have a similar trend as themselves in *Bp* leaves. There has been a gradual increase until peaked in about August and then is projected to decline (can either sharply or steadily) after fully expand. Lignin content in *Fc* leaves also resemble the above tendency as shown in Figure 5.3F.

In Figure 5.3, there were also clear trends of increasing LMA and ConTan in *Qm* leaves (Figure 5.3A and D) as well as increasing C/N and ToPhe in *Fc* leaves (Figure 5.3C and E) over the growing season. Foliar N as it is opposite to C/N, had gradually declined with the process of seasonal changes.

5.4 Discussion

5.4.1 Physical leaf defense characteristics

In general, LMA is known to be positively related to leaf toughness and often studied as an index of physical defense for leaf trait analysis in leaf morphology (Reich et al., 1991; Koike et al. 2006a). LMA usually is decreased with the natural leaf senescence due to the decline of foliar dry mass and the reduction of the nutrient in senescing leaves (Koike, 1990; Hayashi et al., 2005). But one unanticipated finding was that LMA was considerably higher at late leaves of *Bp* (Table 5.1) or latter collected leaves (Table 5.2 and 5.3) under each treatment and it was increased over the growing

season for all three species (Figure 5.3A). Since foliar N was significantly reduced with seasonal changes (Figure 5.3B), the increased LMA is likely due to environmental changes (e.g. eO_3) that induced modification in leaf traits, resulting in a smaller leaf size (Paoletti, 2007).

In our study shown in Figure 5.2A, LMA of *Bp* and *Qm* may be credited for the insignificant effect of eO_3, as we found LMA in *Bp* tended to decrease at eO_3 whereas in *Qm* tended to increase, which indicated that physical defense of leaf (indexed by LMA) was influenced by eO_3. But it is not always the case, as demonstrated previously by Kitao et al. (2009), eO_3 had no effects on leaf morphology.

In addition, LMA of all three species may also be credited for insignificant effect of soil as LMA tended to increase under V soil for all species, suggesting that physical defense was increased with infertile soil conditions. Another possibility of the higher LMA is that decline of the defensive mechanism with seasonal changes may increase the herbivores grazing chances and hence reduce the leaf area and enhance the LMA values. Moreover, ConTan also contribute to leaf toughness (Scalbert and Haslam, 1987), the rise of which decreased the insect growth rate by inhibition of the digestion in insects (Schoonhoven et al., 1998).

Considering the similar differences with LMA we found in ConTan for *Fc* and *Qm* species in Table 5.1, Table 5.2 as well as similar seasonal tendency for *Bp* and *Qm* in Figure 5.3D. It seems that increased ConTan matured the leaves faster and hence toughen the leaves more quickly (Dury et al., 1998) and induced an enhancement on LMA values, eventually increased leaf physical defensive characteristics.

5.4.2 Defensive chemicals in the leaf

5.4.2.1 Birch

As an essential macro-nutrient, N is the major component of various proteins and enzymes that most frequently limits plant growth and energy reserves (Marschner, 2012). Prior studies such as Bryant et al. (1983) and Koike et al (2003; 2006b) have noted the importance of the availability of N and C contents (CNBH), which affects the plant foliar chemistry and in turn would determine the plant defense status.

In *Bp*, foliar nutrients and C/N were significantly affected by eO_3 (Shi et al. 2017). There was also apparent from Figure 5.2B that lower leaf N under V soil at both O_3 treatments. This suggests that adversely environmental changes (eO_3 and V soil in this

study) did hamper the plant growth and decrease the foliar N contents and correspondingly increase the C/N. Retarding the growth by environmental stresses make N absorption will be not assigned more on plant growth but shifted to other allocation such as defense system for instead (Bryant et al., 1983; Hamilton et al., 2001; Novriyanti et al., 2012).

However, it was somewhat unexpected that no soil differences were noted in C/N (Figure 5.2C) though it was noted in N (Figure 5.2B). This may be explained by the fact that the adversely effects of infertile soil was likely able to alter the N contents but was not as significant as eO_3 did to synthesis carbon production to adjust C/N ratio and change defense characteristics for *Bp*. Furthermore, we observed a decrease of N content in late-1 leaves (early-July) at AB but an increase of C/N in both late leaves of *Bp* at ambient under both soils (Table 5.1). This observation could be attributed to the less susceptibility of early leaves to eO_3 as reported by Hoshika et al. (2013b). Besides late leaves, which were indeed younger new leaves, were able to somewhat offset reduced carbon gain in early leaves influenced by eO_3 (Watanabe et al., 2010; Hoshika et al., 2013b).

Acting as a broad spectrum defensive mechanism against herbivores, leaf phenology (total phenolics includes condensed tannins) refers to chemical defense are seasonally increased and responsible for the concentration of herbivory in the spring according to the review of an extensive literatures (e.g. Feeny, 1970; Bryant et al., 1983; Forkner et al., 2004). Seasonal changes of both ConTan and ToPhe in birch *Bp* raised to a peak around mid-summer, then declined in early-autumn and began to rise again in *Bp* (Figure 5.3D, E). This suggests that both ToPhe and ConTan in *Bp* leaves appeared to be suppressed in defoliation. Besides, foliar ConTan and ToPhe were much more by last harvest time, suggesting that leaves were more effective in precipitating on that time and may became less digestible to herbivores (Schultz and Baldwin, 1982).

Although ConTan were significantly decreased by eO_3 under both soil conditions in *Bp* (Figure 5.2D), no significant differences were found between early and late leaves (Table 5.1). This outcome was contrary to that of Sakikawa et al. (2016) who reported that CoTan in early leaves of birch saplings was higher in eO_3. Since the interaction effects Leaf x O_3 x Soil were tested by repeated-measure ANOVA in Table 5.1, infertile soils as well as natural defoliation process induced inhibited accumulation of condensed tannins in *Bp* may offset the increased effects by eO_3. Similar explanation may also apply to the non-significant effects on total phenolics in *Bp* (Table 5.1).

Nonetheless, ConTan was considerably declined by eO_3 while ToPhe insignificantly tended to decline at eO_3 under V soil in *Bp* (Figure 5.2D，2E). This may indicate that the increased nutrient absorption which induced the C/N decreased at eO_3 (Figure 5.2B, C), resulting in the decreased of secondary metabolites (ConTan and ToPhe) in *Bp*. Because secondary metabolites were supposed to be reduced due to the resource allocation is usually priority for plant growth (Herms and Mattson, 1992). In regard to the decreased ConTan by eO_3, eO_3 may also reduce the N availability for production of ConTan since eO_3 diminished plant growth. Moreover, since LMA of *Bp* were also reduced by eO_3 although insignificantly (Figure 5.2A), the secondary metabolites were reduced more than foliar biomass accumulation (Koricheva, 1999; Novriyanti et al., 2012) according to the GDBH.

Lignin is a complex heteropolymer that provides a coating for lignified issues and protect the energetic products of plants from pathogens and herbivores (Moreira-Vilar et al., 2014). As a large molecular weight compounds, lignin is considered as an immobile defense that is metabolically inactive (e.g. Coley, 1988). These properties make lignin have large costs on initial construction but cannot be retrieved upon leaf senescence (Coley et al., 1985; Schoonhoven et al. 1998). Therefore, lignin contents in leaves are usually positively correlated with the leaf lifetime and lignin is common in longer-lived leaves. As a result, fast-growing species with short lived leaves are less defended and would be attached more heavily by herbivores as we expected.

Despite the fact that it did not appear to be the case in our results in both *Bp* and *Fc*. Lignin in both *Bp* and *Fc* were showed a bit complex dynamics with values higher than the initial ones throughout the growing season in our results (Figure 5.3F). This result may be explained by the fact that lignin is a kind of end-product of an available resources in plants (Herms and Mattson, 1992; Novriyanti et al., 2012). Compared to ConTan which is also a kind of immobile defense (Herms and Mattson, 1992), lignin contents reached the highest peak earlier than the ConTan for *Bp* and kept the higher level until ConTan reached the peak (Figure 5.3D, F). Lignin content in *Bp* species was significantly increased by eO_3 as presented in Figure 5.2F, this may be related to the highly sensitivity of *Bp* to eO_3 (Yamaguchi et al., 2011).

In addition that birch is fast-growing species which is less defended; stimulation from eO_3 may accelerate the production of lignin in *Bp* species in order to defense the plants from eO_3 as a sort of self-protection for plants. However, this eO_3-induced temperately strong defense would not last long until other defensive chemicals catch

up. Eventually, in birch species, all the defensive chemicals, including condensed tannins, ToPhe as well as lignin would achieve a relative high content level (Figure 5.3D, E, F) to protect *Bp* seedlings by keeping in a strong defense level in order to make the least loss by herbivory before leaf senescence.

5.4.2.2 Beech

Foliar N was significantly lower (Figure 5.1B) and C/N was correspondingly higher (Figure 5.1C) in beech *Fc* than the other two species. According to combined results in Figure 5.2B and Table 5.2, foliar N in *Fc* which was significantly increased under V soil, having showed a negative dependency to soil nutrients condition. In contrast, C/N was positive dependency to soil nutrients for younger leaves that harvested in late September. I believe that higher N and lower C/N in *Fc* observed under V soil were likely related to higher retranslocation rate of N under infertility soil (Shi et al., 2016a). Because N retranslocated from older leaves to younger leaves more efficiently in order to increase the defensive capacity against the adverse surroundings.

Regulation by naturally process of leaf senescence, foliar N was gradually decreased and C/N was accordingly increased over the seasonal changes in both beech Fc and oak Qm species (Figure 5.3B, C). On the other hand, as C/N was tested significant different between the treatment of AV and EB plus eO_3 showed considerably effects based on ANOVA, the eO_3 effects on the carbon allocation as well as the related C/N should also be taken into account (Table 5.2).

There were clear differences exist in ConTan and ToPhe among different harvest times in *Fc* (Table 5.2). Although LMA in *Fc* was increased over the harvest time and growing season (Table 5.2, Figure 5.3A), chemical defense (ConTan and ToPhe) in *Fc* leaves rose to a peak around mid-summer for those young leaves harvest in early-Aug and then declined in early autumn for those younger leaves harvest in late September according the data in both Table 5.2 and seasonal changes in Figure 5.3D and E.

Our results were consistent with several previous researches such as Rousi et al. (1996), Riipi et al. (2002), and Matsuki et al. (2004). However, these data indicated a rejection of GDBH, suggesting that chemical defensive characteristics are not necessary always rely on the growth rate (Mooney and Gulmon, 1982) and plants would have higher growth rate in absence of defense due to the trade-off between growth and defense occurred on those plants with high growth rate and defense system (Matsuki et al., 2004).

In contrast to earlier findings in birch Bp species, however, ToPhe were tested to be significantly increased by eO_3 in *Fc* (Figure 5.2E), meanwhile ConTan insignificantly increased by eO_3 as well (Figure 5.2D). One possible explanation was that the differentiation between *Bp* and *Fc* were likely concern with their sensitivity to eO_3 and the defensive chemicals perhaps were less susceptible in the species which is more tolerant to eO_3. The other possibility was that those secondary metabolites in *Fc* species were sort of resource allocation that shifted from O_3-induced diminished growth and the adverse environment was able to reduce the cost of defensive chemicals. Because plants prefer to increase the resources availability for producing more secondary metabolites with little trade-off growth in order to increase the defense characteristics (Herms and Mattson, 1992; Novriyanti et al., 2012).

Moreover, I tested that lignin tended to increase with eO_3 in *Fc* (Figure 5.2F) and it was significantly different between leaves harvested in late-May and in early-Aug (Table 5.2). As growth and lignin usually have negatively correlations with each other (Novaes et al., 2010), the increase of lignin by eO_3 may attribute to the low growth since high C/N was observed in early-Aug in Table 5.2. Although C/N was affected by N nutrients in soils as our data as well as many previous researches reported (e.g. Henry et al., 2005), the increased lignin that correlated with C/N was in response to eO_3. Alternatively, the enhancement of lignin with eO_3 may contribute to the enhanced LMA. Elevated O_3-induced modification on cellular level can increase LMA by damaging to the membrane and impair cell extension (Paoletti, 2007). Thus, eO_3 would trigger plant defense reactions as an abiotic elicitor to induce plant signal molecules and hence stimulated the lignin biosynthesis (Sandermann et al., 1998; Paoletti, 2007).

5.4.2.3 Oak

Seasonal changes of both ConTan and ToPhe constantly kept increasing in oak *Qm* (Figure 5.3D, E). With increased trend of C/N (Figure 5.3C) in oak *Qm*, the level of chemical defense might increase over the growing season, regardless the different investment of the carbon allocation (e.g. Lawler et al., 1997). ConTan were tested significantly lower in *Qm* than that in *Bp* and *Fc* (Figure 5.1D) and it was also considerably reduced by infertile soil for younger *Qm* leaves that harvest in late-Sept (Table 5.3). Nabeshima et al. (2001) reported that the concentration of condensed tannins as an index of induced defense, which was negatively related with herbivore activities.

In other words, plant defense would be increased by increasing the grazing of herbivores and hence more ConTan would be synthesized. Therefore, the reduced ConTan in eO_3 and V soil may attribute to the less grazing of herbivores since the surroundings (e.g. eO_3) may also be adverse for herbivores survival. According to the research by Hoshika et al. (2013b), the activity of younger leaves was suppressed by eO_3 in the same species. Consequently, the reduced ConTan in *Qm* by eO_3 was likely due to the constricted production of defensive chemical that was suppressed by eO_3. Taking LMA as an account that tended higher in eO_3 and V soil (Figure 5.2A), the concentration of ConTan were not diluted by foliar biomass but reduced more than biomass accumulation (Koricheva, 1999; Novriyanti et al., 2012).

Furthermore, in contrary to the foliar contents of ConTan, in our comparison results among different species in Figure 5.3E, *Qm* was observed to have significantly higher values on ToPhe than *Bp* and *Fc* species. This was found to be reduced by eO_3 for younger *Qm* leaves harvest in late-Sept grown under V soils (Table 5.3). The decreased ToPhe possibly attributed to the increased C/N and decreased foliar N in younger leaves (Table 5.3). As younger leaves was more vigor and active to be less susceptible on eO_3 (Hoshika et al., 2013b), ToPhe in younger leaves were reduced probably because the adversely environmental changes such as elevated CO_2 or eO_3 in this study reduced the foliar growth rate and possibly inhibited assimilation process of foliar N according to the study of Bloom et al. (2010). As for ToPhe contents in *Qm* leaves were being affected by eO_3 when grown under V soil, it might be related to poor N in V soils and hence further strengthen the inhibition of nitrate assimilation into available N in leaves.

The results of differences among various treatments on foliar defense traits, however, did not show significant changes for oak seedlings (Figure 5.2). Our data are consistent with many previous researches showing that environmental changes (e.g. eO_3 exposure) did not induce a significant variation on both physical and chemical parameters. For instance, N in Scherzer et al. (1998), C/N in Kim et al. (1998), lignin contents in Parsons et al. (2008), and likewise result in for holm oak leaves in Baldantoni et al. (2010). As *Qm* is a tolerant species among the three tree seedlings (Kohno et al., 2005; Yamaguchi et al., 2011), the resource allocation for plant defense might be species specific (Novriyanti et al., 2012).

5.5 Summary

This study provided several plausible understandings of the influence of either eO_3 or soil conditions on related foliar defensive characteristics against herbivores by analysis of leaf phenology, foliar nutrients (C/N) and defensive chemicals (total phenolics and lignin) in projected three species. The results in this study provide the evidences that foliar defensive characteristics are varied upon environmental constrains and also species specific. It might be highly concerned with the species sensitivity to surroundings changes.

In *Bp*, most of foliar defensive characteristics are affected by eO_3 as *Bp* is relatively sensitive to O_3. In *Fc*, foliar nutrients are influenced by soils due to the highly efficient retranslocation rate as determinate growth plants (Shi et al., 2016a). Whereas defensive chemicals are affected more by eO_3 because eO_3 would trigger plants defense reactions and alter the resource allocation of defense chemicals. In *Qm*, as a tolerant species to eO_3, although no effects were detected among O_3 and soil treatments, foliar phenology influenced the available resources for defense characteristics so that *Qm* is further tolerant and competitive to be survival under various adverse environmental changes. Moreover, for some specific cases, the adverse environmental effects on grazing of herbivores should also be taken in to account.

Table 5.1 Mean (±SD) foliar traits and chemical contents of birch *Bp* grown on two soil types with exposure of ambient (A) or elevated O_3 (E) under different leaf types. Soil types include brown forest (B) soil and volcanic ash (V) soil; Leaf types include early-leaf (Early) that harvested in late-May, late-1 that harvest in early-July, and late-2 that harvest after August (include late-Aug). *P* values are the levels of a two-way repeated-measures ANOVA, with different leaf types (Leaf) as within-subjects variable, for the effects of O_3 concentration (O_3) and soil types (Soil) on the leaf traits and chemical contents in birch species. Comparisons were made within each column by general linear model (GLM) analysis or a post hoc Tukey's hsd test. n=3-15. Different letters indicate statistical differences at $P < 0.05$.

Birch *Bp*	Treatment	LMA (g/m2)	N (mg/g)	C/N	Condensed tannins (mg/g)	Total phenolics (mg/g)	Lignin (%)
Early (late-May)	AB	4.490 c ±0.228	4.024 a ±0.133	10.560 c ±0.307	7.517 a ±2.018	23.913 a ±4.248	5.893 a ±0.130
	AV	4.889 bc ±0.534	3.870 ab ±0.289	11.012 bc ±0.816	6.307 a ±1.564	20.194 a ±1.934	5.583 a ±1.744
	EB	5.246 abc ±0.467	3.837 ab ±0.054	11.158 abc ±0.133	7.060 a ±0.914	25.054 a ±0.607	7.250 a ±1.653
	EV	4.855 bc ±0.620	3.899 ab ±0.183	11.166 abc ±0.439	5.664 a ±1.817	22.137 a ±4.965	7.788 a ±0.786
Late-1 (early-July)	AB	5.812 abc ±0.079	3.248 b ±0.108	13.705 a ±0.442	9.673 a ±0.752	32.732 a ±6.592	10.070 a ±2.203
	AV	5.701 abc ±0.909	3.357 ab ±0.030	13.415 ab ±0.112	9.359 a ±0.468	28.609 a ±1.467	9.652 a ±4.747
	EB	5.314 abc ±0.010	3.837 ab ±0.344	11.868 abc ±1.256	8.320 a ±1.242	24.325 a ±3.302	10.737 a ±3.639
	EV	6.091 abc ±1.170	3.428 ab ±0.242	13.229 ab ±1.006	8.253 a ±1.547	27.231 a ±3.924	7.462 a ±2.107
Late-2 (after August)	AB	6.790 ab ±0.556	3.602 ab ±0.113	12.701 abc ±0.439	9.515 a ±1.101	31.202 a ±4.184	8.923 a ±0.981
	AV	7.101 a ±0.480	3.314 ab ±0.158	13.773 a ±0.532	10.004 a ±0.888	42.033 a ±9.250	7.304 a ±0.338
	EB	5.547 abc ±0.404	3.981 ab ±0.191	11.675 abc ±0.686	7.255 a ±0.759	41.531 a ±5.955	8.057 a ±0.696
	EV	6.710 ab ±0.339	3.498 ab ±0.191	13.172 abc ±0.659	7.572 a ±0.854	31.415 a ±4.112	9.575 a ±1.187

Table 5.1 (continued)

P values	df	LMA	N	C/N	Condensed tannins	Total phenolics	Lignin
O_3	2	0.462	0.382	0.434	0.365	0.623	0.836
Soil	2	0.666	0.359	0.366	0.256	0.179	0.211
O_3 x Soil	4	0.730	0.437	0.530	0.705	0.154	0.919
Leaf	3	**0.001**	0.081	**0.019**	0.058	**0.009**	0.112
Leaf x O_3	6	**0.039**	**0.008**	**0.047**	**0.045**	0.472	0.394
Leaf x Soil	6	0.206	0.255	0.310	0.317	0.61	0.217
Leaf x O_3 x Soil	12	0.502	0.391	0.356	**0.032**	**0.031**	0.584

Table 5.2 Mean (±SD) foliar traits and chemical contents of beech *Fc* grown on two soil types with exposure of ambient (A) or elevated O_3 (E) under different collection times. Soil types include brown forest (B) soil and volcanic ash (V) soil; collection times include late-May, early-Aug, and late-Sept. *P* values are the levels of a two-way repeated-measures ANOVA, with different collection times (Time) as within-subjects variable, for the effects of O_3 concentration (O_3) and soil types (Soil) on the leaf traits and chemical contents in beech species. Comparisons were made within each column by general linear model (GLM) analysis or a post hoc Tukey's hsd test. n=3-9. Different letters indicate statistical differences at $P < 0.05$.

Beech *Fc*	Treatment	LMA (g/m^2)	N (mg/g)	C/N	Condensed tannins (mg/g)	Total phenolics (mg/g)	Lignin (%)
late-May	AB	4.847 c ±0.437	3.141 abc ±0.134	13.195 cd ±0.518	6.424 d ±0.317	29.383 d ±0.664	5.040 c ±0.608
	AV	4.584 c ±0.419	3.437 a ±0.069	12.327 d ±0.301	7.488 abcd ±2.373	32.288 abcd ±3.692	6.083 abc ±0.665
	EB	4.813 c ±0.064	3.275 abc ±0.041	13.152 cd ±0.182	7.782 abcd ±0.231	33.814 abcd ±2.854	8.734 ab ±1.281
	EV	4.991 c ±0.714	3.381 ab ±0.192	12.496 cd ±0.676	6.729 cd ±0.193	32.846 cd ±3.177	8.184 ab ±0.364
early-Aug.	AB	6.782 b ±0.331	2.936 abc ±0.009	14.409 abc ±0.170	12.611 a ±2.801	38.027 a ±3.286	7.216 ab ±0.904
	AV	6.791 b ±0.609	3.270 abc ±0.059	12.914 cd ±0.141	11.950 ab ±1.767	36.176 ab ±3.267	8.000 ab ±0.656
	EB	6.867 b ±0.434	3.043 abc ±0.049	13.988 abcd ±0.269	12.520 a ±2.196	39.004 a ±0.893	9.768 a ±2.226
	EV	7.597 ab ±0.310	3.070 abc ±0.182	14.127 abcd ±0.669	11.702 abc ±1.348	36.115 abc ±0.288	9.428 a ±1.307
late-Sept.	AB	8.195 ab ±0.488	2.964 abc ±0.038	14.517 abc ±0.564	7.128 bcd ±0.515	26.044 bcd ±3.058	6.888 abc ±1.161
	AV	8.691 a ±0.895	3.168 abc ±0.107	13.639 bcd ±0.072	9.335 abcd ±0.236	37.692 abcd ±4.495	6.830 abc ±0.290
	EB	7.993 ab ±0.619	2.785 c ±0.079	15.869 a ±0.222	11.400 abcd ±0.893	48.838 abcd ±0.908	9.108 a ±2.274
	EV	8.096 ab ±0.398	2.845 ab ±0.116	15.442 ab ±0.566	11.113 abcd ±1.984	38.681 abcd ±5.717	10.204 a ±0.851

Table 5.2 (continued)

P values	df	LMA	N	C/N	Condensed tannins	Total phenolics	Lignin
O_3	2	0.903	0.276	**0.037**	**0.036**	**0.039**	**0.046**
Soil	2	0.589	**0.007**	**0.017**	0.914	0.912	0.657
O_3 x Soil	4	0.725	**0.025**	0.147	0.588	0.183	0.517
Time	3	**0.001**	**0.046**	**0.029**	**0.038**	**0.049**	**0.040**
Time x O_3	6	**0.046**	0.345	0.296	0.543	0.507	0.718
Time x Soil	6	0.684	0.716	0.750	0.580	0.437	0.878
Time x O_3 x Soil	12	0.458	0.424	0.299	0.630	0.406	0.257

Table 5.3 Mean (±SD) foliar traits and chemical contents of oak *Qm* grown on two soil types with exposure of ambient (A) or elevated O_3 (E) under different collection times. Soil types include brown forest (B) soil and volcanic ash (V) soil; collection times include late-May, early-Aug, and late-Sept. *P* values are the levels of a two-way repeated-measures ANOVA, with different collection times (Time) as within-subjects variable, for the effects of O_3 concentration (O_3) and soil types (Soil) on the leaf traits and chemical contents in oak species. Comparisons were made within each column by general linear model (GLM) analysis or a post hoc Tukey's hsd test. n=3-9. Different letters indicate statistical differences at $P < 0.05$.

Oak *Qm*	Treatment	LMA(g/m^2)	N(mg/g)	C/N	Condensed tannins (mg/g)	Total phenolics (mg/g)	Lignin (%)
late-May	AB	3.141 d ±0.414	3.919 ab ±0.234	10.320 c ±0.570	0.857 cd ±0.155	30.165 c ±2.835	6.477 a ±0.723
	AV	3.327 d ±0.214	4.116 a ±0.403	10.074 c ±0.952	0.530 d ±0.062	39.037 abc ±1.585	8.073 a ±0.909
	EB	3.302 d ±0.289	4.127 a ±0.306	10.104 c ±0.726	0.800 cd ±0.018	33.947 bc ±1.827	9.018 a ±2.753
	EV	4.193 cd ±0.423	3.864 ab ±0.390	10.816 bc ±0.899	0.936 cd ±0.114	33.244 bc ±0.641	8.991 a ±1.078
early-Aug.	AB	6.018 bcd ±0.104	3.476 ab ±0.108	11.952 abc ±0.308	2.507 bcd ±0.255	60.350 a ±10.249	6.895 a ±0.866
	AV	6.451 bcd ±0.244	3.148 b ±0.163	12.974 ab ±0.633	1.434 bcd ±0.375	55.436 ab ±8.366	6.431 a ±0.715
	EB	6.782 bc ±0.603	3.167 b ±0.050	12.950 ab ±0.267	2.583 bcd ±0.512	53.403 abc ±1.506	6.215 a ±1.077
	EV	6.146 bcd ±1.096	3.300 b ±0.161	12.864 ab ±0.660	1.519 bcd ±0.168	59.450 a ±2.160	7.612 a ±0.757
late-Sept.	AB	8.110 b ±0.406	3.504 ab ±0.257	11.883 abc ±0.461	5.217 a ±1.040	41.009 abc ±0.986	8.775 a ±1.659
	AV	8.454 b ±0.415	3.592 ab ±0.089	11.940 abc ±0.520	3.203 b ±0.041	60.766 a ±19.688	7.549 a ±1.394
	EB	8.889 b ±1.161	3.230 b ±0.202	13.467 a ±0.818	2.876 bc ±0.201	48.357 abc ±7.459	6.936 a ±0.077
	EV	9.804 a ±1.450	3.177 b ±0.195	12.951 ab ±0.754	2.266 bcd ±0.803	31.395 bc ±3.084	7.389 a ±0.537

Table 5.3 (continued)

P values	df	LMA	N	C/N	Condensed tannins	Total phenolics	Lignin
O_3	2	0.369	0.585	0.402	0.128	0.305	0.665
Soil	2	0.161	0.59	0.43	**0.046**	0.112	0.693
O_3 x Soil	4	0.911	0.76	0.527	0.496	0.174	0.259
Time	3	**0.001**	**0.042**	**0.049**	**0.007**	**0.027**	0.169
Time x O_3	6	**0.032**	0.619	0.533	**0.041**	0.174	0.308
Time x Soil	6	0.254	0.559	0.458	0.153	0.846	0.507
Time x O_3 x Soil	12	0.106	0.124	0.253	0.572	0.308	0.329

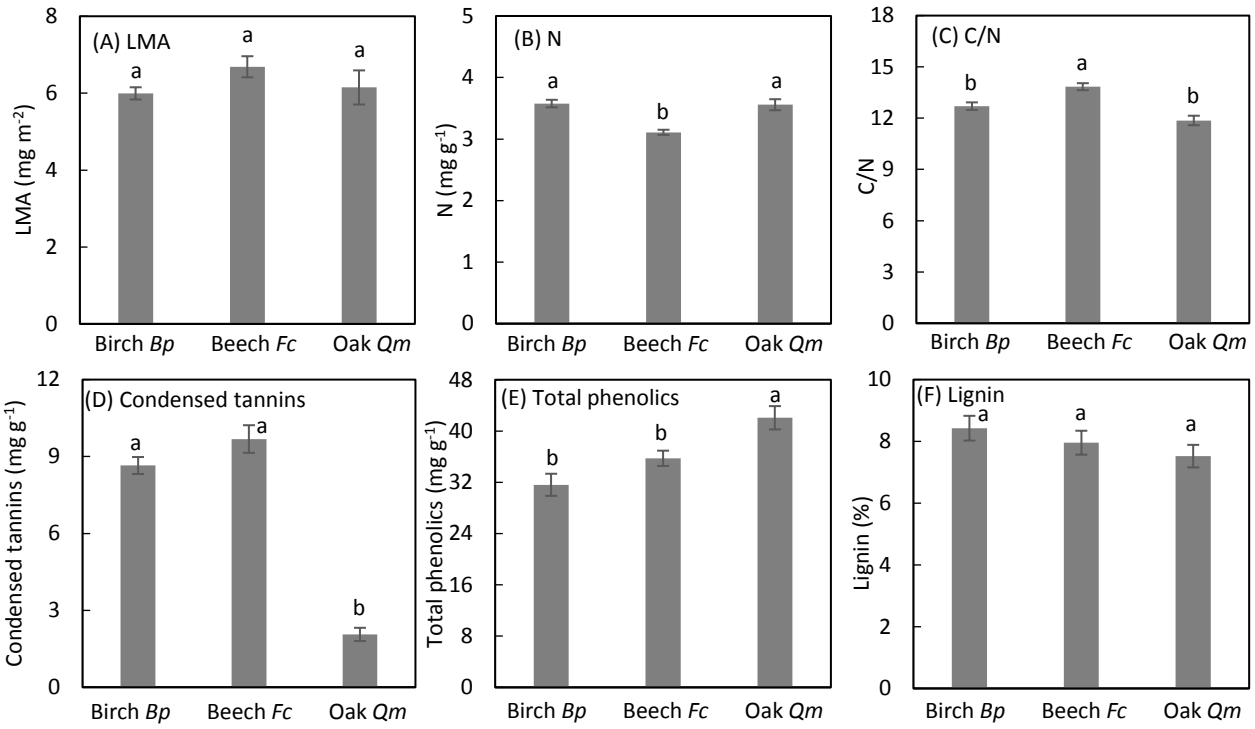
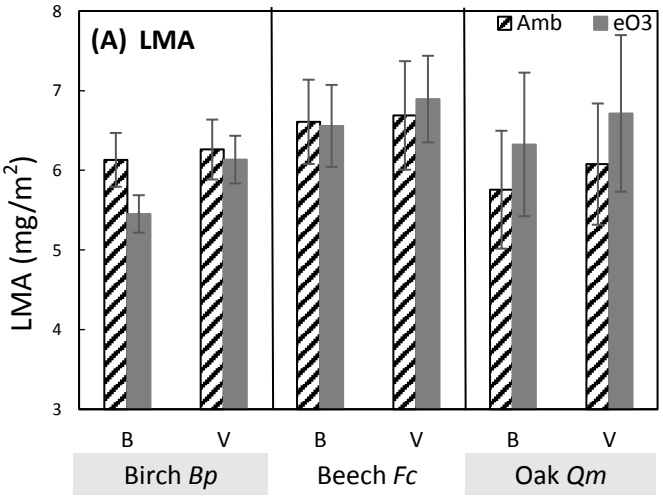
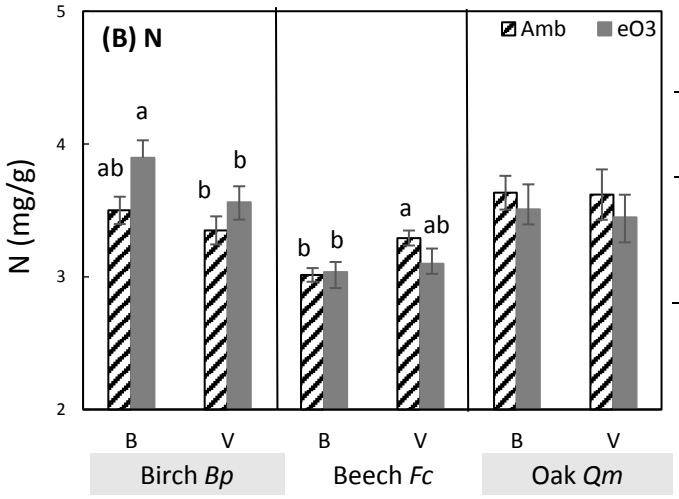


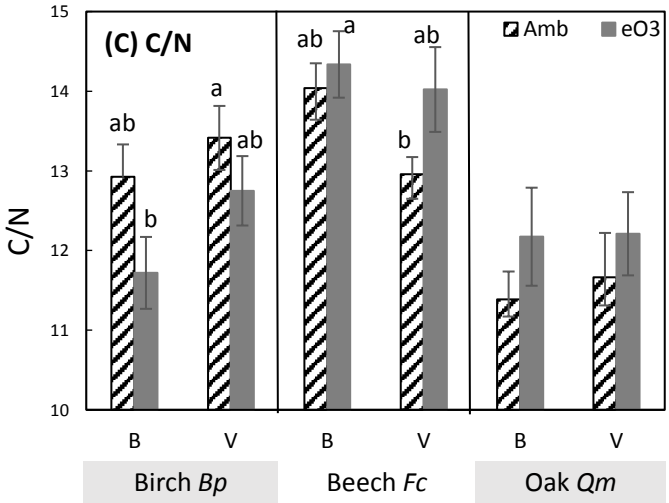
Figure 5.1 Comparisons of leaf traits and defensive chemicals among the three different species (birch *Bp*, beech *Fc* and oak *Qm*). Data are based on the mean values of each species ± SD. Tukey's hsd test was applied among three species. Different letters indicate statistical differences at $P < 0.05$. n=33-72.



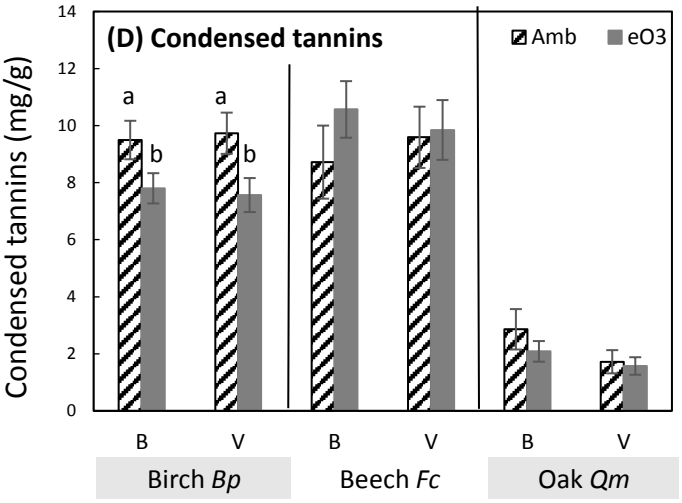
P	Birch *Bp*	Beech *Fc*	Oak *Qm*
O_3	0.207	0.892	0.385
Soil	0.204	0.717	0.829
O_3xSoil	0.386	0.824	0.839



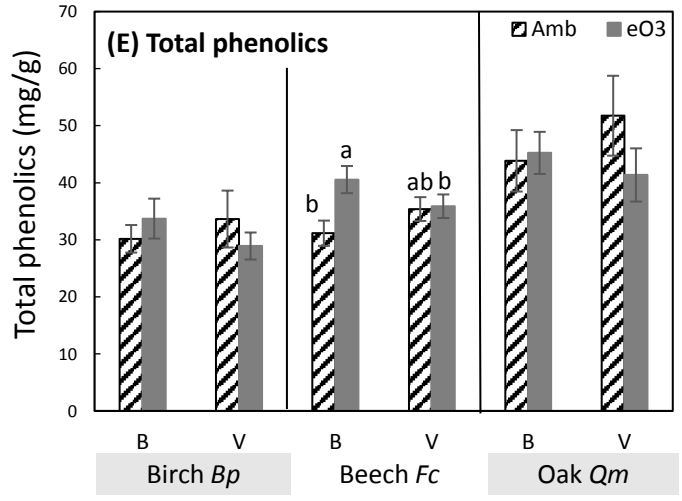
P	Birch *Bp*	Beech *Fc*	Oak *Qm*
O_3	**0.010**	0.281	0.355
Soil	**0.040**	**0.037**	0.807
O_3xSoil	0.430	0.183	0.937



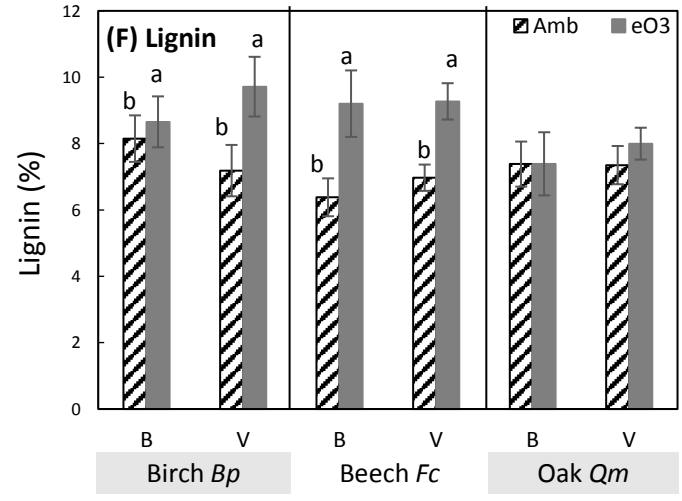
P	Birch *Bp*	Beech *Fc*	Oak *Qm*
O_3	**0.030**	0.089	0.224
Soil	0.077	**0.041**	0.658
O_3xSoil	0.526	0.330	0.705



P	Birch *Bp*	Beech *Fc*	Oak *Qm*
O_3	**0.003**	0.348	0.367
Soil	0.998	0.946	0.075
O_3xSoil	0.710	0.477	0.413



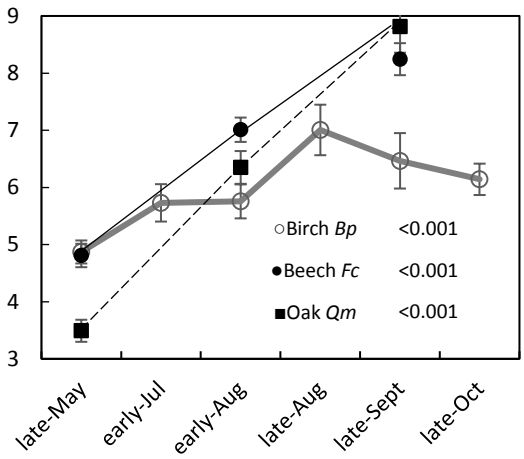
P	Birch *Bp*	Beech *Fc*	Oak *Qm*
O_3	0.865	**0.031**	0.491
Soil	0.848	0.921	0.872
O_3xSoil	0.240	0.051	0.392



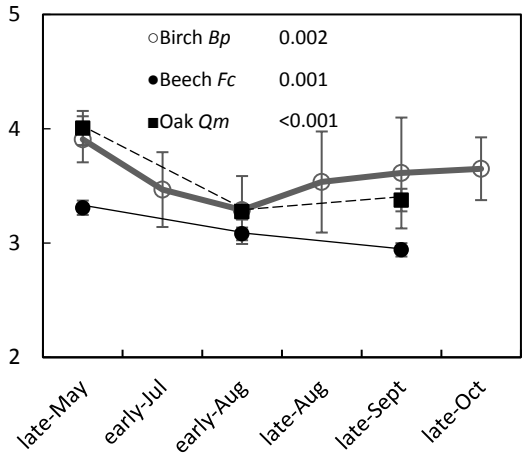
P	Birch *Bp*	Beech *Fc*	Oak *Qm*
O_3	**0.049**	**0.001**	0.628
Soil	0.951	0.626	0.658
O_3xSoil	0.204	0.699	0.730

Figure 5.2 Foliar traits and defensive chemicals under two soil types with free-air O_3 enrichment system in three different species. Data are average values ± SD. Amb = ambient O_3 condition, eO_3 =elevated O_3 condition, B = brown forest soil, V = volcanic ash soil. *P* values results of two-way ANOVA are presented in the table next to each graph, and when significant differences were tested, Tukey's hsd test was followed by to assess the differences among the 4 treatments within each species. n=8-18

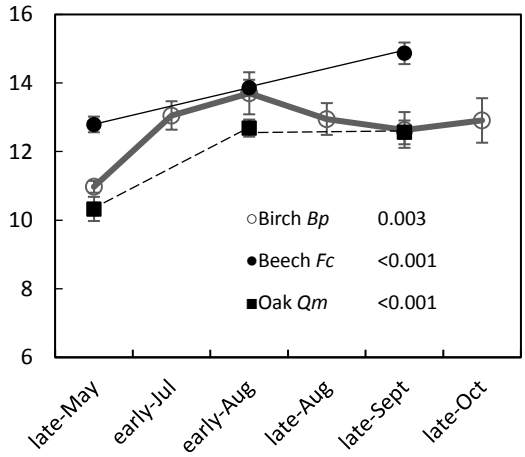
(A) LMA (g/m^2)



(B) N (mg/g)



(C) C/N



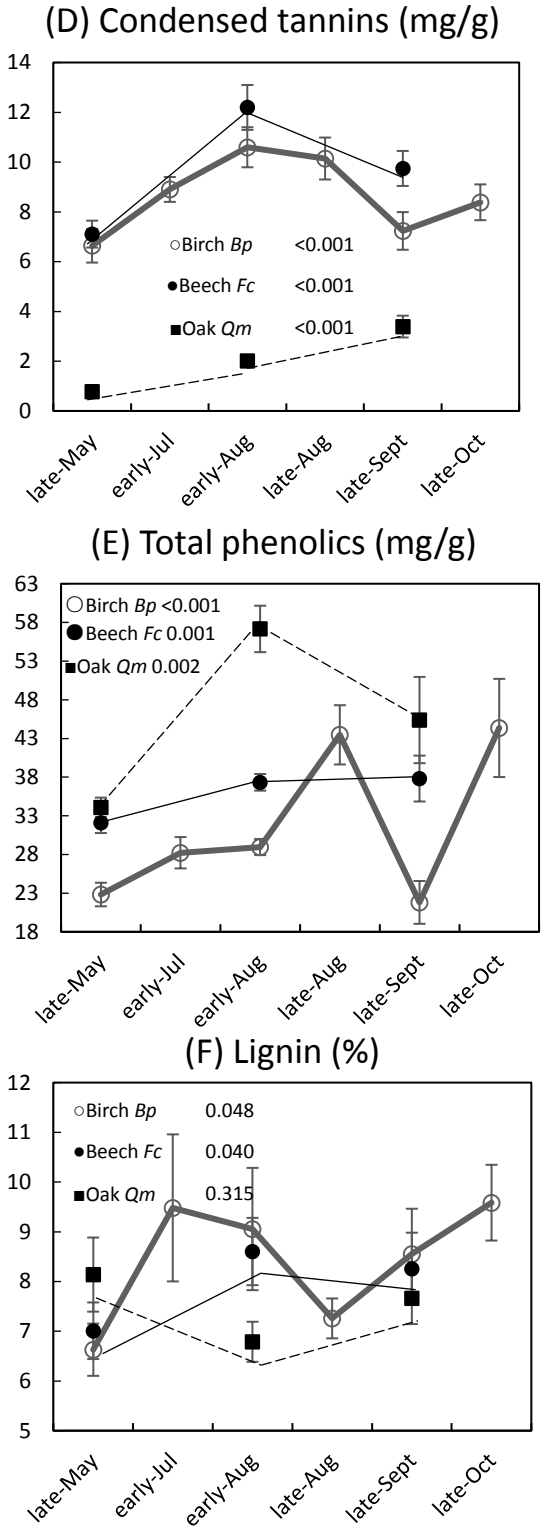


Figure 5.3 Seasonal changes of leaf traits and chemical contents in three different species. Data are represented as mean ± SD of all O_3 and soil treatments, n=12. Thick gray solid lines indicated the seasonal tendency of birch *Bp* species; thin black solid lines indicated the seasonal tendency of beech *Fc* species; thin black dash lines indicated the seasonal tendency of oak *Qm* species. A repeated-measures ANOVA was applied for each species and the corresponding *P* values were indicated.

Appendix 1:

Analysis method of condensed tannins and total phenols

Edited by Shi C.

Chemicals reagents

Methanol (CH_3OH: 99.5%);

Hydrochloride acid (HCl: 30-35%);

1-Butanol ($CH_3(CH_2)_2CH_2OH$: 99%);

Sodium carbonate (Na_2CO_3: solid);

Phenol reagent (Folin-Ciocalteu Reagent);

70% Ethanol (C_2H_5OH: diluted from 99.5% ethanol)

Picture A1.1

Experimental instruments

Degreasing cotton;

Aluminum foil;

Glass marbles;

Small test-tubes (10ml X 150 for 50 samples);

Medium test-tubes (20ml X 50 for 50 samples);

Test-tube stands;

Beaker;

Funnels;

Aspirator (Picture A1.1);

Suction instrument (Picture A1.2);

Vortex mixer;

Magnetic stirrer;

Ultra-sonic automatic washer (Picture A1.3);

Stew pan;

Induction heating (IH cooking);

Disposable cuvettes (Picture A1.4);

UV-VIS spectrophotometer (Picture A1.5)

Picture A1.2

Picture A1. 3

Picture A1.4

Picture A1. 5

Picture A1.6

Procedures

1. *Preparation of leaf samples*

Collected leaf samples should be immediately freeze-dried (FLEX-DRY, FTS Systems, Stony Ridge NY, Picture A1.6) and mill ground to powder.

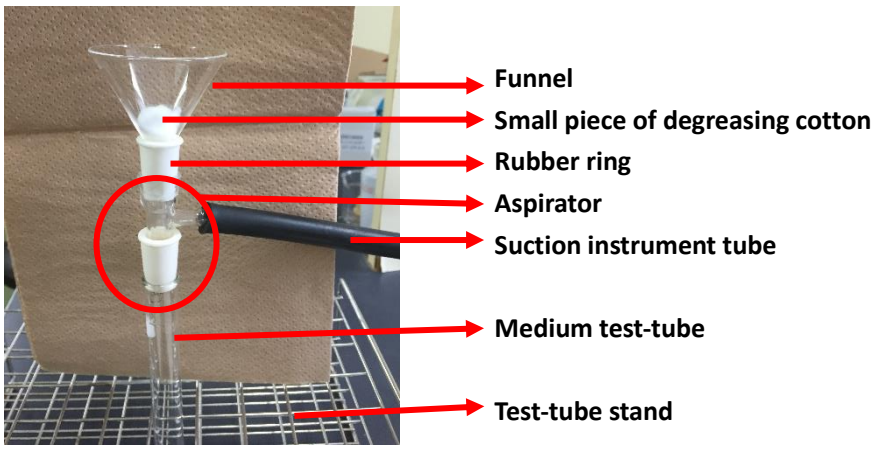
2. *Preparation of filtrate solution*

1) Weigh approximately *20mg* (19.6 ~ 20.5mg) of a freeze-dried sample placed in a small test-tube; Then, add *5ml* *50%* methanol (diluted from 99.5%methanol) into the test-tube.

(Note: More than 2 control filtrates have to be made. Namely, 5ml 50% methanol added directly into a blank test-tube).

2) Put the test-tubes into a test-tube stand and cover with the aluminum foil; Then, place the entire test-tube stand in a ultra-sonic automatic washer with the water over the test-tube stand; Set the ultra-sonic washer as *40 ℃* for *60min*.

3) Medium test-tube, top with an aspirator sealed, is placed on another test-tube stand preparing for the next filtration step: Add a small piece of degreasing cotton into a funnel whose bottom covered with a rubber ring; Place the whole funnel device above the top of the aspirator; Connect with the suction instrument; Turn on the suction instrument and pour the well-washed solution (from step 2) into the funnel to process the filtration to get the filtrate solution (Picture A1.7).



Picture A1.7

3. *Assay of condensed tannins*

1) Add *1ml* filtrate solution and *4ml* *HCl-Butanol solution into a small test-tube; then mix it with a vortex mixer.

*HCl-Butanol solution = HCl : 1-Butanol = 1 : 19 = *15ml* : *285ml*

(Note: More than 2 controls have to be made. Namely, add 1ml blank filtrate and 4ml

HCl-Butanol into a small test-tube).

2) Put the test-tubes into a test-tube stand and every single test-tube should be covered with a glass marble; Then, place the entire test-tube stand in a stew pan with the water over the test-tube stand; Use IH cooker to conduct a water bath heating for about *2 hours*.

(Note: During the water bath heating, water may need to be refilled to keep the water level over the test-tube stand).

3) Solution in the test-tubes are turned into pink (or red wine) color; After the solution is cooled down, add the solution into a disposable cuvette and make a measurement with the UV-VIS spectrophotometer at *550 nm* of the absorption wavelength.

(Note: If the ABS value is over *0.8*, we need to use the control to dilute the solution into 1/2 and re-measure it again; if still over 0.8, then dilute it to 1/3 and so on until it below 0.8; Then the final value equals 2 (or 3..) times of the measured value).

4. *Assay of total phenolics*

1) Make *20%* Na_2CO_3 solution: Add *30g* Na_2CO_3 and *150ml* distilled water into a beaker; Add a stir bar into the beaker and make the solution well mixed with the magnetic stirrer.
2) Make *50%* phenol solution: Dilute the phenol reagent (Folin-Ciocalteu Reagent) to 1/2 phenol solution with distilled water.

(Note: Be attention to the volatile of the phenol reagent!)

3) Add:
   ① *50μl* filtrate solution
   ② *2.25ml* deionized water
   ③ *0.25ml* , 50% phenol solution
   ④ *2.5ml* , 20% Na_2CO_3 solution

   IN ORDER to a small test-tube; then mix it with a vortex mixer.

(Note: a. The above chemicals must be added in order. Because the wrong sequence might be able to terminate the experiment;
b. More than 2 controls have to be made. Namely, add blank filtrate instead of the sample filtrate solution and same afterwards)

4) Stand in the draught cupboard for about *10min*;
5) Solution in the test-tubes are turned into light blue; Add the solution into a

disposable cuvette and make a measurement with the UV-VIS spectrophotometer at *760 nm* of the absorption wavelength.

(Note: If the ABS value is over *0.8*, we need to use the control to dilute the solution into 1/2 and re-measure it again; if still over 0.8, then dilute it to 1/3 and so on until it below 0.8; Then the final value equals 2 (or 3..) times of the measured value).

Clean-up

All the experimental instruments should be rinsed by 70% Ethanol before washed by water.

Calculation

A = Sample mass (mg): about *20mg*

B = ABS of condensed tannin

C = ABS of total phenols

Condensed Tannins (mg/g) = (4.5X**B**/4 + 0.011)/20.255 X 5000 / **A**

Total Phenols (mg/g) = (**C**/2 – 0.003)/12.281 X 100000 / **A**

Appendix 2:

Determination of Lignin in leaf by the Acetyl Bromide Method

Edited by Shi C., improved by Koda K.

Chemical reagents

Ethanol

Benzene

acetyl bromide (C_2H_3BrO)

acetic acid (CH_3COOH)

perchloric acid (70%, $HClO_4$)

sodium hydroxide (2mol/L , NaOH)

Experimental instruments

A sieve with 40 mesh (0.425mm) openings

Filter paper (d=150mm)

Thimble filters (Picture A2.1)

Six sets of Soxlet extraction apparatus (condenser, Soxlet extractor, and flat flask)

A water bath (or an oil bath)

Test tubes with screw cap (with Teflon coat /or liner) (x 12 and even more)

100-ml volumetric flasks (x 6)

UV spectrophotometer (at N363) and related apparatus (e.g. cuvette)

Procedures

1. *Pre-treatment:*

a) The freeze-dried leaf samples are ground into powder and the samples are sieved to obtain 40-60 mesh (0.425mm) – pass fraction (about 100 mg for each sample needed).

b) Several sheets of filter paper should be weighed in advance after they are dried in an oven at 60-70 ºC for (at least) 48 h and then cooled in a desiccator [containing a drying agent like silica gel] at room temperature.

c) Each sample (40 mesh-pass fraction: about 100 mg) is wrapped with a sheet of filter paper (d=150mm).

d) The well-wrapped samples (folded [and stapled when necessary]) are put in an oven

at 60-70 ^{o}C for 48 h.

e) After cooled down in a desiccator, oven-dry mass of each sample and filter paper is weighed. By subtracting the mass of the filter (wrapping) paper, oven-dry mass of each sample (before extraction) is calculated (**M_{before}**).

2. *Extraction by ethanol-benzene:*

a) One liter of ethanol and 2 L of benzene (v/v, 1:2) are mixed and stored in a gallon bottle.

b) Some sets of the pre-treated (well-wrapped) samples are put in a thimble filter (Figure A2.1) and the thimble filters are put in a Soxlet extractor.

c) Boiling stones (5-10 pieces: they are recommended to be put in an oven at 105 ^{o}C overnight before used) and 120+ mL of ethanol-benzene mixture are placed in a flat flask.

d) A condenser, the Soxlet apparatus, and the flat flask are attached (as seen on the left side in Picture A2.2).

Picture A2.1

Picture A2.2

e) Tap water is allowed first to flow into condensers.

f) Extraction is initiated by turning on the switch (first level 4; after boiling is started, it should be controlled) Samples are extracted continuously for 8 h at the first day. After turning off the heating switch, the tap water should be kept running in condensers for another 30 min at least (the solvent will still continue to be evaporated until the Soxlet apparatus are cooling down enough).

(Another tip: Avoid heating at midnight without a person watching by! It may cause fire.)

g) On the second day, all sets of the Soxlet apparatus are detached and new boiling stones (5-10 pieces) are placed in each flat flask.

h) All sets of Soxlet apparatus are attached again to start extraction for another 8 h (extraction will be conducted 8 h×2 d=16 h in total. Extraction should be continued until the color of the solvent (ethanol/benzene) in a Soxlet extractor is gone.

i) The samples are taken out and put in a (fume) hood for 1 day.
(This air drying process is needed before oven dry process; otherwise your sample (containing solvent) will be burned in an oven.)

j) After air-dried, the defatted samples (still wrapped) are put in an oven at 60-70 ^{o}C for 48 h.

k) The oven-dried (wrapped and defatted) samples are cooling down in a desiccator, and then weighed. By subtracting the mass of the filter (wrapping) paper, oven-dry mass of each sample (after extraction) is calculated ($\mathbf{M_{after}}$) By subtracting $\mathbf{M_{after}}$ from $\mathbf{M_{before}}$, mass of extractives in each sample can be calculated.

3. *Acetyl bromide method (Spectrophotometric method)*

a) Beforehand, mother solution (e.g. 100 mL) of acetyl bromide in acetic acid solution (25 w/w%), containing perchloric acid (70%; 4 mL) is prepared.

b) A defatted sample (20 mg) is placed in a test tube.

c) The acetyl bromide solution (25 w/w%) in acetic acid (5ml) is added to the test tube.

d) The test tube is sealed with a screw cap, and then shaken vigorously.

e) The test tubes are put in a water bath at 70 ^{o}C for 30 min to proceed digestion.

f) After digestion, the solution is transferred from a test tube to a 100-mL volumetric flask containing 2 mol/L sodium hydroxide (NaOH, 10mL) and acetic acid (25ml).

g) The flask is rinsed, and then the volume of the solution is made up to 100 mL with acetic acid.

h) Reference sample is made in the same steps as in e), and f), but without the sample inside.

i) UV absorbance of sample ($\mathbf{A_s}$) and reference ($\mathbf{A_b}$) is measured at the wavelength of **280 nm** with UV-spectrophotometer.

4. *Calculation*

a) Lignin content can be calculated, based on the defatted sample mass:

$$\text{Lignin}_{\text{(on defatted)}}\ \% = [100\ (A_s - A_b) \times V] / (aW),$$

where V = volume of the solution, L (100 mL = 0.1 L); a = absorptivity of lignin standard, $Lg^{-1}cm^{-1}$ (around 20 $Lg^{-1}cm^{-1}$); W = weight of sample, g (20 mg = 0.02 g).

b) Lignin content, based on the original sample mass, can be calculated as follows:

$$\text{Lignin}_{\text{(on original)}}\% = \text{Lignin}_{\text{(on defatted)}}\% \times M_{\text{before}} / M_{\text{after}}$$

Waste disposal

Acetyl bromide-based waste has to be quenched with NaOH solution (neutralization) before disposal. AcBr is very reactive and it generates a lot of heat when being neutralized. There cold NaOH solution should be prepared in advance.

Chapter 6: General discussion

6.1 General description of foliar nutrients and secondary metabolites under environmental changes

Environmental changes, including ground-level O_3, atmospheric CO_2 and various soil types in my study, considerably affects dynamics of physiological processes of three kinds of tree species. I found them in both foliar nutrients and their allocation dynamics of retranslocation by changes of the balance between xylem flow and phloem loading, as well as the plant defense characteristics by alternation of secondary compounds. As a result, it will finally have considerable impacts on production of broad-leaved trees forest, especially in temperate regions as the tree species applied in my study are representative of northern Japan forests. The present research extends the knowledge to comprehend the physiological function and stoichiometry of foliar nutrients and plants defensive characteristics in response of high O_3 accompanied with various environmental factors.

It is important to further investigate the nutrients cycling and plant defensive mechanism under O_3 pollutant to achieve the initial target of ecosystem rehabilitation and a sustainable production of temperate broad-leaved forests under environmental changes.

6.1.1 Foliar nutrients and retranslocation rates

Under the environment of eO_3 accompanied with eCO_2, plants have higher water use efficiency and lower O_3 uptake since Gs is reduced by eCO_2 (Norby and Zak, 2011; Koike et al., 2012). As photosynthetic performance is increased by eCO_2 but declined by eO_3, the reduced foliar Ca, Mg, Mn in both oaks (*Qm* and *Qs*) at eO_3 are attributed to the declined photosynthesis function as well as eO_3-induced stomata sluggishness (Hoshika et al., 2012b). Increase of starch at eCO_2 as well as the lower Gs are due to the stomata limitation of carbon assimilation (Grantz, 1989). Alternatively, the starch enhancement stems from a feedback mechanism that decrease the production of sucrose in cytosol, thereby triose phosphate exported from chloroplast, can result in an accumulation of starch in chloroplast (Sharkey, 1985; Katny et a., 2005).

As water soluble elements, P, N and K are able to mobile in phloem and retranslocated from aged leaves to young ones easily (Marschner, 2012). As lower Gs,

induced by eO_3 and/or by eCO_2, alters the absorption of water soluble elements with older leaves (Hoshika et al., 2013c), leaf P is decreased in older leaves. As eCO_2 increases the P demand for leaf flushing, reduction of P in leaves may be attributed to P consumption by leaf flush. Unlike leaf P, foliar N dynamics more depends on photo flux density (Hikosaka et al., 1994; Koike et al., 2007), and foliar N may distribute to chlorophyll for photosynthetic carbon fixation in light limited condition, which suggests that foliar N highly connects with photosynthesis process. Therefore, the decreased foliar N may due to the fact that oaks need not to accumulate much N for carbon fixation because CO_2 surrounding parts of plants are enough to high concentration for photosynthesis process. As a result, the decreased foliar N may attribute to the reduced demand of N at eCO_2 and the reduction of foliar N at eCO_2 is also likely related to the constrains of nutrients supply from soils (Koike et al., 2007; Noby and Zak, 2011).

Leaf K is still under unclear since no clearly differences are observed at eO_3 and eCO_2 in my data. Moreover, my data also provide evidence to indicate that the decreased photosynthesis rate by eO_3 may slightly be recovered by the combined effects of eO_3 and eCO_2. This may bring an increase of biomass in *Om* and *Os* at eO_3 and eCO_2 (Kitao et al. 2015). What's more, investigation of foliar N and Mg, as major indices in assessing eO_3 on oaks (*Qs* and *Qm*), would be of use to discriminate O_3 and CO_2 in field studies according to the principal component analysis (PCA) results provided in this study (Shi et al. 2016b).

Retranslocation rates of foliar nutrients can be constrained by elements mobility. Generally, as water soluble elements (N, P, K, Mg and Ni) are mobile in phloem (Marschner, 2012), they can easily translocate from aged to young leaves during process of leaf senescence or degradation of growth light condition for the plants. On the contrary, as non-mobile elements (Ca, Mn, and Al, Cr, Fe, Al) are basically poorly mobile in phloem (Schulze et al., 2005; Marschner, 2012), they are remained in older leaves and can give rise to a deficiency in younger leaves. Referring the calculation of retranslocation rate, it can be a positive value for water soluble elements and a negative for non-mobiles.

Under the environment of eO_3 accompanied with various soil types, leaf senescence is accelerated by eO_3 and nutrients retranslate more from old to young leaves with poor fertility soil due to the occurrence of nutrients conservation. As a result, retranslocation rate of foliar elements (ERE) is expected to increase in fertile soil but to

decrease as responses to eO_3. In fact, our data indicated that the significantly differences in foliar nutrients contents as well as their acquisition and loss are varied upon different environmental combinations and O_3 exposure duration, and are also species specific.

There are significantly higher nutrients retranslocation rates can be observed at eO_3 with one-year exposure, such as higher NRE, PRE and AlRE at eO_3 in birch *Bp* species as well as higher AlRE in beech *Fc* species. This is because mature leaves which are also younger leaves, are more tolerant to O_3 stress compared to senescing leaves (Hoshika et al., 2013b). As a result, nutrients in senescing leaves are easily affected by eO_3 and thereby lead a higher rate of retranslocation. In addition, higher ERE of Ca, K, Mn and Al in *Bp* species have been found at eO_3 in rich fertility soil, suggesting that sensitivity of growth and photosynthetic factors to eO_3 are able to be enhanced under fertile soil with higher available N (Yamaguchi et al., 2007). Moreover, PRE, NRE as well as CaRE in *Qm* are markedly affected by soils, especially higher at V soil. This may result from the high depletion-driven loss in acidic soil as N output is negative correlated with soil acidification (Dise and Wright, 1995). Retranslocation of other mineral elements (Ca, K, Mg, Mn and Fe, Al) have been most sensitive to both eO_3 and soil types in *Fc*, therefore *Fc* may be applied as an index species for investigation of mineral nutrient retranslocation in field survey for short-term O_3 exposure.

Foliar element contents are usually highly related with the corresponding foliar retranslocation rates. Based on my data within two-growing seasons, the effects of eO_3 and soil treatments on the retranslocation rate can be detected in those elements basically detected to have significantly treatment effects on foliar contents (Ca, K, Mg, Mn, P and Al) among 3 species. I believe those different environmental changes that promote plants growth are also able to facilitate the retranslocation rate of those relative elements and vice versa (Nambiar and Fife, 1991). According to the soil chemical determination conducted in my study, soil elements (Ca, K, Mg, Mn, N, Ni, P and Al, Cr, Fe) exhibited considerable differences among different types (B, V and S soils) with two-year O_3 exposure. Regarding of the same conditions for both seedlings and experimental site, these differences of surface soil are probably because of the elemental function of each species. Moreover, KRE and PRE at eO_3 are increased at nutrient deficient soil in order to improve the competition at species-level so as to overcome the O_3 stress (Aerts and Chapin, 2000).

With regard to foliar elements contents and those relationships, N is detected to be one of the elements pair that had negative correlations independent with the species,

such as N-Fe, N-Mn, N-Ca, N-Al and N-Fe. This is because N uptake is constrained by the accumulation of those minerals which can induce the decrease of leaf water potential, giving rise to a lower Gs and photosynthetic rate (Rao et al., 1987; Kayama and Koike, 2015). At the same times, N-K is positive correlated. Consequently, I consider that the uptake of N is further easier to be suppressed by various mineral elements when it correlated with K, as foliar K is highly connected with the hormone regulation in the plants (Conti and Geiger, 1982). But foliar K is independent with Mn in all studied seedlings. With regard to the PCA results, K and Mn can be regarded as the indices in assessing the effects of O_3 and soil on long-term growth monitoring in seedlings of the projected species. Additionally, leaf N is independent with Mg, Ni, P and Cr in studied species, which is also supported by the conclusion from another study in Chapter 2 that N-Mg can be used as a pair of major indices in assessing the O_3 effects in a free-air enrichment system (Shi et al., 2016b).

6.1.2 Foliar defense and secondary metabolites

In my study, foliar defense characteristics are varied upon environmental changes and it is also highly concerned with species sensitivity to environmental changes. For a certain species, the higher sensitivity to environmental differences, the more susceptible defense mechanism (indexed as secondary metabolites) to surroundings changes. For instance, *Bp* is relatively sensitive to eO_3, the defensive chemicals (e.g. secondary metabolites: SM) thereby are easily affected by eO_3.

Moreover, as plants defensive parameters, N and C/N are affected by soil conditions while secondary metabolites are influenced by eO_3. The influence of soils on foliar contents (C and N) can be explained by the higher retranslocation rates under relatively poor soil fertility. Indeed, defensive chemicals (SM) are affected by eO_3 because eO_3 can raise the defense reactions to regulate the resource allocation on defensive chemicals.

In addition, foliar phenology, as reprehensive of the secondary metabolites in plants, affects the resource availability for maintain plant defense mechanism, in order to make plants be competitive to survive under various harsh environmental conditions.

6.2 Species specific characteristics

Based on overall results throughout my study, both stoichiometry and physiological functions have considerable responses on both eO_3 and soil conditions for those involving species applied in my experiments. The effects of O_3 and soil varied across species with different shoot growth patterns. Referring the species-specific effects of O_3 on plants species, as I introduced in Chapter 1 in Table 1.2, O_3 sensitivity of the relative species utilized in my study (which have been shown in bold) can be sequenced from high to low as: *Fc* > *Bp* > *Qs* > *Qm*. But on basis of my study, this is not always the case, especially for those immobile foliar nutrients in oak species.

With regard to plants shoot growth pattern for each species, birch shows heterophyllous type shoot growth which is similar to an indeterminate shoot growth pattern (Koike, 1995; Hoshika et al., 2013b) and beech typically shows a determinate shoot growth pattern (Kitao et al., 2009). Although oak is usually in between them, considering as a semi-determinate shoot growth pattern (Holbrook and Zwieniecki, 2011) or flush and succeeding shoot growth (Kikuzawa 1983), it is hard to have a clear-cut on classification of shoot growth patterns and plenty of overlaps do exist in between classes.

With regard to successional characteristic for each species, birch shows an early-successional heterophyllous species with two leaf types (early and late), while beech is late-successional species, and oak shows in between, regarding as mid-successional species. Besides, birch as a shorter lifespan within its indeterminate shoot growth (Coley, 1988; Koike, 1988; 2004; Matsuki and Koike, 2006) is relatively susceptible to herbivores in my study.

6.2.1 Birch

Considering the effects of eO_3, it has commonly been assumed that exposure of eO_3 can accelerate leaf senescence and give rise to a lower Gs because of eO_3-induced stomata sluggishness (Yamaguchi et al., 2011; Hoshika et al., 2015). As a result, it is anticipated that eO_3 is detrimental on plants' photosynthetic performances and nutrient dynamics, resulting in a decrease of retranslocation rate (Uddling et al., 2006). In birch species, foliar MnRE as well as leaf contents of Mg and Mn are decreased at eO_3, supporting the point of view that eO_3 is detrimental to plants growth and decline the

ERE. Besides, since I detected a positive correlations between foliar contents of Mg and Mn in birch, there is no surprise that Mg and Mn have similar responds to eO_3 in birch leaves, especially so with long-term O_3 exposure.

Contrarily, since O_3 affects adversely more on old leaves (senescing leaves) in birch species as younger leaves (mature leaves) are more able to avoid from O_3, (Hoshika et al., 2013b), NRE, PRE and AlRE, as highly concerning with the difference between mature leaves and senescing leaves, are enhanced at eO_3 for birch species.

On the other hand, soil nutrients condition considerable affects the foliar K contents and I found leaf K was decreased by poor fertility soil condition. As toxic elements accumulate largely in infertility soil, K uptake in birch leaves is likely concerning with the accumulation of Mg, Ni, Cr in the serpentine soil (S soil). Generally, both early- and late- successional species may retranslocate less from old leaves to other organs under infertile soil due to the nutrients conservation system works in plants (Killingbeck, 2004; Koike 2004), which is able to result in an enhancement of retranslocation rate of foliar element in birch, beech and oak species. Consequently, increased ERE of foliar K and/or foliar P under S soil were observed in every projected species including *Bp*, *Fc* and *Qm*.

Foliar N as highly connected with leaf defense, is decreased with eO_3, which has important implication for the adversely effects of eO_3 on plants growth, suggesting that environmental stress cannot make N uptake be assigned more on plant growth but be transferred to defense system for instead (Bryant et al., 1983; Noriyanti et al., 2012). When eO_3 accompanied with poor fertility soil, as the adverse environmental conditions, are likely to reduce the N availability for production of defense chemicals (condensed tannins: ConTan and total phenolics: ToPhe), making them decline in birch.

Seasonal changes of SM in birch leaves appear to be accumulated over growth and be suppressed at beginning of defoliation and then be raised once more before winter. Since SM are reduced more than foliar biomass accumulation on basis of GDBH, inhibition effects of environmental changes (eO_3 and/or poor fertile soil) and natural defoliation process on chemical defense are more dominant than physical defense (LMA; Leaf area per mass) in birch species to avoid herbivores.

In order to defense eO_3 and turn-on the self-protection, *Bp* as a fast-growing species with less defensed, may accelerate the lignin production under the stimulation of eO_3. As a result, lignin in *Bp* as an end product of available resources (Herms and Mattson, 1992), can reach the peak content by eO_3 within shorter period compared to ConTan,

though both of them are immobile defensive compounds with large molecular.

In conclusion, defensive chemicals (ConTan, ToPhe and lignin) in birch species, typically achieve a relative high content level to protect *Bp* seedling by keeping in a strong defense capacity on purpose to make the loss by herbivores as less as possible before senescence.

6.2.2 Beech

Beech with determinate shoot growth pattern is known as the most sensitive species to eO_3 among the three species applied in my study. Accordingly, the retranslocation rates of various foliar mineral elements (Ca, K, Mg, Mn and Fe, Al) are also shown more efficient and more sensitive to eO_3 for beech species compared to other species. As the sensitive species to eO_3, NRE and PRE in beech leaves are reduced due to leaching in senescing leaves at eO_3 (Helmisaari and Mälkönen, 1989). However, NRE is increased at S soil, suggesting that more N would prefer to allocate in younger (mature) leaves for further growth, especially for the plants grown under poor fertility soil. This result is also applied for defense mechanism. Higher NRE under relatively infertile soil may lead to a higher N and lower C/N, which results in an enhancement of defensive capacity for *Fc* against the adverse environmental changes.

In my context, nutrients dynamics in beech are also considerably affected by soil conditions. As KRE, CaRE, MnRE and AlRE at eO_3 are found to get higher values at B soil than that at V or S soil, I consider that the higher ERE at eO_3 under high fertility soil is a consequence of relatively lower retentions of elements in senescing leaves. Because old leaves are indeed more susceptible to eO_3 (Hoshika et al., 2013) and the sensitivity of growth and photosynthetic parameters to O_3 in *Fc* can be increased by higher soil N availability (Yamaguchi et al., 2007).

Moreover, either eO_3 or nutrient-limit soil can somehow inhibit the photosynthesis process as well as growth rate, especially for the O_3-sensitive beech species with a longer O_3 exposure. As a result, many elements in leaf contents (e.g. K) as well as their dynamics of retranslocation rates (e.g. MgRE, CaRE) are further decreased after longer exposure of eO_3. But the decreased of MgRE and CaRE may be also attributed to the hormonal alternation regulated by K dynamics since KRE at eO_3 is enhanced under B soil.

In addition, there is another possibility supported by Kayama and Koike (2015) that reduced foliar contents are highly connected with the accumulation of the toxic

elements (e.g. Mg, Ni and Al, Cr) in leaves under poor fertility soil as they can reduce the leaf water potential and make photosynthetic rate decrease. Despite the fact that merely decreased Al content is detected in beech leaves for both eO_3 and infertile soil after the observation of longer-O_3 exposure. I consider that beech species has a strong detoxification mechanism to protect itself being away from long-term negative environmental changes and thereby suppress the uptake of other toxic elements meanwhile keep Al in a low content since low-level of Al has beneficial effects on plant growth (Marschner, 2012) as found by Wanatabe et al. (2008).

With regard to beech defense, although foliar physical defense (LMA) kept increasing over growing season, chemical defense (ConTan and ToPhe) rose to a peak around mid-summer and then declined in early-autumn. This suggests that chemical defense are not necessary always respond to the growth rate, and *Fc* would get higher growth rate in absence of defense since the trade-off between growth and defense generally happened on the plants with higher growth rate and higher defense system (Matsuki et al., 2004; Ngubeni et al., 2016).

In contrast to the results of birch species that SM are decreased at eO_3, defense chemicals (ConTan, ToPhe and lignin) in *Fc* are increased by eO_3. This might be related with the different sensitivity to eO_3 for the two species and defensive chemicals perhaps become more defensible in the species that is more tolerant to eO_3. Alternatively, SM as sort of resource allocations in beech species is shifted from O_3-induced decline of growth. Because plants may increase the resource availability to produce more SM with little trade-off growth, plants improve the defense capacity (Herms and Mattson, 1992; Novriyanti et al., 2012).

In addition, the enhancement of lignin contents in beech leaves at eO_3 may also contribute to the enhancement of physical defense (LMA). Elevated O_3-induced cellular-level alternation can result in enhancement of LMA by damage to membrane and cell extension. Consequently, O_3 can give rise to plant defense reactions as an abiotic elicitor that triggers plant signal molecules and hence stimulates the lignin biosynthesis (Sandermann et al., 1998; Paoletti, 2007).

6.2.3 Oak

Oaks (*Quercus* sp.) are regarded as the most tolerant species to eO_3 among the projected tree seedlings in my study. For the oak family, *Qm* is considered as a more tolerant one to eO_3 than *Qs*. Effects of eO_3 accompanied with eCO_2 and various soil

types on oak leaves are both investigated in my study.

Generally speaking, Gs is reduced by both elevated eO_3 and eCO_2. Because on one hand, plants need not to open the stomata widely at eCO_2 so as to make lower O_3 uptake and keep higher water use efficiency (e.g. Koike et al., 2012); on the other hand, stomata sluggishness occurred when under eO_3 and lead to a lower Gs, especially works on old leaves due to younger leaves have more capacity of avoidance of eO_3-effects (Hoshika et al., 2012b; 2013b). Consequently, the decrease of foliar nutrients contents (Ca, Mg, Mn) in both oak species are due to the eO_3-induced lower Gs and diminish of photosynthetic function. Moreover, relatively lower leaf Mg in *Qs* than that in *Qm* at eO_3 could be associated with the higher susceptibility of *Qs* to eO_3. The higher leaf Mn in *Qm* independent with the both gas conditions and flushed leaves might be due to the higher Gs of *Qm*.

Despite the fact that *Qs* is considered to be more sensitive to eO_3 compared to *Qm* as summarized by Kohno et al. (2005) and Yamaguchi et al. (2011), the trends alternation of foliar nutrients contents under combined effects of $e(O_3+CO_2)$ in my data suggest that *Qs* has a higher ability to recover from O_3-damage and perhaps become more tolerant to eO_3 than *Qm* when involves the effect of eCO_2.

With respect to *Qm* under eO_3 accompanied with different soil types, *Qm* is fairly tolerant to eO_3 on both foliar nutrients and their retranslocation rates. But the ERE of *Qm* leaves for certain elements are considerably affected by soil condition.

According to my data, higher NRE, PRE as well as CaRE can be associated with the high depletion-driven loss in poor nutrient soils. Nevertheless, lower FeRE and AlRE are probably due to the interactions of O_3 x Soil because eO_3 impacts can be aggravated by high N soils and may lead to a lower ERE (Yamaguchi et al., 2007).

Moreover, under long-term O_3 exposure can somehow give rise to alter the retranslocation rate ultimately. Foliar contents and retranslocation rates for most of the elements are dramatically affected by interactions of O_3 x Soil. Effects of the accumulation of toxic elements in soils can reduce the photosynthetic rate at eO_3 and hence decrease the uptake of nutrients (e.g. Ca) in *Qm* leaves.

As a result, for those immobile elements (e.g. Ca, Mn and Cr, Al), reductions of ERE are obviously connected with the higher accumulation in senescing leaves induced by infertile soil at eO_3. With respect to the compositive results of ERE in *Qm*, oak is the least sensitive one to O_3 among the studied species and only a few relationships of foliar elements are tested within oak species. In spite the fact that oak is relatively more

susceptive to eO_3 and also more sensitive to soils on dynamics of immobile foliar elements compared to birch and beech, especially under long-term O_3 exposure observation.

Regarding defensive chemicals in *Qm*, although chemical defense (indexed by SM) is ever-increasing accompanied with the constantly increase of C/N over seasonal changes, there is no significant differences among various environmental changes on foliar defense characteristics in my results. Considering oak as the least sensitive species to O_3 among the three tree seedlings, either eO_3 or infertile soil may not induce the variations on foliar defense characteristics. Furthermore, oak is found containing higher foliar ToPhe compared to *Bp* and *Fc*, indicating that SM as resource allocation for plant defense is species specific.

6.3 Conclusions

With consideration of the compositive impacts of environmental changes (O_3, CO_2 and soils) on the foliar elements as well as leaf defense characteristics, I will make comparisons among different species and make brief conclusions as follow:

1) As regards effects of O_3 and CO_2 on two oak species of genus *Quercus*, *Qs* has relatively higher susceptibility to eO_3 compared to *Qm*, which is in agreement with the previous researches of Kohno et al. (2005) and Yamaguchi et al. (2011). The current study provides additional evidences that *Qs* as a higher susceptible oak species to O_3 may also has higher ability for being recovery from O_3 damages and may become less susceptible to eO_3 when involves of eCO_2.
2) As regards effects of O_3 and soils on different species, beech species with determinate shoot growth pattern showed a higher sensitivity to either eO_3 or soils on foliar nutrients uptake as well as leaf retranslocation rates. However, for long-term monitoring on plant growth with eO_3-exposure, the dynamics allocation of immobile elements in leaves can become more susceptible to eO_3 and soils in oak *Qm* leaves, even though *Qm* is commonly considered as the most tolerant one to eO_3 among the trees species applied in my study.
3) As regards effects of O_3 and soils on foliage regardless species, my study further proves that detrimental effect of eO_3 is detected more on older leaves (or senescing leaves) since younger leaves (or mature leaves) have more capacity of avoidance of O_3-damage (Hoshika et al., 2013b), resulting in a lower foliar content and a higher

retranslocation rate. Besides, plants would retranslocate more elements from old leaves to other tissues grown under poor fertility soil as a nutrients conservation policy, which results in a higher value on foliar retranslocation rate.

4) As regards indexes of foliar nutrients in assessing the environmental changes, my observations are on basis of the principle component analysis conducted in my study to profile the elements. Under eO_3 accompanied with eCO_2, N and Mg can become a pair of indexes for evaluating the response to the two-gas environmental changes on oak species (*Qm* and *Qs*); Under eO_3 accompanied with three soils (B, V, and S soils), K and Mn can be regarded as indexes for evaluating the O_3 and soils effects on *Bp, Fc* and *Qm*. Besides, the above nutrients indexes are tested to be independent with each other within each species according to the results of elemental relationships in my study.
5) As regards foliar defense characteristics, chemical defense of birch and beech are influenced by eO_3 and/or soils, while oak (*Qm*) is non-effects. This is in agreement with the properties of species sensitivity. But, for each species in any case, resource allocation is undoubtfully in charge of the defense reaction. On one aspect, SM as the available resources can be altered by adversely environmental changes when species is susceptible. On the other aspect, SM can also affect the available resources when species is tolerant and make it further competitive to be survival under adversely environmental changes.

6.4 Future perspectives

In this study, I attempted to concentrate on interpreting the effects of environmental changes on three representative broad-leaved forest species in cool temperate region of Japan by investigating the physiological functions and stoichiometry alternations of foliar nutrients and defensive chemicals. My study set out to provide many evidences that both O_3 and soil properties play the essential roles on foliar elements stoichiometry and physiological processes for Bp, *Fc* and *Om*. But I believe more researches need to be carried out for further study in the near future:

1) In terms of the physiological study on eO_3 accompanied with eCO_2 in *Qm*, if the demands from young developing tissues as well as the nutrients allocations as a whole plant can be taken into account, the understanding of dynamics of mineral nutrients mobility in the phloem could be more developed.

2) On basis of the stoichiometry study on eO_3 accompanied with various soils in birch, beech and oak species, if the dynamics allocation of nutrients as well as their trade-off between leaves and soils can be further studies, it will be of help on better explaining the nutrients interrelations functions for each species.
3) With consideration of the foliar defense chemicals on eO_3 and two different soils in the three tree seedlings, although chemical defense, which indexed by secondary metabolites, is notedly decreased by adversely environmental changes, there are no considerable variations on physical defense. If the debate about whether adversely environmental changes affects or not on behaviors of herbivory can be move forward, this would be a fruitful area for further work about plant defense.

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