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Leaf defense capacity of Japanese elm (*Ulmus davidiana* var. *japonica*) seedlings subjected to a nitrogen loading and insect herbivore dynamics in a free air ozone-enriched environment

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Abstract

Japanese elm (*Ulmus davidiana* var. *japonica*) is a native species in cool temperate forests in Japan. We investigated growth, physiological reactions, and leaf defense capacity of Japanese elm seedlings under nitrogen (N) loading ($45.3 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) and seasonal insect dynamics in a free-air ozone (O_3)-enriched environment (about $54.5 \text{ nmol O}_3 \text{ mol}^{-1}$) over a growing season. Higher leaf N content and lower condensed tannin content under N loading and lower condensed tannin content in elevated O_3 were observed, suggesting that both N loading and elevated O_3 decreased the leaf defense capacity and that N loading further enhanced the leaf quality as food resource of insect herbivores. Two major herbivores were observed on the plants, elm leaf-beetle (*Pyrrhalta maculicollis*) and elm sawfly (*Arge captiva*). The peak number of observed insects was decreased by N loading. Visible foliar injury caused by N loading might directly induce the reduction of number of the observed elm sawfly individuals. While elevated O_3 slightly suppressed the chemical defense capacity, significantly lower number of elm leaf-beetle was observed in elevated O_3 . We conclude that N loading and elevated O_3 can alter not only the leaf defense capacity of Japanese elm seedlings, but also the dynamics of elm leaf-beetle and sawfly herbivores.

Keywords: Japanese elm, elevated ozone, nitrogen deposition, nitrogen, phenolics, tannins, insect dynamics

Abbreviations

C_{mass} – carbon content per unit leaf mass; C/N ratio – carbon to nitrogen ratio; Chl – chlorophyll; F_v/F_m – maximum quantum yield of PSII; LMA – leaf mass area; N – nitrogen; N_{mass} – nitrogen content per unit leaf mass; T/R ratio – top to root ratio; RF ratio – relative fine root ratio.

Introduction

The ongoing climate change may induce decline of forest ecosystems in several regions of the globe (Allen et al. 2010; Cayan et al. 2010; Wang et al. 2013; Steigvilė et al. 2018), with negative direct impacts on trees and changes in the outbreaks and dynamics of forest insects (Breshears et al. 2005; Jactel et al. 2012; De Lucia et al. 2012) and tree pathogens (Moore and Allard 2008; Masuya 2016). Hence, studies are needed to clarify the effects of atmospheric changes, such as global warming and air pollution, on the relationship between trees and insect dynamics (Santini et al. 2004; Ghelardini et al. 2010; Lindroth 2010; Chappelka and Grulke 2016).

Nitrogen (N) deposition and ground-level ozone (O_3) concentrations are elevated in northeast Asia due to the economic development (Galloway et al. 2008; Ainworth et al. 2012; Hatakeyama 2017; Akimoto 2017; Li et al. 2018; Tian et al. 2018). Higher levels of N deposition and O_3 may also occur in the future (Yamaji et al. 2008; Fiore et al. 2012; Liu et al. 2018). In particular, $(NH_4)_2SO_4$ deposition has been of concern in northeast Asia (Hatakeyama 2017; Yamaguchi et al. 2014b; Matsumura and Izuta 2017; Liu et al. 2018). Oxidated sulfur species and SO_4^{2-} , which can easily be transported long distance, are mainly emitted from industrial sources in Asia (Aikawa et al. 2013; Noguchi and Yamaguchi 2019), whereas ammonia and NH_4^+ particle result from other sources including fertilizers used in agricultural practice and livestock fields (UNEP 2019). On the other hand, the current levels of the ground-level O_3 often suppress growth of trees (Chappelka and Grulke 2016; Li et al. 2018) and change herbivores dynamics (Lindroth 2010; Jamieson et al. 2017). Many studies have previously evaluated the air pollutant impacts on tree growth and/or plant-insect interactions using closed and open-top chambers (e.g. Richard et al. 1988; Jordan et al. 1991; Barger et al. 1992; Kainulainen et al. 1993; Lindroth 2010). Experiments in closed and semi-closed facilities are more suitable for mechanistic studies, however, they are less suitable for studies dealing with ecological aspects such as plant-insect interactions (Paoletti et al. 2007; 2017).

N loading can increase the damage of plants by insect herbivores (Throop and Lerdau 2004). N loading can increase leaf N content and photosynthetic rate to some extent (Sun et al. 2018), whereas it can decrease carbon (C) based defense chemicals (Coley et al. 1985; Koike et al. 2006). Excess N loading can induce suppression of photosynthesis at the individual tree level (Sun et al. 2018) and decline of forests at the ecosystem level (Aer et al. 1998). Higher impacts of $(NH_4)_2SO_4$ deposition on leaves of vegetation through nitrate ammonium deposition, was found (Smart et al. 2004). Since the forest canopy tends to be subjected to relatively higher N deposition, the effect of N deposition on the foliage surface is of concern (Lindberg

and Lovett 1992; Yamaguchi et al. 2014b). Besides, SO_4^{2-} was found to reduce chlorophylls in soybean grown in the presence of H_2SO_4 (Chevone et al. 1986). However, Yamaguchi et al. (2014b) have reported that SO_4^{2-} concentration was not significantly increased in four Asian forest tree species grown under dry particle $(\text{NH}_4)_2\text{SO}_4$ loading. O_3 absorbed via stomata directly inhibits photosynthesis, C-based metabolism and leaf mechanical strength of plants (Li et al. 2016; Watanabe et al. 2018), with potential decreases in the leaf defense capacity and changes in plant–insect relationships (Lindroth 2010; Agathokleous et al. 2017a). The impacts of $(\text{NH}_4)_2\text{SO}_4$ deposition and elevated O_3 on the relationship between plants and insects should be studied concurrently. Nonetheless, there is only one study on the combined effects of $(\text{NH}_4)_2\text{SO}_4$ and O_3 on plant–insect interaction, using a crop plant as a model (Agathokleous et al. 2019a), and, to the best of our knowledge, no study with tree species.

Japanese elm (*Ulmus davidiana* var. *japonica*) is an ecological and economically important tree species in cool temperate forests in Japan (Kon and Okitsu 1995; 1999). The susceptibility of this species to O_3 remains unknown, while it has a moderate susceptibility or resistance to pests (Masuya et al. 2010; Büchel et al. 2016). Recently, the appearance of several types of Ascomycete fungi and declining symptoms of Japanese elm trees were reported in Japan (e.g. Fukui et al. 2007; Yamaguchi et al. 2014a; Masuya et al. 2010; Masuya 2016; Koike 2016). These may relate to changing insect damages induced by environmental changes (Santini et al. 2004; Santini and Faccoli 2015), especially N deposition and O_3 that can change plant-insect interaction and consequently contribute in the decline of forest trees (Koike et al. 2013; Koike 2016). Only three studies have evaluated the leaf defense capacity of Japanese trees in relation to insect dynamics in free-air O_3 -concentration enrichment (FACE) systems (Sakikawa et al. 2016; Agathokleous et al. 2017a; ElEla et al. 2018), and none of them concerns Japanese elm, despite previous researches have reported that grazing activities of elm leaf beetles was increased by elevated O_3 (Richard et al. 1988; Barger et al. 1992).

The aim of this study was to investigate the growth and leaf defense capacity of Japanese elm seedlings treated with N loading and associated insect herbivore dynamics over a growing season in a FACE system. We expected that Japanese elm seedlings will have higher leaf N content due to N loading. The chemical leaf defense capacity depends on C-based photosynthetic products, and N deposition can alter interactions among organisms via higher concentrations of N and lower concentrations of C-based compounds in the plant leaves (Throop and Lerdau 2004; Lindroth 2010). Previous studies have reported that elm leaves with higher N contents were suitable food source for insects, however the leaf defense capacities were not

assessed (Young and Hall 1986; Richard et al. 1988). We expected that this elm may be tolerant to moderate elevation of O₃ levels, similarly to the American elm (Santamour Jr. 1969; Walters and Reich 1989). Therefore, we hypothesized that high N loading, and not O₃, can be the driver of plant-insect interactions, via changes in the phytochemistry, when O₃ concentration is only moderately elevated.

Materials and Methods

Site and plant

This experiment was conducted in the FACE system at Sapporo Experimental Forest of Hokkaido University, Japan (43°04'N, 141°20'E, 15 m a.s.l.), from June to November in 2017. The meteorological data of this experimental site were monitored by the Japan Meteorological Agency, at a nearby station (43°03.6'N, 141°19.7'E). Mean temperature and the total precipitation from May to October 2017 were 17.3 °C and 681 mm, respectively. Snow-free period is May to mid November.

Two-year-old elm (*Ulmus davidiana* var. *japonica*) seedlings were obtained from Hokkaido Horti-Green Co Ltd; seedlings were raised in a nursery under full sunlight at Naganuma town near Sapporo. Seedlings planted in brown forest soil (Dystric Cambisols) in the FACE sites in mid October in the previous year of the treatment. The mean ± s.e. of initial seedling height was 45.5 ± 3.4 cm.

Treatments of N loading and O₃

Regarding the N loading, there were two targeted treatments; 0 kg N ha⁻¹ yr⁻¹ (N0) and 50 kg N ha⁻¹ yr⁻¹ (N50). The amount of N in N50 represents the observed maximum value in Japan (Ministry of the Environment 2004). Given the concerns about the impacts of N deposition on the foliar surface of forest trees canopy (Lindberg and Lovett 1992; Yamaguchi et al. 2014b), N, in the form of ammonium sulfate ((NH₄)₂SO₄), was applied to the foliage using a hand sprayer. A 0.75 % solution of (NH₄)₂SO₄ was applied onto crown leaves of each seedling every approximately 3 days from June to September 2017. The N loading area value was estimated based on the measured ellipse area of the crown leaves in each seedling and the calculated N concentration in the solution. The total amount of N loading was approximately 45.3 kg N ha⁻¹ during the experimental period.

Regarding O₃ treatments, the ambient O₃ (AOZ) and elevated O₃ (EOZ, ≈1.5×AOZ) were set with tree plot replicates for each treatment randomly located in the experimental forest site. The size of treatment-circular plots was 6.5 m in diameter. O₃ was generated from pure oxygen and it diluted with ambient air in a pressurized tank. Then, diluted O₃ released into the rings by 2.5 m Teflon tubes, which were suspended vertically, and two horizontal Teflon tubes, which were fixed around the plots. Ozone monitors (Model 202,

2B Technologies, Boulder, CO, USA, and Aeroqual Ltd., Auckland, NZ) and a Hioki LR5042-20 (HIOKI E.E. Corporation, Nagano, JP) were used for O₃ analysis and data recording. Details of the exposure systems in field station, as well as the plot design, can be found in Agathokleous et al. (2017b). The mean $\pm$ SD monthly O₃ concentrations during the daytime (07:00 to 17:00) of the months May-October were 41.5 $\pm$ 1.19, 36.2 $\pm$ 1.19, 39.0 $\pm$ 0.96, 42.8 $\pm$ 2.27, 42.4 $\pm$ 1.23, 39.3 $\pm$ 1.01 nmol mol⁻¹ in AOZ plots (average= 40.2 nmol mol⁻¹), and 48.1 $\pm$ 2.46, 53.0 $\pm$ 1.21, 63.1 $\pm$ 1.60, 60.7 $\pm$ 1.61, 54.5 $\pm$ 1.44, 47.8 $\pm$ 1.92 nmol mol⁻¹ in EOZ plots (average= 54.5 nmol mol⁻¹), respectively.

Overall, there were 4 treatments (AOZ + N0, AOZ + N50, EOZ + N0, EOZ + N50) with 3 plot replications each (n=3). Eight seedlings were planted in each plot, and half of them (n=4) were subjected to each treatment, for a total of 12 seedlings per treatment.

Measurements

Growth of seedlings

The height and diameter at bottom of the elm seedlings were determined on 1 May and 12 October 2017 using a measuring tape (1 mm gradient) and a Vernier calliper (Mitsutoyo, Kanagawa, Japan). The diameter was taken as the mean of two crosswise measurements at the stem base. Height and diameter growth of the seedlings were calculated as the difference between the final value and the initial value.

Chlorophyll-fluorescence

A pulse amplitude modulated (PAM) fluorescence system (PAM-2000, Walz, Germany) was used to obtain the chlorophyll (Chl) fluorescence reactions of leaves. Based on the basic Chl fluorescence parameters, the maximum quantum yield of PS II (F_v/F_m) can be derived from the equation $F_v/F_m = (F_m - F_o)/F_m$ (Murchie and Lawson 2013). The measurement was conducted from 0:00 to 2:00 on 15 September 2017, when elm leaves have completed their maturation. Sunset occurred at 17:45, therefore, approximately 6 hours was the duration of the dark adaptation of plants (Murchie and Lawson 2013).

Chlorophyll, LMA, leaf carbon and nitrogen content

Ten leaf disks per plant (diameter 5.5 mm) were obtained from mature and non-grazing leaves with a leaf punch (Kokuyo Ltd. Tokyo) on 5th September 2017. Five leaf disks were immediately frozen by liquid N after sampling and they were used for chlorophyll extraction with DMSO (Shinano et al. 1996) determined by a spectrophotometer (GeneSpec II, Hitachi, Tokyo). The remaining 5 disks were put to dry-oven at 65°C for 48 hours. Then, the dry mass of each leaf disk was measured and the LMA was calculated (g m⁻²). These disks were used to measure mass-based leaf C and N contents (C_{mass} , N_{mass}) with a NC

analyzer (Vario EL II, Elementar Elementar Analys system, Germany). The balance of leaf C and N in leaves was calculated as C to N ratio ($C/N \text{ ratio} = C_{\text{mass}}/N_{\text{mass}}$).

Leaf defense chemicals

Three to five leaves without any grazing damages of each plant were sampled on 6th September to measure the total phenolics and the condensed tannin content (hereafter tannin). Collected leaves were immediately frozen by liquid N, then they were put into a freeze-dry machine (EYELA, PDM-2100, Tokyo, Japan). The total phenolics amount was measured by the Folin-Ciocalteu method (Folin and Ciocalteu 1927) modified by Julkunen-Titto (1985), and condensed tannin was measured by butanol-hydrochloric acid-iron assay method (Bate-Smith 1977), based on previous studies (Matsuki et al. 2004; Matsuki and Koike 2006), with use of a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan).

Dry mass

The whole plants were dug out after the senescence was over, at the end of November 2017, and soil around root was washed and eliminated. Plants were separated in different organs; stem, branches, coarse root and fine root (diameter < 2.0 mm). All organs were put into the dry oven at 75°C for 7 days to determine the dry mass. With use of dry mass of each organ, shoot to root ratio (S/R ratio) and relative fine root ratio (RF ratio) were obtained using the equations $S/R \text{ ratio} = (\text{stem} + \text{branches})/(\text{coarse root} + \text{fine root})$ and $RF \text{ ratio} = 100 \times (\text{fine root})/(\text{coarse root} + \text{fine root})$.

Seasonal observation of insect dynamics

The seasonal trends of insect fauna visiting and grazing of the elm seedlings were observed for all the plants and all the plots. Species identification was conducted based on Shimura (2005) and Shinohara et al. (2009). The number of individuals in each insect species was monitored on 6 and 19 Jun, 13 and 20 July, 4 and 11 August, 5 and 19 September in 2017.

Statistical analysis

For each response variable, data were averaged per experimental unit, thus, resulting in three values per O₃ treatment (one per ring) per N treatment for statistical analysis. To approximate a normal distribution, the data of each response variable, except the number of insects, were transformed with a Box-Cox transformation (Box and Cox 1964), as described previously (Agathokleous et al. 2016a). Data of the number of insects and number of insect eggs were log-transformed. The data of each response variable were analyzed by a general linear model (GLM) randomized by plot; O₃ and N were the predictors. The number of leaf beetle individuals was analyzed for the observations on 11 August, when the insect

population reached its maximum according to the seasonal dynamics, and that of elm sawfly was analyzed for the observations on 20 July and 19 September (population peaks), using GLM randomized by plot; O₃, N, and Time were the predictors. F_v/F_m data could not satisfactorily fit a Gaussian distribution and thus the data were subjected to Mann-Whitney U test. MS EXCEL 2010 (Microsoft ©) and STATISTICA v.10 (StatSoft Inc. ©) software were used for data processing and statistics.

Results

Growth and dry mass

There were no significant effects of O₃ on the growth and above-ground dry mass (Table 1). However, EOZ significantly increased RF ratio ($P<0.05$) and marginally increased fine root dry mass ($P=0.05$). On the other hand, N50 significantly decreased the total dry mass of Japanese elm seedlings ($P<0.05$). N50 marginally decreased the dry mass of coarse root ($P=0.05$), and, although non-significant, there was a trend for decreasing the dry mass of above-ground ($P=0.07$). N50 significantly increased the S/R ratio 125.6% compared to N0, independently of O₃ ($P<0.05$). There were no significant interaction effects of EOZ and N50 (Table 1).

Leaf capacities and Chl responses

While C_{mass} was not affected by any treatments, N_{mass} was increased by N50 ($P=0.05$, Fig. 1). Significant changes in LMA and C/N ratio were not observed. Leaf tannin content was significantly decreased by N50 (28.4%, $P<0.01$) and marginally decreased by EOZ ($P=0.06$) whereas total phenolic content was not altered by any treatment. Leaf Chl contents and F_v/F_m were not significantly changed by any treatment (Fig. 2).

Insect dynamics

We observed two major herbivores (Supplementary Materials, Fig. 1S): the elm leaf-beetle (*Pyrrhalta maculicollis*) and the elm sawfly (*Arge captiva*). The seasonal trend of elm leaf-beetle population dynamics was similar among treatments (Fig. 3); the initial dynamics of the population were less than 5 individuals until 20 July. Maximum number of individuals was observed on 11 August, and then the population declined from 5 September. Most obvious difference in individual numbers between treatments was observed on 11 August. On that day, number of elm leaf-beetle on EOZ plants was 3.3 times lower than on AOZ plants, independently of N treatment (Fig. 4A, $P<0.05$).

On the other hand, seasonal trends of elm sawfly population varied with those of elm leaf-beetle and between treatments (Fig. 5); elm sawfly was not observed up to 13 July (across treatments). Except for AOZ + N50, the number of elm sawfly increased on 20 July, with a following decline up to 11 August. After that, however, the number of elm sawfly individuals again increased up to 19 September. The periods of re-increment varied among treatments; the earliest re-increment of individuals was observed in EOZ + N0 (Fig. 5). At the two peaks of the population of elm sawfly, there was a significant effect of N loading (Fig. 4B-C). At the first peak, the elm sawfly population in N50 was 9.4 times lower than that in N0, independently of O₃ treatment (Fig. 4B, $P < 0.01$). The elm sawfly population in September showed the same pattern with that in July (Fig. 4C, $P < 0.05$).

Eggs of elm sawfly were observed on 5 September, while there were no significant differences among treatments. The mean number $\pm$ SD of leaves with the oviposited eggs in EOZ was 11.3 ± 4.67 , which was approximately 3.7 times higher than in AOZ, independently of N treatment ($F = 4.8$, $P = 0.06$). However, there were no significant effects of N50 and no significant N*O₃ interaction (Supplementary Materials, Fig. 2S).

Discussion

Growth responses

N loading promotes tree growth to some extent because it is a fundamental plant nutrient (e.g. Aber et al. 1998). However, excess loading may suppress physiological performance (Sun et al. 2018) and decrease the net primary production of forests (Aber et al. 1998; Galloway et al. 2008). N loading decreased the total dry mass of Japanese elm seedlings in this study, suggesting that the ammonium sulfate loading induced severe stress in the studied plants. Many studies have reported that N loading increased leaf N, as in our experiment, but decreased other nutrient cations such as K, Ca, and Mg and inhibited physiological functions (Nakaji et al. 2001; Elvir et al. 2006; Mao et al. 2018). NH₄⁺-N spray application can lead to toxic concentrations in the leaves and acceleration of the weathering of the leaf cuticle (Van Hove et al. 1991).

Sprayed ammonium sulfate solution provided an extra amount of NH₄⁺ and SO₄²⁻. Previous studies have reported that an excess NH₄⁺ application to leaves could cause physiological disturbances in leaves such as N metabolism and hormonal balance (Britto and Kronzucker 2002; Yamaguchi et al. 2010). In addition, SO₄²⁻, as sulfuric acid, can negatively affect and cause visible foliar injuries and suppress physiological activities of woody species (Van Hove et al. 1991; Matsumura and Izuta 2017). We also

observed visible symptoms at leaf edges in N50 plots. Thus, the present N loading degraded quality as food resources for insect herbivores in this study.

On the other hand, it is suggested that O₃ did not induce severe stress in the studied plants and that plants were tolerant to EOZ due to a number of reasons. This postulation relies upon the fact that most physiological and growth traits as well as the dry mass of Japanese elm seedlings were not significantly affected by EOZ, whereas tannins, as major defense compounds, were increased by EOZ. In addition, fine roots and RF ratio of Japanese elm seedlings were increased by EOZ. As the main role of fine roots is absorbing nutrient and water, the leaf nutrition can be enhanced if the fine root production or allocation is increased (Fujita et al. 2018; Wang et al. 2018). Furthermore, O₃ stress can change C allocation, resulting in altered S/R ratio and fine root production, in a dose-dependent manner (Yamaji et al. 2003; Agathokleous et al. 2016b; 2019b). In this study, the S/R ratio was not altered by EOZ.

Plant and insect responses to N loading

Japanese elm was reported to be resistant or moderately susceptible to insect damage and pathogen (Masuya et al. 2016). We expected that the investigated elm may allocate photosynthates more to plant defense chemicals due to indeterminate shoot developing pattern; developing shoot with leaf flush and continuously expanding of few leaves (Matsuki and Koike 2006). In this study, however, N50 significantly decreased the leaf tannin contents in the investigated elm. Results of dry mass may indicate that N50 reduced photosynthate production, resulting in fewer tannins which are C-based defense compounds. Given that CN balance hypothesis supports these results, the surplus of leaf N might be utilized to produce N-based secondary compounds such as alkaloid rather than C-based compounds (Braynt et al. 1983; Matsuki et al. 2004). A meta-analysis study has investigated the responses of leaf secondary metabolism under N loading (Koricheva et al. 1998; Valkama et al. 2007), suggesting that N loading decreased not only C-based secondary compounds, including tannins, but also phenylpropanoid-derived compounds. Iriti and Franco (2009) have pointed out the relationship between alkaloid synthetic response and N balance under elevated O₃, although there is still lack of empirical data verifying this hypothesis. For further understanding the plant-insect dynamics in elevated O₃, future studies should investigate how plants allocate the surplus of N to several defense compounds.

Insect herbivores prefer grazing leaves with high N content (Lindroth 2010; De Lucia et al. 2012). However, only a limited number of elm sawfly individuals inhabited the plants in N50 plots even though N50 increased N contents and suppressed leaf defense. Noteworthy, a recent field experiment with

cauliflower plants grown in N50 revealed that leaves had highly increased content of nitrates, and further laboratory assays showed that insects fed with such leaves displayed increased mortality (Agathokleous et al. 2019a). The grazing pattern observed in this study may be attributed to the accumulation of sprayed ammonium sulfate, causing visible injuries at leaf edges and perhaps decreasing the food quality for elm sawfly. Given that excess amount of N loading can induce severe foliar injuries, the injured leaves may also be avoided by the herbivores that would be potentially negatively affected (Hughes et al. 1985; Dohmen 1988; Agathokleous et al. 2019a). Our results may also indicate that the elm sawfly feeding dynamics could be suppressed directly by N50 loading regardless of leaf defense capacities.

Plant and insect responses to ozone exposure

Regarding EOZ effects, however, C/N ratio in leaves was not affected by EOZ. Previous studies have reported that there was a relatively high deviation in the O₃ responses of defense compounds such as C-based secondary compounds and phenylpropanoid-derived compounds (Koricheva et al. 1998). EOZ led to significantly lower number of beetles appeared on 11 August even though leaf tannin content was lower. In fact, this phenomenon is in agreement with previous studies in this research field, but with different tree species, where different insect species avoided EOZ sites of FACE systems (Sakikawa et al. 2016; Agathokleous et al. 2018; ElEla Abu et al. 2018). In particular, investigations in the FACE plots showed that a coleopteran leaf beetle (*Agelastica coerulea*) preferred leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) in AOZ over those in EOZ, whereas laboratory assays showed the opposite result. These results indicated that the direct O₃ effects on beetle dynamics and/or indirect effects such as plant-emitted biogenic volatile organic compounds (BVOCs) may explain the field observations (Agathokleous et al. 2017a; Masui and Koike 2018; Fitzky et al. 2019). Shinohara et al. (2009) have reported that the adult elm sawfly are observed from the end of May to the middle of September, and the larvae are observed up to early October in Hokkaido. The feeding damage period is generally observed twice a year, in agreement with our experiment.

Interestingly, there was no difference in the number of sawfly insects between AOZ and EOZ on 20 July (the first peak of the population), whereas there were more eggs in EOZ than in AOZ on 5 September, i.e. two weeks before the second observed peak. This may indicate that there was an increased oviposition in EOZ compared to AOZ. In fact, an analysis of the results of over 200 published studies suggested that leaf feeders can perform better on host plants that are stressed than on plants that are non-stressed, in support to the plant vigor hypothesis (Galway et al., 2004). In agreement to these, adult

beetles of a different species (*Agelastica coerulea*) inhabiting birch trees in this FACE system were found to have higher activities of total antioxidants and lower detoxifying hydrolase such as α - and β -esterases in EOZ than in AOZ, although direct EOZ impacts on insects and indirect effects via plants cannot be separated (Eie et al., 2018). Similarly to O₃, leaf number with oviposited eggs was not affected by N despite there was a lower number of insects on N50 plants than on N0 plants on 20 July and September 19. Therefore, it seems that females had no higher preference for oviposition on N0 plants than on N50 plants. However, in this study, we did not evaluate the insect dynamics per gender or calculate the gender ratio, therefore, further studies will be needed to clarify the effects of O₃ on the population composition from a gender point of view (Richard et al. 1988; Barger et al. 1992; Hummel et al. 1998). It is important to also note that this research does not provide any empirical evidence for differences in population dynamics among treatments due to insect competition (the population dynamics were also asynchronous for the leaf beetle and sawfly). Studies on the role of inter- and intra-specific competition of herbivore insects would be needed, especially because the *per capita rate of population increase*, which measures species fitness, displays a biphasic response to population density (Saitanis and Agathokleous 2019).

Conclusion

Leaf defense capacities of the studied elm seedlings and population dynamics of two insect herbivore species were studied in response to ammonium sulfate loading and O₃ in a FACE system. Increased N deposition may adversely affect this elm species independently from O₃. While foliar N treatment suppressed chemical defense capacities, it could decrease the appearance of elm sawfly on the plants. In conclusion, the results of this study show that O₃ levels 1.5 times the current ambient O₃ levels in Hokkaido, northern Japan, did not adversely affect this elm species; however, they could affect the dynamics of insect herbivores, an issue of ecological significance and potential unpredicted sequences.

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Table 1

Means ($\pm$ SE) of growth, dry mass increment, shoot to root (S/R) ratio, and relative fine root (RF) ratio of elm seedlings treated with 0 (N0) or 50 (N50) kg N ha⁻¹ yr⁻¹ (given as (NH₄)₂SO₄) and ambient (AOZ) or elevated (EOZ) ozone. The data were analyzed by General Linear Model (GLM) at a level of significance $\alpha=0.05$.

	Treatment				P value of GLM		
	AOZ + N0	AOZ + N50	EOZ + N0	EOZ + N50	O ₃	N	O ₃ x N
Height (cm)	17.1 $\pm$ 1.23	17.7 $\pm$ 1.97	22.6 $\pm$ 3.19	22.3 $\pm$ 4.45	n.s.	n.s.	n.s.
Diameter (mm)	2.45 $\pm$ 0.09	2.23 $\pm$ 0.30	2.91 $\pm$ 0.63	2.46 $\pm$ 0.03	n.s.	n.s.	n.s.
Stem (g)	6.07 $\pm$ 0.27	5.58 $\pm$ 1.11	6.32 $\pm$ 0.86	6.60 $\pm$ 0.24	n.s.	n.s.	n.s.
Branches (g)	2.77 $\pm$ 0.34	3.38 $\pm$ 0.69	3.99 $\pm$ 0.60	3.31 $\pm$ 0.42	n.s.	n.s.	n.s.
Above-ground (g)	8.84 $\pm$ 0.43	8.97 $\pm$ 1.73	10.30 $\pm$ 1.34	9.91 $\pm$ 0.42	n.s.	0.07	n.s.
Coarse root (g)	9.21 $\pm$ 0.25	6.91 $\pm$ 0.85	9.65 $\pm$ 1.89	7.37 $\pm$ 0.77	n.s.	0.05	n.s.
Fine root (g)	0.73 $\pm$ 0.15	0.72 $\pm$ 0.11	1.25 $\pm$ 0.07	0.96 $\pm$ 0.25	0.05	n.s.	n.s.
Below-ground (g)	9.93 $\pm$ 0.22	7.63 $\pm$ 0.88	10.90 $\pm$ 1.93	8.34 $\pm$ 0.54	n.s.	n.s.	n.s.
Total dry mass (g)	18.8 $\pm$ 0.34	16.6 $\pm$ 2.60	21.2 $\pm$ 3.23	18.3 $\pm$ 0.90	n.s.	*	n.s.
S/R ratio (g g ⁻¹)	0.90 $\pm$ 0.06	1.19 $\pm$ 0.11	1.00 $\pm$ 0.06	1.20 $\pm$ 0.05	n.s.	*	n.s.
RF ratio (%)	7.33 $\pm$ 1.48	9.62 $\pm$ 1.39	12.09 $\pm$ 1.70	11.98 $\pm$ 3.72	*	n.s.	n.s.

Captions

Fig. 1

Means ($\pm$ SE) of leaf capacities related of elm seedlings treated with 0 (N0) or 50 (N50) kg N ha⁻¹ yr⁻¹ (given as (NH₄)₂SO₄) and ambient (AOZ) or elevated (EOZ) ozone. The data were analyzed by General Linear Model (GLM) at a level of significance $\alpha=0.05$.

Fig. 2

Means ($\pm$ SE) of maximum quantum yield of PSII (F_v/F_m) and chlorophyll contents (Chl a, Chl b, Chl a+b) of elm seedlings treated with 0 (N0) or 50 (N50) kg N ha⁻¹ yr⁻¹ (given as (NH₄)₂SO₄) and ambient (AOZ) or elevated (EOZ) ozone. The data of F_v/F_m were analysed by Mann-Whitney U test, whereas the data of chlorophyll contents were analyzed by General Linear Model (GLM) at a level of significance $\alpha=0.05$.

Fig. 3

Number of individuals of leaf beetle (*Pyrrhalta maculicollis*) grazing on elm seedlings treated with 0 (N0) or 50 (N50) kg N ha⁻¹ yr⁻¹ (given as (NH₄)₂SO₄) and ambient (AOZ) or elevated (EOZ) ozone. Each point and error bar mean the mean value $\pm$ SE of the population for each treatment (n=3).

Fig. 4

Means ($\pm$ SE) of the observed total number of leaf beetle (*Pyrrhalta maculicollis*, A) on 11 August, and elm sawfly (*Arge captiva*, B,C) on 20 July and 19 September on elm seedlings treated with 0 (N0) or 50 (N50) kg N ha⁻¹ yr⁻¹ (given as (NH₄)₂SO₄) and ambient (AOZ) or elevated (EOZ) ozone. The data were analyzed by General Linear Model (GLM) at a level of significance $\alpha=0.05$.

Fig. 5

Number of individuals of elm sawfly (*Arge captiva*) grazing on Japanese elm seedlings treated with 0 (N0) or 50 (N50) kg N ha⁻¹ yr⁻¹ (given as (NH₄)₂SO₄) and ambient (AOZ) or elevated (EOZ) ozone. Each point and error bar mean the mean value $\pm$ SE of the population for each treatment (n=3).

Fig.1

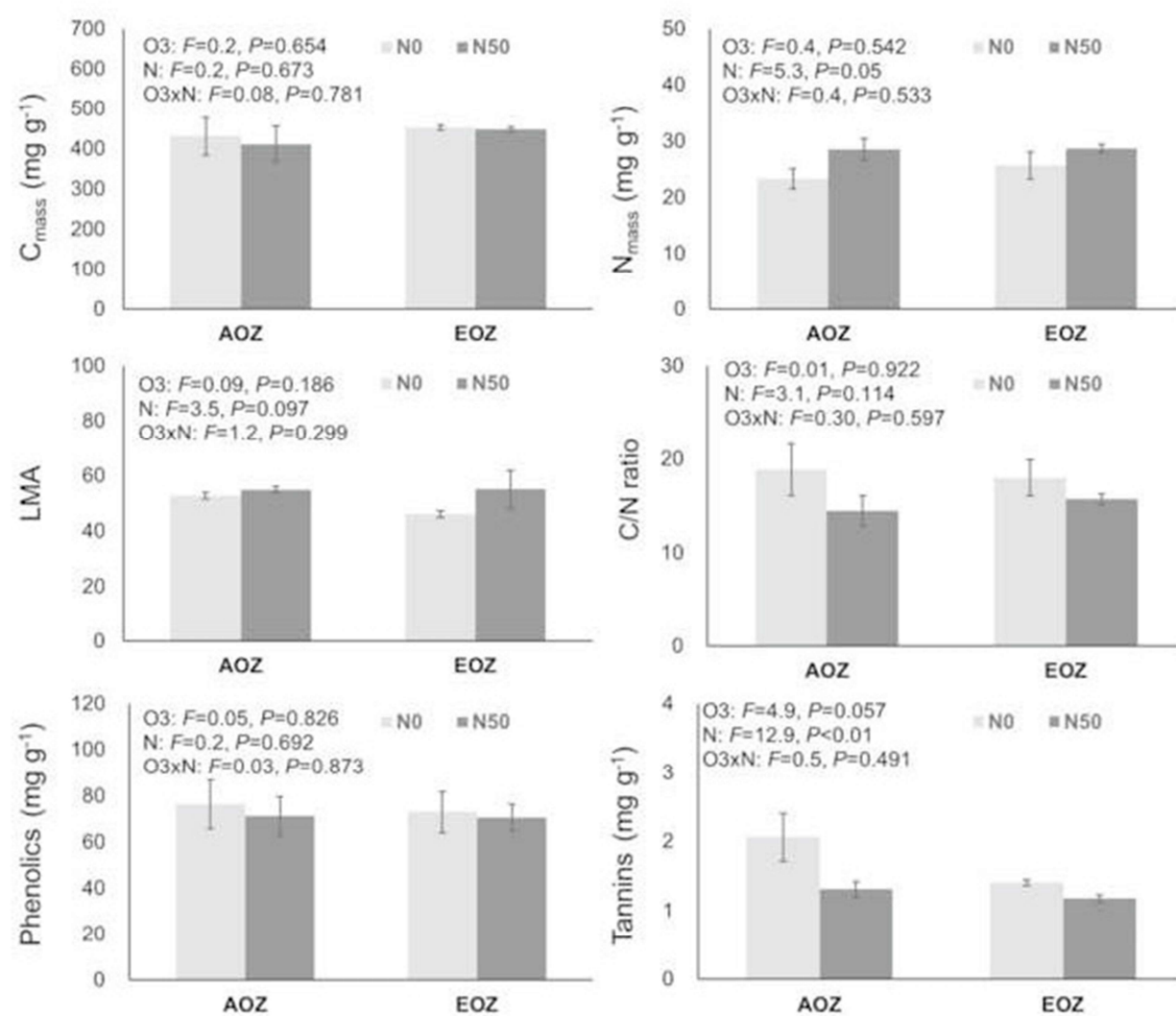
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Fig.2

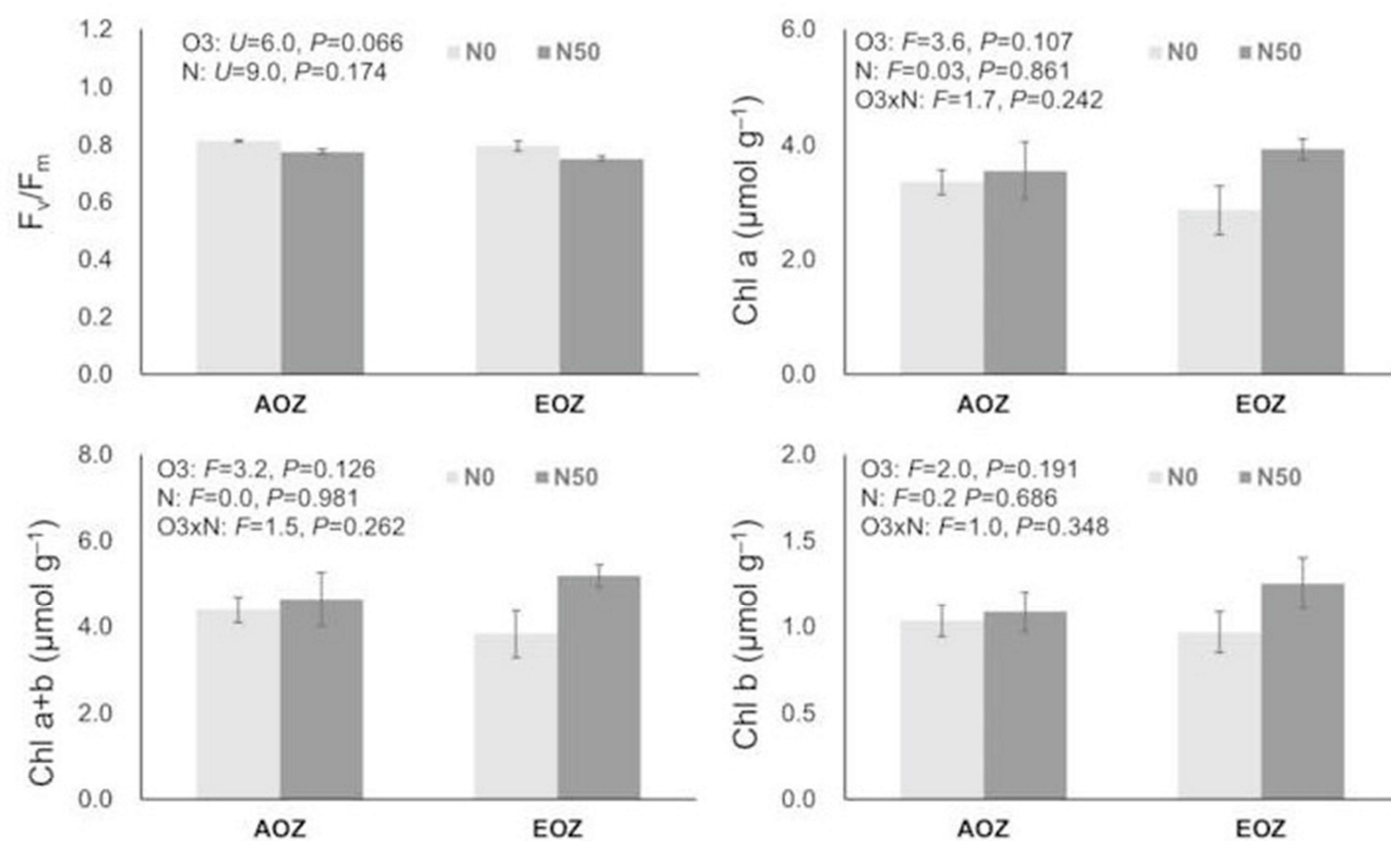
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Fig.3

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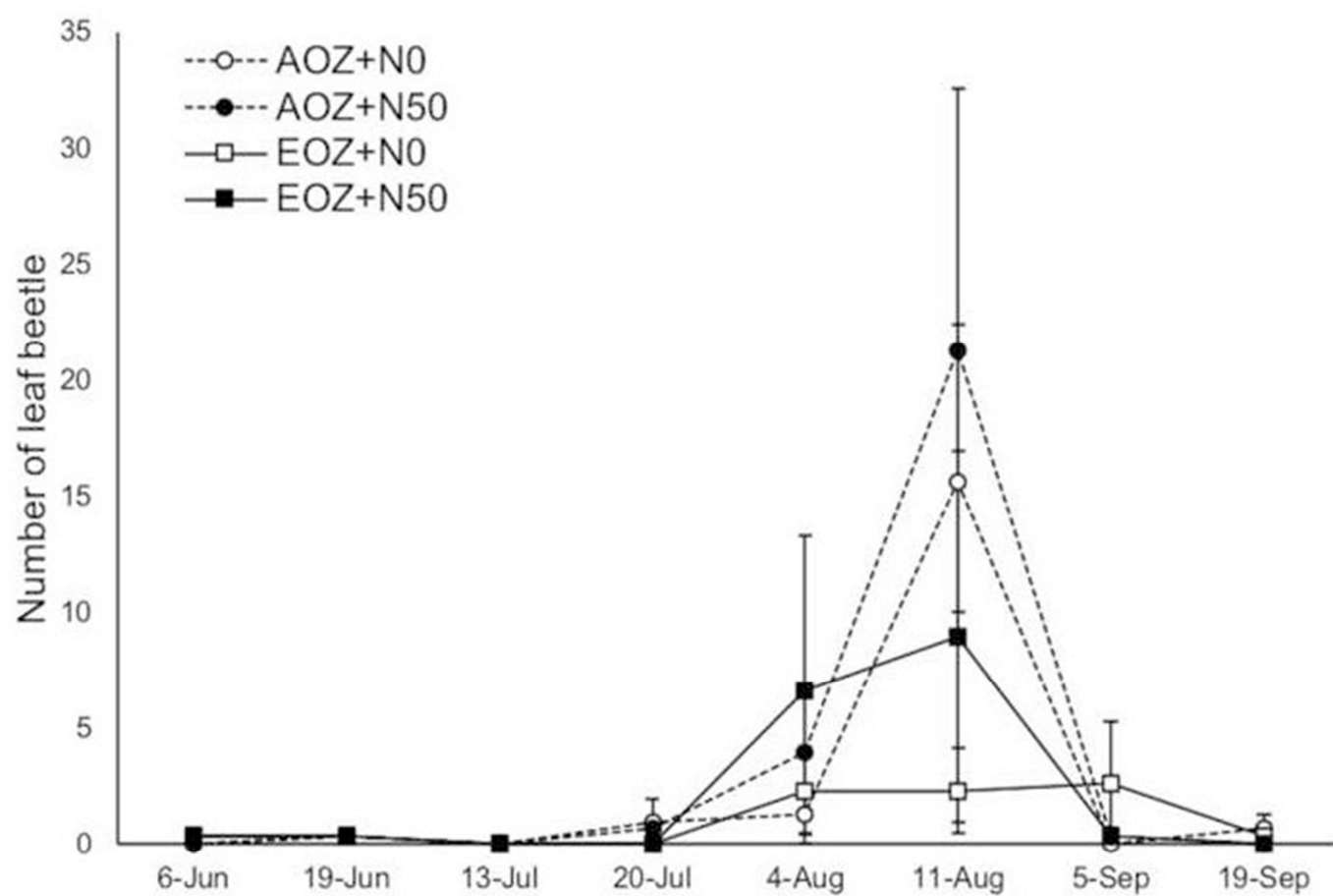


Fig. 4

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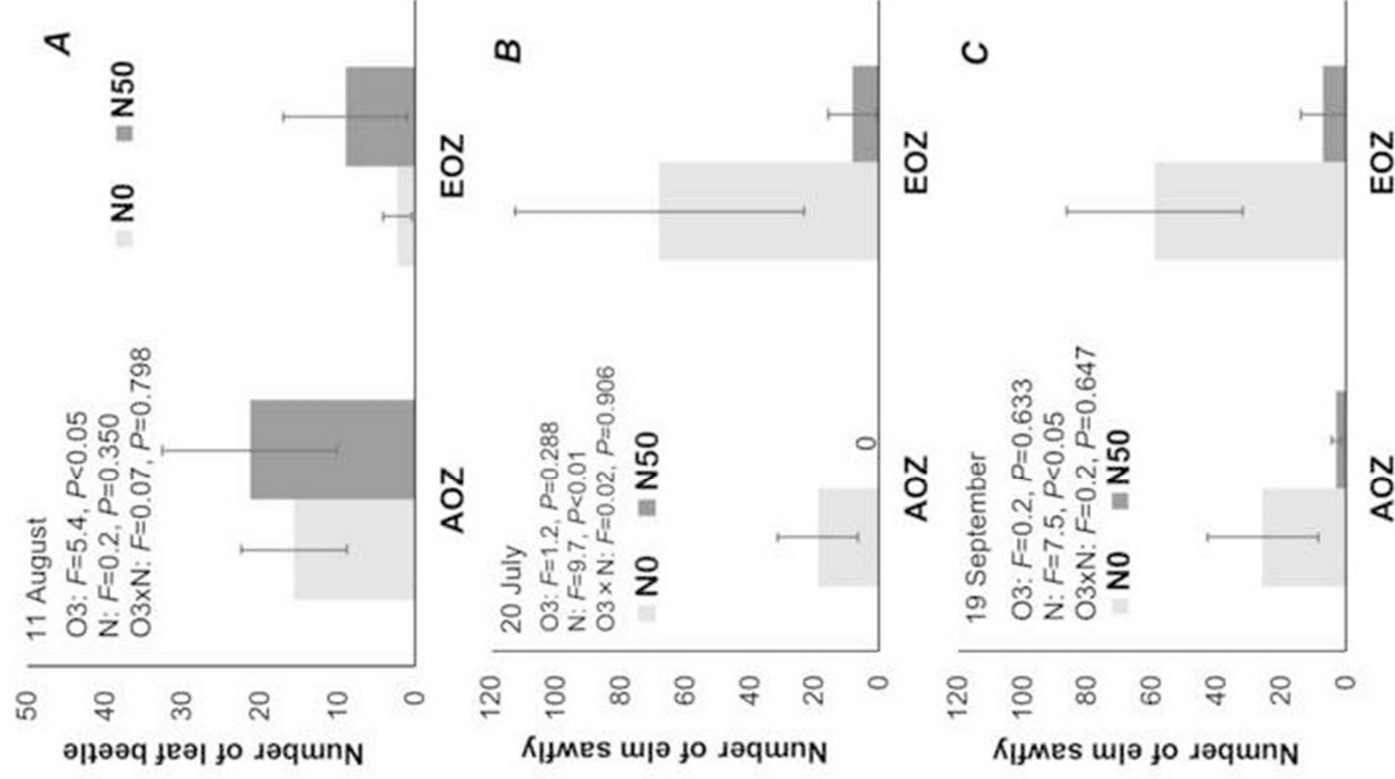


Fig.5

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