



Title	Leaf defense capacity of Japanese elm ( <i>Ulmus davidiana</i> var. <i>japonica</i> ) seedlings subjected to a nitrogen loading and insect herbivore dynamics in a free air ozone-enriched environment
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1 **Leaf defense capacity of Japanese elm (*Ulmus davidiana* var. *japonica*) seedlings**  
2 **subjected to a nitrogen loading and insect herbivore dynamics in a free air ozone-**  
3 **enriched environment**

4  
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31 **Abstract**

32 Japanese elm (*Ulmus davidiana* var. *japonica*) is a native species in cool temperate forests in Japan. We  
33 investigated growth, physiological reactions, and leaf defense capacity of Japanese elm seedlings under  
34 nitrogen (N) loading (45.3 kg N ha<sup>-1</sup> yr<sup>-1</sup>) and seasonal insect dynamics in a free-air ozone (O<sub>3</sub>)-enriched  
35 environment (about 54.5 nmol O<sub>3</sub> mol<sup>-1</sup>) over a growing season. Higher leaf N content and lower condensed  
36 tannin content under N loading and lower condensed tannin content in elevated O<sub>3</sub> were observed,  
37 suggesting that both N loading and elevated O<sub>3</sub> decreased the leaf defense capacity and that N loading  
38 further enhanced the leaf quality as food resource of insect herbivores. Two major herbivores were observed  
39 on the plants, elm leaf-beetle (*Pyrrhalta maculicollis*) and elm sawfly (*Arge captiva*). The peak number of  
40 observed insects was decreased by N loading. Visible foliar injury caused by N loading might directly  
41 induce the reduction of number of the observed elm sawfly individuals. While elevated O<sub>3</sub> slightly  
42 suppressed the chemical defense capacity, significantly lower number of elm leaf-beetle was observed in  
43 elevated O<sub>3</sub>. We conclude that N loading and elevated O<sub>3</sub> can alter not only the leaf defense capacity of  
44 Japanese elm seedlings, but also the dynamics of elm leaf-beetle and sawfly herbivores.

45

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

48

49 **Abbreviations**

50 C<sub>mass</sub> – carbon content per unit leaf mass; C/N ratio – carbon to nitrogen ratio; Chl – chlorophyll; F<sub>v</sub>/F<sub>m</sub> –  
51 maximum quantum yield of PSII; LMA – leaf mass area; N – nitrogen; N<sub>mass</sub> – nitrogen content per unit  
52 leaf mass; T/R ratio – top to root ratio; RF ratio – relative fine root ratio.

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## 60 **Introduction**

61 The ongoing climate change may induce decline of forest ecosystems in several regions of the globe  
62 (Allen et al. 2010; Cayan et al. 2010; Wang et al. 2013; Steigvilė et al. 2018), with negative direct impacts  
63 on trees and changes in the outbreaks and dynamics of forest insects (Breshears et al. 2005; Jactel et al.  
64 2012; De Lucia et al. 2012) and tree pathogens (Moore and Allard 2008; Masuya 2016). Hence, studies are  
65 needed to clarify the effects of atmospheric changes, such as global warming and air pollution, on the  
66 relationship between trees and insect dynamics (Santini et al. 2004; Ghelardini et al. 2010; Lindroth 2010;  
67 Chappelka and Grulke 2016).

68 Nitrogen (N) deposition and ground-level ozone (O<sub>3</sub>) concentrations are elevated in northeast Asia due  
69 to the economic development (Galloway et al. 2008; Ainworth et al. 2012; Hatakeyama 2017; Akimoto  
70 2017; Li et al. 2018; Tian et al. 2018). Higher levels of N deposition and O<sub>3</sub> may also occur in the future  
71 (Yamaji et al. 2008; Fiore et al. 2012; Liu et al. 2018). In particular, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> deposition has been of  
72 concern in northeast Asia (Hatakeyama 2017; Yamaguchi et al. 2014b; Matsumura and Izuta 2017; Liu et  
73 al. 2018). Oxidated sulfur species and SO<sub>4</sub><sup>2-</sup>, which can easily be transported long distance, are mainly  
74 emitted from industrial sources in Asia (Aikawa et al. 2013; Noguchi and Yamaguchi 2019), whereas  
75 ammonia and NH<sub>4</sub><sup>+</sup> particle result from other sources including fertilizers used in agricultural practice and  
76 livestock fields (UNEP 2019). On the other hand, the current levels of the ground-level O<sub>3</sub> often suppress  
77 growth of trees (Chappelka and Grulke 2016; Li et al. 2018) and change herbivores dynamics (Lindroth  
78 2010; Jamieson et al. 2017). Many studies have previously evaluated the air pollutant impacts on tree  
79 growth and/or plant-insect interactions using closed and open-top chambers (e.g. Richard et al. 1988; Jordan  
80 et al. 1991; Barger et al. 1992; Kainulainen et al. 1993; Lindroth 2010). Experiments in closed and semi-  
81 closed facilities are more suitable for mechanistic studies, however, they are less suitable for studies dealing  
82 with ecological aspects such as plant-insect interactions (Paoletti et al. 2007; 2017).

83 N loading can increase the damage of plants by insect herbivores (Throop and Lerdau 2004). N loading  
84 can increase leaf N content and photosynthetic rate to some extent (Sun et al. 2018), whereas it can decrease  
85 carbon (C) based defense chemicals (Coley et al. 1985; Koike et al. 2006). Excess N loading can induce  
86 suppression of photosynthesis at the individual tree level (Sun et al. 2018) and decline of forests at the  
87 ecosystem level (Aer et al. 1998). Higher impacts of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> deposition on leaves of vegetation through  
88 nitrate ammonium deposition, was found (Smart et al. 2004). Since the forest canopy tends to be subjected  
89 to relatively higher N deposition, the effect of N deposition on the foliage surface is of concern (Lindberg

90 and Lovett 1992; Yamaguchi et al. 2014b). Besides,  $\text{SO}_4^{2-}$  was found to reduce chlorophylls in soybean  
91 grown in the presence of  $\text{H}_2\text{SO}_4$  (Chevone et al. 1986). However, Yamaguchi et al. (2014b) have reported  
92 that  $\text{SO}_4^{2-}$  concentration was not significantly increased in four Asian forest tree species grown under dry  
93 particle  $(\text{NH}_4)_2\text{SO}_4$  loading.  $\text{O}_3$  absorbed via stomata directly inhibits photosynthesis, C-based metabolism  
94 and leaf mechanical strength of plants (Li et al. 2016; Watanabe et al. 2018), with potential decreases in the  
95 leaf defense capacity and changes in plant–insect relationships (Lindroth 2010; Agathokleous et al. 2017a).  
96 The impacts of  $(\text{NH}_4)_2\text{SO}_4$  deposition and elevated  $\text{O}_3$  on the relationship between plants and insects should  
97 be studied concurrently. Nonetheless, there is only one study on the combined effects of  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{O}_3$   
98 on plant–insect interaction, using a crop plant as a model (Agathokleous et al. 2019a), and, to the best of  
99 our knowledge, no study with tree species.

100 Japanese elm (*Ulmus davidiana* var. *japonica*) is an ecological and economically important tree  
101 species in cool temperate forests in Japan (Kon and Okitsu 1995; 1999). The susceptibility of this species  
102 to  $\text{O}_3$  remains unknown, while it has a moderate susceptibility or resistance to pests (Masuya et al. 2010;  
103 Büchel et al. 2016). Recently, the appearance of several types of Ascomycete fungi and declining symptoms  
104 of Japanese elm trees were reported in Japan (e.g. Fukui et al. 2007; Yamaguchi et al. 2014a; Masuya et al.  
105 2010; Masuya 2016; Koike 2016). These may relate to changing insect damages induced by environmental  
106 changes (Santini et al. 2004; Santini and Faccoli 2015), especially N deposition and  $\text{O}_3$  that can change  
107 plant-insect interaction and consequently contribute in the decline of forest trees (Koike et al. 2013; Koike  
108 2016). Only three studies have evaluated the leaf defense capacity of Japanese trees in relation to insect  
109 dynamics in free-air  $\text{O}_3$ -concentration enrichment (FACE) systems (Sakikawa et al. 2016; Agathokleous et  
110 al. 2017a; EIEla et al. 2018), and none of them concerns Japanese elm, despite previous researches have  
111 reported that grazing activities of elm leaf beetles was increased by elevated  $\text{O}_3$  (Richard et al. 1988; Barger  
112 et al. 1992).

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

120 assessed (Young and Hall 1986; Richard et al. 1988). We expected that this elm may be tolerant to moderate  
121 elevation of O<sub>3</sub> levels, similarly to the American elm (Santamour Jr. 1969; Walters and Reich 1989).  
122 Therefore, we hypothesized that high N loading, and not O<sub>3</sub>, can be the driver of plant-insect interactions,  
123 via changes in the phytochemistry, when O<sub>3</sub> concentration is only moderately elevated.

## 124 **Materials and Methods**

### 125 **Site and plant**

126 This experiment was conducted in the FACE system at Sapporo Experimental Forest of Hokkaido  
127 University, Japan (43°04'N, 141°20'E, 15 m a.s.l.), from June to November in 2017. The meteorological  
128 data of this experimental site were monitored by the Japan Meteorological Agency, at a nearby station  
129 (43°03.6'N, 141°19.7'E). Mean temperature and the total precipitation from May to October 2017 were  
130 17.3 °C and 681 mm, respectively. Snow-free period is May to mid November.

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

### 135 **Treatments of N loading and O<sub>3</sub>**

136 Regarding the N loading, there were two targeted treatments; 0 kg N ha<sup>-1</sup> yr<sup>-1</sup> (N0) and 50 kg N ha<sup>-1</sup>  
137 yr<sup>-1</sup> (N50). The amount of N in N50 represents the observed maximum value in Japan (Ministry of the  
138 Environment 2004). Given the concerns about the impacts of N deposition on the foliar surface of forest  
139 trees canopy (Lindberg and Lovett 1992; Yamaguchi et al. 2014b), N, in the form of ammonium sulfate  
140 ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), was applied to the foliage using a hand sprayer. A 0.75 % solution of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was applied  
141 onto crown leaves of each seedling every approximately 3 days from June to September 2017. The N  
142 loading area value was estimated based on the measured ellipse area of the crown leaves in each seedling  
143 and the calculated N concentration in the solution. The total amount of N loading was approximately 45.3  
144 kg N ha<sup>-1</sup> during the experimental period.

145 Regarding O<sub>3</sub> treatments, the ambient O<sub>3</sub> (AOZ) and elevated O<sub>3</sub> (EOZ, ≈1.5×AOZ) were set with tree  
146 plot replicates for each treatment randomly located in the experimental forest site. The size of treatment-  
147 circular plots was 6.5 m in diameter. O<sub>3</sub> was generated from pure oxygen and it diluted with ambient air in  
148 a pressurized tank. Then, diluted O<sub>3</sub> released into the rings by 2.5 m Teflon tubes, which were suspended  
149 vertically, and two horizontal Teflon tubes, which were fixed around the plots. Ozone monitors (Model 202,

150 2B Technologies, Boulder, CO, USA, and Aeroqual Ltd., Auckland, NZ) and a Hioki LR5042-20 (HIOKI  
151 E.E. Corporation, Nagano, JP) were used for O<sub>3</sub> analysis and data recording. Details of the exposure systems  
152 in field station, as well as the plot design, can be found in Agathokleous et al. (2017b). The mean ± SD  
153 monthly O<sub>3</sub> concentrations during the daytime (07:00 to 17:00) of the months May-October were 41.5 ±  
154 1.19, 36.2 ± 1.19, 39.0 ± 0.96, 42.8 ± 2.27, 42.4 ± 1.23, 39.3 ± 1.01 nmol mol<sup>-1</sup> in AOZ plots (average=  
155 40.2 nmol mol<sup>-1</sup>), and 48.1 ± 2.46, 53.0 ± 1.21, 63.1 ± 1.60, 60.7 ± 1.61, 54.5 ± 1.44, 47.8 ± 1.92 nmol mol<sup>-1</sup>  
156 in EOZ plots (average= 54.5 nmol mol<sup>-1</sup>), respectively.

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

## 160 **Measurements**

### 161 **Growth of seedlings**

162 The height and diameter at bottom of the elm seedlings were determined on 1 May and 12 October  
163 2017 using a measuring tape (1 mm gradient) and a Vernier calliper (Mitsutoyo, Kanagawa, Japan). The  
164 diameter was taken as the mean of two crosswise measurements at the stem base. Height and diameter  
165 growth of the seedlings were calculated as the difference between the final value and the initial value.

### 166 **Chlorophyll-fluorescence**

167 A pulse amplitude modulated (PAM) fluorescence system (PAM-2000, Walz, Germany) was used to  
168 obtain the chlorophyll (Chl) fluorescence reactions of leaves. Based on the basic Chl fluorescence  
169 parameters, the maximum quantum yield of PS II ( $F_v/F_m$ ) can be derived from the equation  $F_v/F_m = (F_m -$   
170  $F_o)/F_m$  (Murchie and Lawson 2013). The measurement was conducted from 0:00 to 2:00 on 15 September  
171 2017, when elm leaves have completed their maturation. Sunset occurred at 17:45, therefore, approximately  
172 6 hours was the duration of the dark adaptation of plants (Murchie and Lawson 2013).

### 173 **Chlorophyll, LMA, leaf carbon and nitrogen content**

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

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

## 182 **Leaf defense chemicals**

183 Three to five leaves without any grazing damages of each plant were sampled on 6<sup>th</sup> September to  
184 measure the total phenolics and the condensed tannin content (hereafter tannin). Collected leaves were  
185 immediately frozen by liquid N, then they were put into a freeze-dry machine (EYELA, PDM-2100, Tokyo,  
186 Japan). The total phenolics amount was measured by the Folin-Ciocalten method (Folin and Ciocalteu  
187 1927) modified by Julkunen-Titto (1985), and condensed tannin was measured by butanol-hydrochloric  
188 acid-iron assay method (Bate-Smith 1977), based on previous studies (Matsuki et al. 2004; Matsuki and  
189 Koike 2006), with use of a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan).

## 190 **Dry mass**

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

## 197 **Seasonal observation of insect dynamics**

198 The seasonal trends of insect fauna visiting and grazing of the elm seedlings were observed for all the  
199 plants and all the plots. Species identification was conducted based on Shimura (2005) and Shinohara et al.  
200 (2009). The number of individuals in each insect species was monitored on 6 and 19 Jun, 13 and 20 July, 4  
201 and 11 August, 5 and 19 September in 2017.

## 202 **Statistical analysis**

203 For each response variable, data were averaged per experimental unit, thus, resulting in three values  
204 per O<sub>3</sub> treatment (one per ring) per N treatment for statistical analysis. To approximate a normal distribution,  
205 the data of each response variable, except the number of insects, were transformed with a Box-Cox  
206 transformation (Box and Cox 1964), as described previously (Agathokleous et al. 2016a). Data of the  
207 number of insects and number of insect eggs were log-transformed. The data of each response variable  
208 were analyzed by a general linear model (GLM) randomized by plot; O<sub>3</sub> and N were the predictors. The  
209 number of leaf beetle individuals was analyzed for the observations on 11 August, when the insect



210 population reached its maximum according to the seasonal dynamics, and that of elm sawfly was analyzed  
211 for the observations on 20 July and 19 September (population peaks), using GLM randomized by plot; O<sub>3</sub>,  
212 N, and Time were the predictors. F<sub>v</sub>/F<sub>m</sub> data could not satisfactorily fit a Gaussian distribution and thus the  
213 data were subjected to Mann-Whitney U test. MS EXCEL 2010 (Microsoft ©) and STATISTICA v.10  
214 (StatSoft Inc. ©) software were used for data processing and statistics.

## 215 **Results**

### 216 **Growth and dry mass**

217 There were no significant effects of O<sub>3</sub> on the growth and above-ground dry mass (Table 1).  
218 However, EOZ significantly increased RF ratio ( $P<0.05$ ) and marginally increased fine root dry mass  
219 ( $P=0.05$ ). On the other hand, N50 significantly decreased the total dry mass of Japanese elm seedlings  
220 ( $P<0.05$ ). N50 marginally decreased the dry mass of coarse root ( $P=0.05$ ), and, although non-significant,  
221 there was a trend for decreasing the dry mass of above-ground ( $P=0.07$ ). N50 significantly increased the  
222 S/R ratio 125.6% compared to N0, independently of O<sub>3</sub> ( $P<0.05$ ). There were no significant interaction  
223 effects of EOZ and N50 (Table 1).

### 224 **Leaf capacities and Chl responses**

225 While C<sub>mass</sub> was not affected by any treatments, N<sub>mass</sub> was increased by N50 ( $P=0.05$ , Fig. 1).  
226 Significant changes in LMA and C/N ratio were not observed. Leaf tannin content was significantly  
227 decreased by N50 (28.4%,  $P<0.01$ ) and marginally decreased by EOZ ( $P=0.06$ ) whereas total phenolic  
228 content was not altered by any treatment. Leaf Chl contents and F<sub>v</sub>/F<sub>m</sub> were not significantly changed by  
229 any treatment (Fig. 2).

### 230 **Insect dynamics**

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

238 On the other hand, seasonal trends of elm sawfly population varied with those of elm leaf-beetle and  
239 between treatments (Fig. 5); elm sawfly was not observed up to 13 July (across treatments). Except for  
240 AOZ + N50, the number of elm sawfly increased on 20 July, with a following decline up to 11 August.  
241 After that, however, the number of elm sawfly individuals again increased up to 19 September. The  
242 periods of re-increment varied among treatments; the earliest re-increment of individuals was observed in  
243 EOZ + N0 (Fig. 5). At the two peaks of the population of elm sawfly, there was a significant effect of N  
244 loading (Fig. 4B-C). At the first peak, the elm sawfly population in N50 was 9.4 times lower than that in  
245 N0, independently of O<sub>3</sub> treatment (Fig. 4B,  $P < 0.01$ ). The elm sawfly population in September showed  
246 the same pattern with that in July (Fig. 4C,  $P < 0.05$ ).

247 Eggs of elm sawfly were observed on 5 September, while there were no significant differences  
248 among treatments. The mean number  $\pm$  SD of leaves with the oviposited eggs in EOZ was  $11.3 \pm 4.67$ ,  
249 which was approximately 3.7 times higher than in AOZ, independently of N treatment ( $F = 4.8$ ,  $P = 0.06$ ).  
250 However, there were no significant effects of N50 and no significant N\*O<sub>3</sub> interaction (Supplementary  
251 Materials, Fig. 2S).

## 252 **Discussion**

### 253 **Growth responses**

254 N loading promotes tree growth to some extent because it is a fundamental plant nutrient (e.g. Aber et  
255 al. 1998). However, excess loading may suppress physiological performance (Sun et al. 2018) and  
256 decrease the net primary production of forests (Aber et al. 1998; Galloway et al. 2008). N loading  
257 decreased the total dry mass of Japanese elm seedlings in this study, suggesting that the ammonium  
258 sulfate loading induced severe stress in the studied plants. Many studies have reported that N loading  
259 increased leaf N, as in our experiment, but decreased other nutrient cations such as K, Ca, and Mg and  
260 inhibited physiological functions (Nakaji et al. 2001; Elvir et al. 2006; Mao et al. 2018). NH<sub>4</sub><sup>+</sup>-N spray  
261 application can lead to toxic concentrations in the leaves and acceleration of the weathering of the leaf  
262 cuticle (Van Hove et al. 1991).

263 Sprayed ammonium sulfate solution provided an extra amount of NH<sub>4</sub><sup>+</sup> and SO<sub>4</sub><sup>2-</sup>. Previous studies  
264 have reported that an excess NH<sub>4</sub><sup>+</sup> application to leaves could cause physiological disturbances in leaves  
265 such as N metabolism and hormonal balance (Britto and Kronzucker 2002; Yamaguchi et al. 2010). In  
266 addition, SO<sub>4</sub><sup>2-</sup>, as sulfuric acid, can negatively affect and cause visible foliar injuries and suppress  
267 physiological activities of woody species (Van Hove et al. 1991; Matsumura and Izuta 2017). We also

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

270 On the other hand, it is suggested that O<sub>3</sub> did not induce severe stress in the studied plants and that  
271 plants were tolerant to EOZ due to a number of reasons. This postulation relies upon the fact that most  
272 physiological and growth traits as well as the dry mass of Japanese elm seedlings were not significantly  
273 affected by EOZ, whereas tannins, as major defense compounds, were increased by EOZ. In addition, fine  
274 roots and RF ratio of Japanese elm seedlings were increased by EOZ. As the main role of fine roots is  
275 absorbing nutrient and water, the leaf nutrition can be enhanced if the fine root production or allocation is  
276 increased (Fujita et al. 2018; Wang et al. 2018). Furthermore, O<sub>3</sub> stress can change C allocation, resulting  
277 in altered S/R ratio and fine root production, in a dose-dependent manner (Yamaji et al. 2003;  
278 Agathokleous et al. 2016b; 2019b). In this study, the S/R ratio was not altered by EOZ.

#### 279 **Plant and insect responses to N loading**

280 Japanese elm was reported to be resistant or moderately susceptible to insect damage and pathogen  
281 (Masuya et al. 2016). We expected that the investigated elm may allocate photosynthates more to plant  
282 defense chemicals due to indeterminate shoot developing pattern; developing shoot with leaf flush and  
283 continuously expanding of few leaves (Matsuki and Koike 2006). In this study, however, N50 significantly  
284 decreased the leaf tannin contents in the investigated elm. Results of dry mass may indicate that N50  
285 reduced photosynthate production, resulting in fewer tannins which are C-based defense compounds.  
286 Given that CN balance hypothesis supports these results, the surplus of leaf N might be utilized to  
287 produce N-based secondary compounds such as alkaloid rather than C-based compounds (Braynt et al.  
288 1983; Matsuki et al. 2004). A meta-analysis study has investigated the responses of leaf secondary  
289 metabolism under N loading (Koricheva et al. 1998; Valkama et al. 2007), suggesting that N loading  
290 decreased not only C-based secondary compounds, including tannins, but also phenylpropanoid-derived  
291 compounds. Iriti and Franco (2009) have pointed out the relationship between alkaloid synthetic response  
292 and N balance under elevated O<sub>3</sub>, although there is still lack of empirical data verifying this hypothesis.  
293 For further understanding the plant-insect dynamics in elevated O<sub>3</sub>, future studies should investigate how  
294 plants allocate the surplus of N to several defense compounds.

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

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

### 306 **Plant and insect responses to ozone exposure**

307       Regarding EOZ effects, however, C/N ratio in leaves was not affected by EOZ. Previous studies  
308 have reported that there was a relatively high deviation in the O<sub>3</sub> responses of defense compounds such as  
309 C-based secondary compounds and phenylpropanoid-derived compounds (Koricheva et al. 1998). EOZ  
310 led to significantly lower number of beetles appeared on 11 August even though leaf tannin content was  
311 lower. In fact, this phenomenon is in agreement with previous studies in this research field, but with  
312 different tree species, where different insect species avoided EOZ sites of FACE systems (Sakikawa et al.  
313 2016; Agathokleous et al. 2018; EIEla Abu et al. 2018). In particular, investigations in the FACE plots  
314 showed that a coleopteran leaf beetle (*Agelastica coerulea*) preferred leaves of Japanese white birch  
315 (*Betula platyphylla* var. *japonica*) in AOZ over those in EOZ, whereas laboratory assays showed the  
316 opposite result. These results indicated that the direct O<sub>3</sub> effects on beetle dynamics and/or indirect effects  
317 such as plant-emitted biogenic volatile organic compounds (BVOCs) may explain the field observations  
318 (Agathokleous et al. 2017a; Masui and Koike 2018; Fitzky et al. 2019). Shinohara et al. (2009) have  
319 reported that the adult elm sawfly are observed from the end of May to the middle of September, and the  
320 larvae are observed up to early October in Hokkaido. The feeding damage period is generally observed  
321 twice a year, in agreement with our experiment.

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

328 beetles of a different species (*Agelastica coerulea*) inhabiting birch trees in this FACE system were found  
329 to have higher activities of total antioxidants and lower detoxifying hydrolase such as  $\alpha$ - and  $\beta$ -esterases  
330 in EOZ than in AOZ, although direct EOZ impacts on insects and indirect effects via plants cannot be  
331 separated (EIEla et al., 2018). Similarly to O<sub>3</sub>, leaf number with oviposited eggs was not affected by N  
332 despite there was a lower number of insects on N50 plants than on N0 plants on 20 July and September  
333 19. Therefore, it seems that females had no higher preference for oviposition on N0 plants than on N50  
334 plants. However, in this study, we did not evaluate the insect dynamics per gender or calculate the gender  
335 ratio, therefore, further studies will be needed to clarify the effects of O<sub>3</sub> on the population composition  
336 from a gender point of view (Richard et al. 1988; Barger et al. 1992; Hummel et al. 1998). It is important  
337 to also note that this research does not provide any empirical evidence for differences in population  
338 dynamics among treatments due to insect competition (the population dynamics were also asynchronous  
339 for the leaf beetle and sawfly). Studies on the role of inter- and intra-specific competition of herbivore  
340 insects would be needed, especially because the *per capita rate of population increase*, which measures  
341 species fitness, displays a biphasic response to population density (Saitanis and Agathokleous 2019).

#### 342 **Conclusion**

343 Leaf defense capacities of the studied elm seedlings and population dynamics of two insect herbivore  
344 species were studied in response to ammonium sulfate loading and O<sub>3</sub> in a FACE system. Increased N  
345 deposition may adversely affect this elm species independently from O<sub>3</sub>. While foliar N treatment  
346 suppressed chemical defense capacities, it could decrease the appearance of elm sawfly on the plants. In  
347 conclusion, the results of this study show that O<sub>3</sub> levels 1.5 times the current ambient O<sub>3</sub> levels in  
348 Hokkaido, northern Japan, did not adversely affect this elm species; however, they could affect the  
349 dynamics of insect herbivores, an issue of ecological significance and potential unpredicted sequences.

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355 (JSPS). JSPS is a non-profit, independent administrative institution.

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598 **Table 1**

599 Means ( $\pm$ SE) of growth, dry mass increment, shoot to root (S/R) ratio, and relative fine  
 600 root (RF) ratio of elm seedlings treated with 0 (N0) or 50 (N50) kg N ha<sup>-1</sup> yr<sup>-1</sup> (given as  
 601 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) and ambient (AOZ) or elevated (EOZ) ozone. The data were analyzed by  
 602 General Linear Model (GLM) at a level of significance  $\alpha=0.05$ .

603

	Treatment				P value of GLM		
	AOZ + N0	AOZ + N50	EOZ + N0	EOZ + N50	O <sub>3</sub>	N	O <sub>3</sub> 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 <sup>-1</sup> )	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.

604

605

606 **Captions**

607 **Fig. 1**

608 Means ( $\pm$ SE) of leaf capacities related of elm seedlings treated with 0 (N0) or 50 (N50)  
609 kg N ha<sup>-1</sup> yr<sup>-1</sup> (given as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) and ambient (AOZ) or elevated (EOZ) ozone. The  
610 data were analyzed by General Linear Model (GLM) at a level of significance  $\alpha=0.05$ .

611 **Fig. 2**

612 Means ( $\pm$ SE) of maximum quantum yield of PSII ( $F_v/F_m$ ) and chlorophyll contents (Chl  
613 a, Chl b, Chl a+b) of elm seedlings treated with 0 (N0) or 50 (N50) kg N ha<sup>-1</sup> yr<sup>-1</sup> (given  
614 as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) and ambient (AOZ) or elevated (EOZ) ozone. The data of  $F_v/F_m$  were  
615 analysed by Mann-Whitney U test, whereas the data of chlorophyll contents were  
616 analyzed by General Linear Model (GLM) at a level of significance  $\alpha=0.05$ .

617 **Fig. 3**

618 Number of individuals of leaf beetle (*Pyrrhalta maculicollis*) grazing on elm seedlings  
619 treated with 0 (N0) or 50 (N50) kg N ha<sup>-1</sup> yr<sup>-1</sup> (given as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) and ambient (AOZ)  
620 or elevated (EOZ) ozone. Each point and error bar mean the mean value  $\pm$  SE of the  
621 population for each treatment (n=3).

622 **Fig. 4**

623 Means ( $\pm$ SE) of the observed total number of leaf beetle (*Pyrrhalta maculicollis*, A) on  
624 11 August, and elm sawfly (*Arge captiva*, B,C) on 20 July and 19 September on elm  
625 seedlings treated with 0 (N0) or 50 (N50) kg N ha<sup>-1</sup> yr<sup>-1</sup> (given as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) and ambient  
626 (AOZ) or elevated (EOZ) ozone. The data were analyzed by General Linear Model  
627 (GLM) at a level of significance  $\alpha=0.05$ .

628 **Fig. 5**

629 Number of individuals of elm sawfly (*Arge captiva*) grazing on Japanese elm seedlings  
630 treated with 0 (N0) or 50 (N50) kg N ha<sup>-1</sup> yr<sup>-1</sup> (given as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) and ambient (AOZ)  
631 or elevated (EOZ) ozone. Each point and error bar mean the mean value  $\pm$  SE of the  
632 population for each treatment (n=3).

633

Fig.1

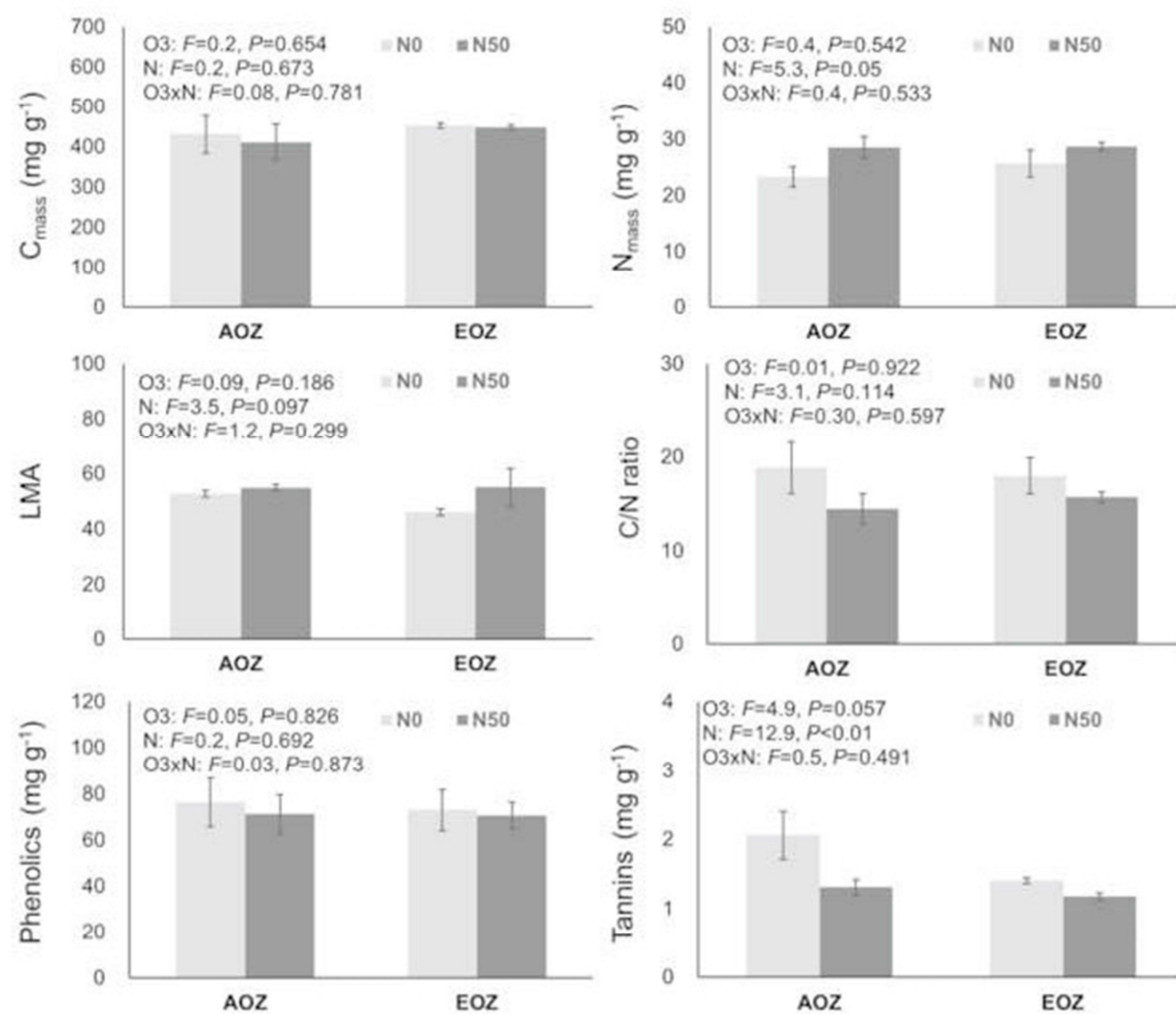
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Fig.2

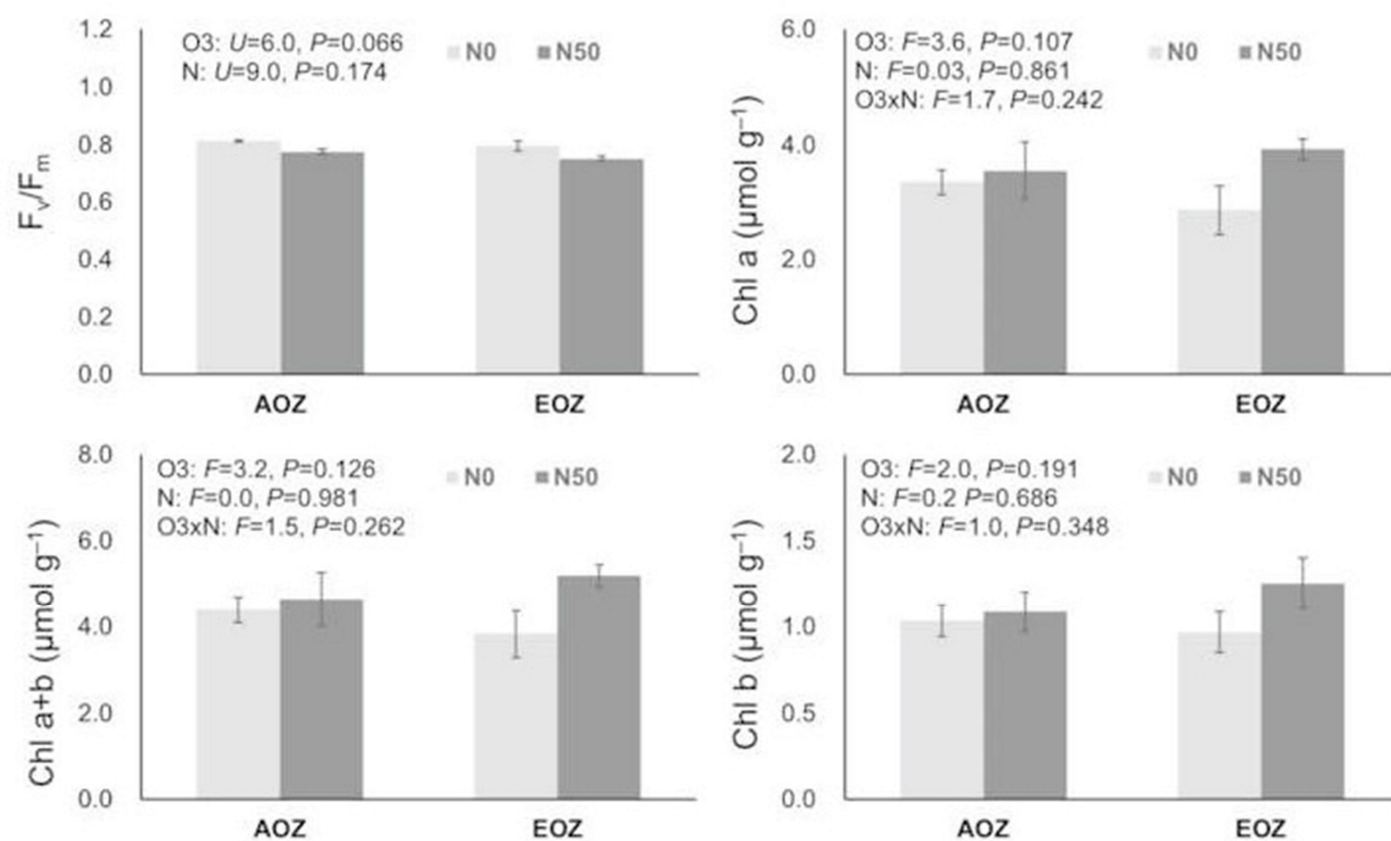
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Fig.3

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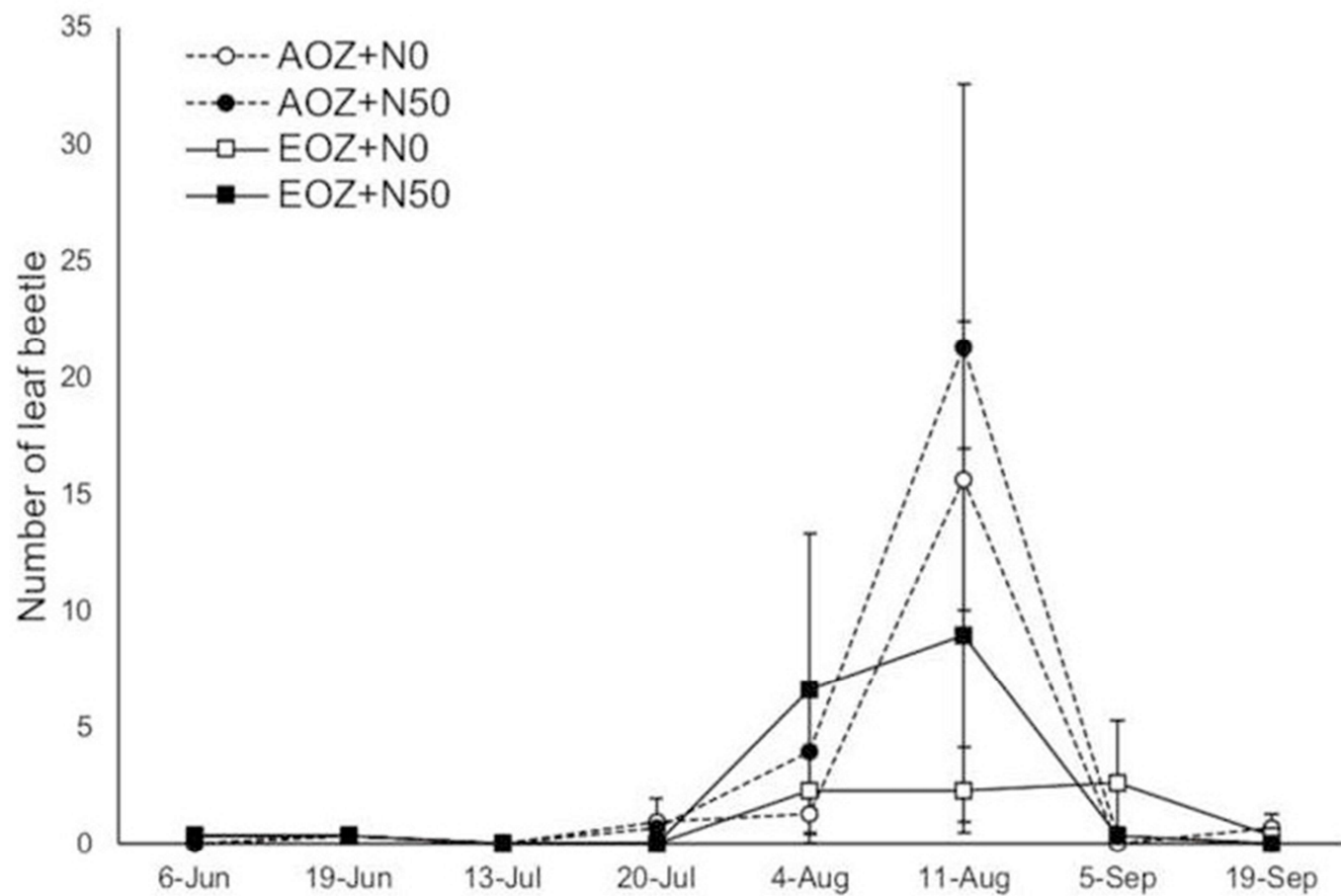


Fig.4

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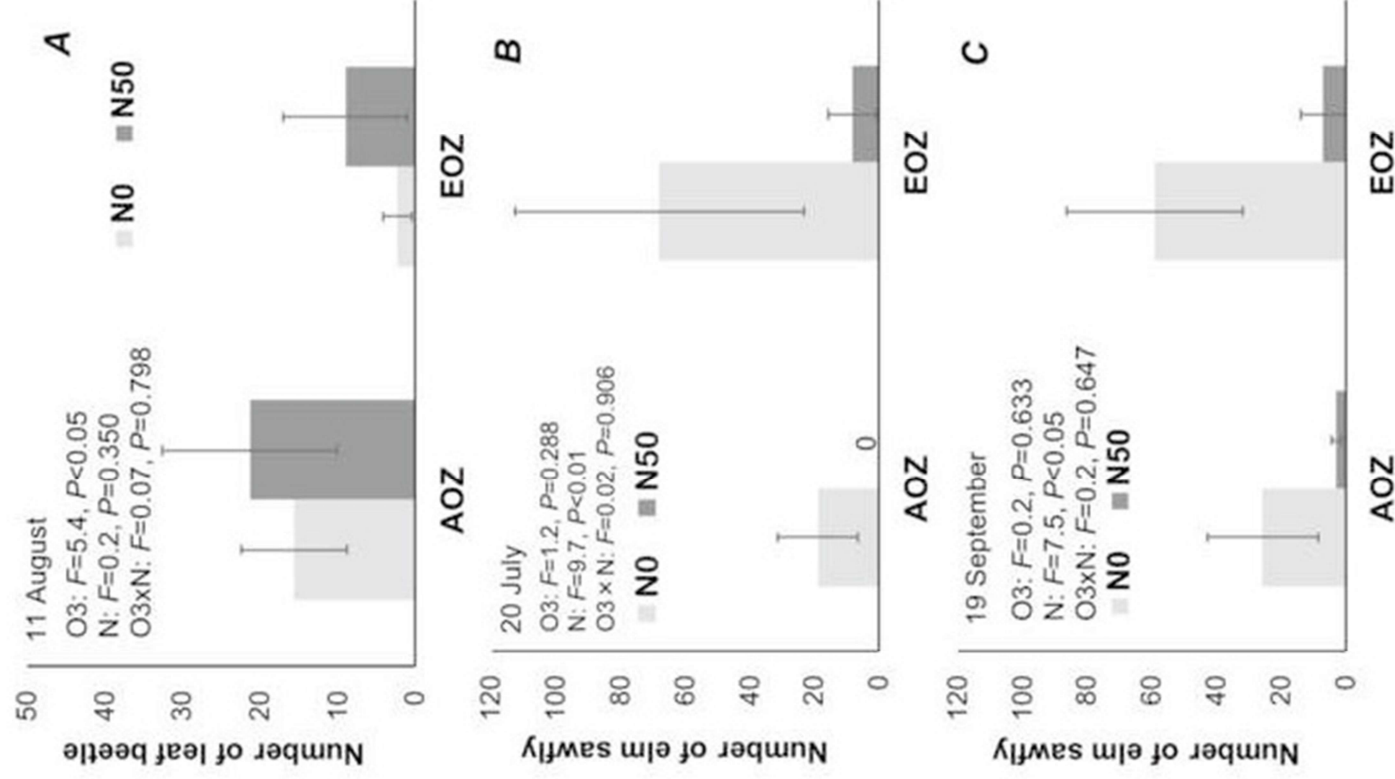


Fig.5

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