

1 Feeding of leaf beetle to two species of alder grown under two different soils in a Free Air 2 CO₂ Enrichment

3
4 Noboru Masui^{1,2)}, Yoko Watanabe^{3,4)}, Hiroyuki Tobita^{5*)}, Takayoshi Koike^{3,4)}

- 5
6 1) School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka 422-8526, Japan
7 2) Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
8 3) Field Science Center for Northern Biosphere, Hokkaido University, Sapporo 060-0809, Japan
9 4) Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
10 5) Forestry and Forest Products Research Institute, Ibaraki 305-8687, Japan

11 *: corresponding author

12 13 Abstract

14 The effects of elevated CO₂ concentration on the defensive ability of two *Alnus* species (*A. maximowiczii*,
15 *A. hirsuta*) against herbivory attacks (alder leaf beetle; *Agelastica coerulea*) were investigated using a
16 free-air CO₂ enrichment. Elevated CO₂ significantly affected consumed leaf area index (CLI) of *A.*
17 *maximowiczii* but soil fertility did not, while more significant effects on CLI of *A. hirsuta* were found in
18 both CO₂ and soil fertility. As found in natural conditions, *A. hirsuta* was grazed five times more than *A.*
19 *maximowiczii* in both CO₂ levels, which was explained with the low condensed tannin (CT) concentration
20 of the leaves. The value of leaf mass per area in fertile soil was 10 g m⁻² lower than that in infertile soil
21 under ambient CO₂ in July. Leaf C/N ratio was not affected by elevated CO₂ for both species but that of *A.*
22 *hirsuta* was little higher in infertile soil when compared to fertile soil in July. CT of *A. maximowiczii*
23 tended to increase in September under elevated CO₂. CT of *A. hirsuta* was higher in infertile soil than in
24 fertile soil in July, and showed an overall decrease in September compared to July. Nitrogen-fixing activity
25 by a nodule of *Frankia* sp. on *A. maximowiczii* was higher in elevated CO₂ treatments than in ambient CO₂
26 independent of soil fertility. As a result of changes in these parameters, except for fertile soil, the peak of
27 the herbivorous damage was later in elevated CO₂ for the *Alnus* species than in the control.

28
29 Keywords: Elevated CO₂, Nitrogen deposition, Plant defense, *Alnus hirsuta*, *Alnus maximowiczii*

1 **Introduction**

2 Nitrogen deposition and ever-increasing atmospheric CO₂ concentrations affect plant defense capacity
3 through changes in the growing environment (e.g., Lindroth 2010; Sun et al. 2020; Jamlöki et al. 2021).

4 Plants use a variety of feeding defenses to protect their leaves, which are photosynthetic products, from
5 feeding damage by leaf-feeding insects (Schoonhoven et al. 2005). The defense ability of plants tends to be
6 lower in species with short leaf longevity and in fertile soil condition (Temperton et al. 2003; Mizumachi et
7 al. 2004; Schoonhoven et al. 2005), such as Betulaceae trees (Koike et al. 2006a; Matsuki and Koike 2006).

8 Broadleaved trees defend themselves by the physical defense such as tough leaf with high leaf mass per
9 area (LMA) and/or trichome, as well as chemical defense such as phenolics and condensed tannin (CT)
10 originated from photosynthates (Bauer et al. 1991; Schoonhoven et al. 2005; Koike et al. 2006a; Fürstenberg-
11 Hägg et al. 2013). Particularly in *Alnus* trees, they did not have significant developed trichomes in some
12 research (Matsuki et al. 2004; Kitahashi 2005), thus LMA is more important physical defense in *Alnus* trees.
13 Except for alder, phytotron experiments have shown that leaves of deciduous broadleaf trees (birch, oak,
14 maple) grown under high CO₂ and infertile soil conditions have an increased ability to defend themselves
15 against insect herbivory (Koike et al. 2006b, 2015). This was due to an increase in the leaf C/N ratio, an
16 increase in the amount of phenolic compounds, and an increase in LMA as a measure of leaf hardness
17 (Koike et al. 2006a), which supports the well-known “Carbon Nutrient Balance (CNB)” hypothesis (Bryant
18 et al. 1983; Schoonhoven et al. 2005). However, this hypothesis does not hold true for all broadleaved trees.
19 One reason for this is that alder trees are infected by *Frankia* sp. (nitrogen (N₂)-fixing bacteria) in their roots,
20 forming nodules, which are called as actinorhizal plants and have symbiotic N₂-fixing ability (Huss-Danell
21 1997; Tobita et al. 2005, 2013a, 2013b). With this symbiotic trait, alders have been used as trees for greening
22 bare ground (Yamanaka and Okabe 2008; Lefrançois et al. 2010; Tobita et al. 2013b). *Alnus maximowiczii* is
23 woody shrub and used for greening in high altitudes and cold regions, while *A. hirsuta* is tall tree and used in
24 lowland and temperate regions (Tanahashi and Shimizu 2007).

25 *Frankia* sp. inoculated with alder reduce atmospheric N₂ to ammonia (NH₃) in root nodules for fixation
26 in the tree, which increases the foliar N content and correspondingly decrease C/N ratio (Tobita et al. 2010,
27 2013a). At this time, plants translocate photosynthetic products as rewards from the leaves to the *Frankia* sp.
28 Namely, in alder trees, the increase in leaf C/N ratio may be moderated by the consumption of photosynthetic
29 products in the leaves by the nodule even in high CO₂ environments (Tobita et al. 2011, 2013b). In an
30 experiment with *A. hirsuta*, the C/N ratio of leaves did not increase even under elevated CO₂ with phytotron
31 (Koike et al. 2006b). Alder trees in elevated CO₂ with phytotron did not change much the defense capacity
32 such as CT, C/N ratio, and LMA in *Alnus pendula*, while defense capacity increased in *A. japonica*. The
33 response of alder to insect feeding damage is not necessarily the same among trees of genus *Alnus*. In *A.*
34 *pendula*, however, N₂-fixing activity by unit mass (g) of nodule under high CO₂ condition was increased,
35 suggesting that the photosynthetic products increased and were promptly translocated to the nodule in roots.
36 In terms of N₂-fixing per unit mass of nodule, *A. maximowiczii* and *A. hirsuta* showed little change on N₂-
37 fixing activity between CO₂ treatments (Tobita et al. 2013b). However, the number of nodules per individual
38 of *A. maximowiczii* and *A. hirsuta* increase thus the total amount of N₂-fixing was higher in a whole of

39 individual under high CO₂ condition (Tobita et al. 2005, 2010).

40 These results were obtained under regulated environmental conditions with phytotron, and it is necessary
41 to verify how insect feeding damage changes under high CO₂ conditions in the field, where feeding damages
42 actually occur. Furthermore, since the data were obtained in a controlled environment with intensive pest
43 management, no examination of induced defenses was conducted. Although herbivores are roughly classified
44 into generalist (polyphagy) vs. specialist (oligophagous), most alders (*Alnus* spp.) are usually fed by
45 oligophagous herbivore: alder leaf beetle (*Agelastica coerulea*) (Oikawa et al. 2012; Sakikawa et al. 2016;
46 Masui et al. 2022).

47 The question that arises here is how the plant defense capacity of alder trees (*Alnus* spp.) and the N₂-fixing
48 activity of root nodule change under high CO₂ in the field. As leaves are eaten, photosynthetic production is
49 expected to decrease and the N₂-fixing activity per unit mass of root nodule also decreases in a whole of
50 individual. The objective of this study was to elucidate changes specific difference in the feeding rate of two
51 species of alder trees (*A. maximowiczii* vs. *A. hirsuta*), the plant defense capacity of leaves, and the N₂-fixing
52 activity of *Frankia* sp. in nodules under predicted future high CO₂ conditions.

53

54 **Materials and Methods**

55 **1) Study site**

56 The CO₂ fumigation study was conducted with a Free-Air CO₂ Enrichment (FACE) located experimental
57 nursery of the Sapporo Experiment Forest of Hokkaido University (43.07N; 141.34E, 52m a.s.l.). The FACE
58 was made following the design of the system at Davos, Switzerland (Hättenschwiler et al. 2002), details are
59 offered by Eguchi et al. (2008). There were six circular FACE plots, each 6.5 m in diameter, surrounded by
60 5.5-m-tall dispersal pipes. Three circular plots were maintained at an elevated [CO₂] of 500 ppm/v (hereafter
61 eCO₂), and the other three were used as ambient plots at 370 ppm/v of CO₂ (aCO₂) as shown in Koike et al.
62 (2015).

63

64 **2) Plant materials**

65 The material plants were 2-year-old seedlings of *Alnus maximowiczii* and *A. hirsuta* obtained from the Oji-
66 Horti-green Co. Ltd., Sapporo in early April, 2006 before leaf unfolding. The seed origin was Kuriyama-
67 town near Sapporo. At mid-April, the CO₂ treatment was started when 95% seedlings of two species opened
68 winter buds. *A. maximowiczii* flushed leaves and kept most of them until late September in Sapporo but *A.*
69 *hirsuta* started to shed half of leaves by mid-August but simultaneously emerges leaves by late September as
70 found by Kikuzawa (1980); and leaf longevity of *A. maximowiczii* is about more than 20 days longer than
71 that of *A. hirsuta*.

72 Mean seedling height of *A. maximowiczii* and *A. hirsuta* was 16.6 ± 1.8 cm and 18.5 ± 1.2 cm,
73 respectively. We observed small nodules (about 1~2 mm in diameter) of *Frankia* sp. in the roots of all
74 seedlings. The former species flushes most leaves and continues to develop few leaves by early August while
75 the latter species develops leaves sequentially by early September and has a relation with a symbiotic
76 actinomycete (*Frankia* sp.) (Tobita et al. 2011). To avoid the influence of roots of other tree species, the

77 black-color-pots (31 cm in diameter; 30.5 cm deep) were filled with poor nutrients (infertile=hereafter Si; a
78 mixture of brown forest soil and the Akadama well-weathered volcanic ash soil at a volume ratio of 1:2) and
79 rich nutrient (fertile Sf; brown forest soil) and one seedling was planted in each pot on 15 April 2006. The
80 nutrient condition of the two soil was as follows: Nitrogen concentration was 30.1 ± 0.20 mg/g in Sf and
81 18.0 ± 0.20 mg/g in Si ($n = 6$). Phosphorus concentration was 3.48 ± 0.25 mg/g in Sf and 1.58 ± 0.17 mg/g in
82 Si ($n = 6$). Potassium concentration was 21.32 ± 3.15 mg/g in Sf and 12.55 ± 1.54 mg/g in Si ($n = 6$). CO₂
83 fumigation to potted alder started on 27 April 2006. The number of seedlings in each plot treated with CO₂
84 and nutrients was five. Pipe water was supplied adequately to the pots for avoiding drought stress (about one
85 or two times per week).

86 Shoot phenology was observed for 5 seedlings at 3-day intervals and included observations of bud opening,
87 defoliation, time of insect damage, and time of shoot elongation cessation in 2006. The leaf expansion period
88 was observed for about 7 days in late April for both species, tending to be a few days earlier in *A. hirsuta*
89 than in *A. maximowiczii*, but there was no statistical difference among treatments. One shoot per individual
90 tree was targeted, which grew from the top at planting seedlings. Leaf sampling is shown in the below 5).

91

92 **3) Insects**

93 Based on former observation (Oikawa et al. 2012), alder trees were fed by only alder leaf beetle (*Agelastica*
94 *coerulea*) in the study site. We determined leaf sampling time for chemical analyses as follows: life cycle of
95 leaf beetle was previously reported (Masui et al. 2023a) and illustrated in Figure 1: Two generations per year;
96 adult overwinters; adults emerge when leaves begin to unfold and lay eggs in clusters on the abaxial side of
97 leaves. Adults chew the leaf whereas the larvae feed by scraping the leaf surface, resulting in skeletonized
98 leaves. The mature larva forms a small chamber in the soil and pupates, becoming an adult in about two
99 weeks. After hatching, the adults eat the leaves and burrow under deciduous leaves or in the soil at shallow
100 depths by late September to overwinter. During experiment, only alder leaf beetle was found in the sites.

101

102 **4) Census of insect feeding damage**

103 To evaluate the feeding rate, the total consumed leaf area per individual was visually observed from late May
104 to mid-September in 2006 at 3-day intervals in principle. The Consumed Leaf-area Index (CLI; %) was
105 calculated dividing the number of attached leaves with approximately 1/3 or more damaged area on the shoot
106 (in the middle of the crown of alder seedlings) by the total number of attached leaves on the entire shoot.
107 Shoot length was 18 cm in *A. maximowiczii* and 25 cm in *A. hirsuta* at the end of the measurement. In the
108 latter species, sylleptic shoots occurred more frequently and the leaf area in this shoot was also included in
109 the CLI calculation. The feeding damage rate of *A. maximowiczii* was clearly smaller than that of *A. hirsuta*.
110 Therefore, in order to make a relative comparison of the feeding damage of the two tree species, the peak of
111 feeding damage rate among alders, approximately 85% of an individual *A. hirsuta* in the end of July, was
112 correlated to 100%. The other CLI values were also corrected based on the corrected peak of the *A. hirsuta*.
113 For example, when the peak CLI of *A. hirsuta* was 85.0% (corrected to 100%) in eCO₂×Si, 40% and 75% of
114 other CLI values are corrected to 47.1% and 88.2%, respectively.

115

116 **5) Measurement of leaf traits**

117 To evaluate the ability to defend against insect feeding, intact leaves were collected and analyzed in late July
118 (before the peak of feeding damage) and mid-September (after the peak). For leaf trait determination,
119 complete expanded leaves in both species were sampled in the 3rd ~5th leaves counted from shoot top.
120 Collected leaves from 5 seedlings per each were punched into discs with a leaf punch (10 mm diameter;
121 Fujiwara Scientific, CO. Ltd., Tokyo), discs were dried in an oven at 60°C for 2 days, LMA was measured as
122 for physical defense (Koike et al. 2006b), and nitrogen and carbon were analyzed with a NC analyzer (NC-
123 900, Sumica-Shimadzu; Osaka) to calculate the C/N ratio.

124 The remaining leaves after disc punching were dried in a freeze-dryer (FLEXI-DRY, FTS system, MA
125 U.S.A.), ground in a mill, and the total phenolic and CT contents were determined (Matsuki et al. 2004). To
126 evaluate the N₂-fixing activity of nodule (*Frankia* sp.), both species of alder seedlings (two individuals per
127 one FACE ring) were dug up in late September, and the acetylene reduction method (GC-2014, Shimadzu,
128 Kyoto) was performed to estimate nitrogenase activity per unit mass of nodule (Tobita et al. 2013a). The
129 dug-up seedlings were divided into each organ (leaves, stem + branches, root, and nodules), dried, and
130 weighed dry mass. The leaf area was measured via a scanner (Epson GT-X770).

131

132 **Statistical analysis**

133 Two-way analysis of variance was conducted (parameters: CO₂, soil, and their interactions), with $P < 0.05$
134 and $P < 0.10$, indicating a significant difference and a trend, respectively. To evaluate the effects of two fixed
135 factors (CO₂ level and soil fertility) on CLI, liner mixed model was conducted with a random factor
136 (observation day; 29-May to 26-Sep). All statistical analysis was conducted using software R ver. 3.6.3
137 (www.r-project.org/).

138

139 **Results**

140 Total number of emerged leaves of both alders were larger in eCO₂×Sf than those in aCO₂×Si. This trend was
141 much clear in *A. hirsuta* but not in *A. maximowiczii*. In late September, number of leaves attached to main
142 shoot was about 6 for *A. maximowiczii* and 8~11 for *A. hirsuta* independent of soil fertility (data not shown).
143 Number of attached, fallen and grazed leaves of *A. maximowiczii* was almost same independent of treatment.
144 Attached leaf number in *A. hirsuta* at eCO₂ was larger than that at aCO₂ ($P < 0.05$). Figure 2 shows CLI per
145 tree individual. In both species, feeding damage was mainly caused by adults and larvae of alder leaf beetle
146 (hereafter referred to as adults and larvae). A comparison of feeding damage at its peak of CLI around mid-
147 August showed that *A. maximowiczii* was about 1/5 of that of *A. hirsuta* (Fig. 2). From the result of liner mixed
148 model, CLI of *A. maximowiczii* was affected by CO₂ ($P < 0.05$) but not by soil fertility ($P = 0.5$). In case of *A.*
149 *hirsuta*, CO₂, soil fertility and this interaction (CO₂ × soil) significantly affected the CLI ($P < 0.001$). When
150 focusing on each season, larval feeding damage became more pronounced in *A. maximowiczii* in late July and
151 in *A. hirsuta* in mid-July. Feeding damage by larvae to leaves in *A. maximowiczii* was primarily suppressed at
152 eCO₂ until mid-August. In contrast, difference in CLI of *A. hirsuta* derived from soil fertility was large under

153 aCO₂ but small under eCO₂. As a result, feeding damage by the larvae was smallest in aCO₂ × Sf plots and
154 largest in aCO₂ × Si plots. However, from mid-August, there was a similar trend in both *Alnus* tree species;
155 CLI values were higher under eCO₂.

156 LMA was not affected by CO₂ treatment for both species, although LMA of both species in July, LMA of
157 *A. maximowiczii* was about 8 g m⁻² larger than that of *A. hirsuta* in September (Fig. 3). However, LMA was
158 not affected by soil fertility. In *A. hirsuta*, the value of LMA under Si in July was about 10 g m⁻² larger than
159 that of Sf ($P < 0.05$); LMA at both CO₂ levels in September tended to be slightly lower than in July ($P =$
160 0.12). Only *A. maximowiczii* in July, Sf slightly increased leaf C/N ratio ($P < 0.10$). However, for the rests,
161 no statistical difference in C/N ratio was found in both species but no effect of CO₂ treatment was found for
162 both species (Fig. 4). On the other hand, in *A. hirsuta*, there is a little higher trend that the values were
163 higher in Si than in Sf when the CO₂ level was low (i.e., aCO₂) in July. In *A. hirsuta*, the values were
164 generally lower in September compared to July.

165 Total phenolic concentrations (ToPhe) in leaves did not differ between treatments (Fig. 5), with *A.*
166 *maximowiczii* at about 50 mg g⁻¹, about half that of *A. hirsuta*, which was measured in July. In September,
167 there were no difference between species, and *A. maximowiczii* tended to be slightly lower concentrations
168 than in July. In *A. hirsuta*, on the other hand, the concentration decreased to about half of that in July. CT
169 ranged from 4 to 8 mg g⁻¹ in *A. maximowiczii* and from 0.8 to 2.0 mg g⁻¹ in *A. hirsuta*, namely CT in *A.*
170 *maximowiczii* was 4-5 times higher than in *A. hirsuta* (Fig. 6). When compared to the aCO₂, CT of *A.*
171 *maximowiczii* was tended to be higher at eCO₂ in September, this trend was not found under Si condition in
172 July. Especially in September, CT was higher in Sf soil under aCO₂ but there was no effect by soil fertility
173 under eCO₂, although eCO₂ showed the highest value of CT in both CO₂ levels. In *A. hirsuta*, there was an
174 interactive effect of CO₂ and soil fertility on CT in July ($P = 0.08$). There were no differences in the rests. *A.*
175 *hirsuta* produced more leaves after leaf beetle feeding. Nitrogenase activity of *A. maximowiczii* seemed to be
176 a little higher in eCO₂ independent of soil fertility ($P = 0.14$), while that of *A. hirsuta* was unaffected by
177 eCO₂ and soil fertility (Fig. 7).

178

179 Discussion

180 The plant defense capacity of leaves increases generally under eCO₂ with an increase in photosynthesis, and
181 foliar quality as food for insects decreased with increased LMA and C/N ratio (e.g., Lindroth 2010; Jamlóki et
182 al. 2021). As a result, generally the larvae need to eat more leaves to grow, which is a process with more severe
183 herbivorous damages under eCO₂ (e.g., Schoonhoven et al. 2005; Koike et al. 2015). At peak time, the CLI per
184 tree individual finally increased in eCO₂ treatments for both *Alnus* species (Fig. 2). This phenomenon in *A.*
185 *hirsuta* seedlings in phytotron was opposite to other deciduous trees (birch, maple oak) that have a negative
186 relation between CO₂ level and survival rate of herbivore insects (Koike et al. 2006b). Although the expectation
187 was that feeding damage by “new adults” (Fig. 1) after August would be suppressed by eCO₂ (Schoonhoven
188 et al., 2005), the results did not support our original expectation. In *A. hirsuta* grown under phytotron and
189 shade condition, the concentration of ToPhe was suggested to play a more important role than CT in feeding
190 defense (Koike et al. 2001, 2006b).

191 The leaf traits (Figs. 3~6) did not explain why feeding damage increased under eCO₂ for both species in this
192 study. We made a new hypothesis based on biogenic volatile organic compounds (BVOCs) as an attractant
193 signal against insects (Masui et al. 2023b) including *A. coerulea* (Masui et al. 2022). The eCO₂ has the potential
194 to alter biological communication via BVOCs. Usually, BVOCs are physiologically synthesized with
195 photosynthetic products such as acetyl-CoA and glyceraldehyde 3-phosphate, pyruvic acid (Rosenkranz and
196 Schnitzler 2013), thus eCO₂ can accelerate the emission of BVOCs through the increased photosynthetic rate
197 (Staudt et al. 2001; Lupitu et al. 2022). Furthermore, herbivorous damage alters the emission of plant volatiles
198 such as green leaf volatiles and terpenoids, called “herbivorous induced plant volatiles (HIPVs)” (Douma et
199 al. 2019), which affects subsequent herbivorous attacks in some cases by attractive/repellent function of HIPVs
200 against insects (Gish et al. 2015; Masui et al. 2023b). *A. hirsuta* which exhibits lower defensive ability against
201 alder leaf beetle (*A. coerulea*) has more possibility to be affected by herbivores in the emission of HIPVs. Thus,
202 there may be gaps between *A. maximowiczii* and *A. hirsuta* in continuous scheme of attracting *A. coerulea*
203 under changing environmental conditions. Further studies should be needed to focus on the effects of eCO₂
204 and soil nutrients on plant-insect communication via plant volatiles.

205 In the case of *A. maximowiczii* at FACE (eCO₂), leaf C/N ratio decreased in Si soils, and this trend was also
206 expected by the other study (Tobita et al. 2013b). However, there was no change in the C/N ratio of *A. hirsuta*
207 by a symbiotic actinomycete (*Frankia* sp.) and subsequent feeding damage. In the eCO₂, the peak period of
208 feeding damage by leaf beetle was delayed compared to the aCO₂ (Fig. 2). On the other hand, *A. hirsuta* grown
209 in aCO₂ × Si, the severe feeding damage that started in late July may have triggered an induced defense and
210 suppressed feeding damage (Tscharntke et al. 2001; Mostafa et al. 2022), however, this did not differ much
211 from the eCO₂.

212 In *A. maximowiczii* and *A. hirsuta* grown in eCO₂, feeding damage was more severe in late August (Fig. 2).
213 In September, there was no difference of ToPhe between CO₂ treatments in both species (Fig. 5). The aCO₂ ×
214 Sf treatment had higher CT in *A. maximowiczii*, but only 2/3 of that in eCO₂. This trend was also observed in
215 *A. hirsuta* (Fig. 6). However, this could not explain the high feeding damage rate of both alder species grown
216 in eCO₂ from late August. Although we did not observe trichome, we should consider physically induced
217 defense such as an increase in trichomes in *Alnus incana* (Bauer et al. 1991).

218 In July, while CT of *A. hirsuta* were not affected or a little higher in the Si under aCO₂, these values were a
219 little higher in the Sf under eCO₂ (Fig. 6). This suggests that the leaves of *A. hirsuta* were a relatively
220 unattractive food source for leaf beetle in the later condition (eCO₂ × Sf). In that season, the peak of adult
221 and larval feeding in *A. hirsuta* had passed but newly born adult feeding already started (Fig 1 and 2).
222 However, CT decreased toward September (Fig. 6), which may be responsible for increased CLI in eCO₂.
223 Since the CLI of *A. maximowiczii* was about 1/5 of that of *A. hirsuta*, it is considered that *A. maximowiczii*
224 less susceptible to feeding damage than *A. hirsuta*. A trade-off relationship between defense and growth is
225 considered to be established to some extent in terms of carbon compound investment (Züst and Agrawal
226 2017).

227 The effects of soil nutrition on N₂-fixing activity per unit mass of nodule were not found in both species
228 (Fig .7), which did not support the original prediction of high activity under Si. In *A. maximowiczii*, it was

229 higher in the eCO₂, while in *A. hirsuta* it did not increase in the eCO₂. Although V_{cmax} decreased at eCO₂
230 (Tobita et al. 2010), increased photosynthetic production in *A. maximowiczii* under eCO₂ may have increased
231 the translocation of photosynthetic products to belowground and increased N₂-fixing activity (Feng et al.
232 2004). On the other hand, the photosynthetic products of *A. hirsuta*, which increased under eCO₂, were
233 successively consumed above ground by subsequent leaf production and induced defense following feeding
234 damage (Tobita et al., 2013a), implying that translocation to the nodules hardly increased. The amount of
235 leaves per individual *A. hirsuta* turned to decrease under eCO₂ conditions (e.g., Koike 1995), suggesting that
236 the decrease in leaf area (mass) due to feeding damage reduced the photosynthetic production of *A. hirsuta*.

237 In terms of tree response to feeding damage, it is expected that there will be cases of induced defense as
238 producing CT as for *A. maximowiczii*, as suggested by long leaf longevity (Kikuzawa 1980). Also, it is
239 considered reduced investment in defense but re-growth in *A. hirsuta*. These differences may depend on the
240 species-specific traits such as leaf phenology, tree physiologies, the degree of feeding damage, etc. However,
241 for making clear on these predictions, it will be necessary to incorporate experiments using low-shading insect
242 protective nets for vegetables to exclude adult leaf beetles as the main herbivore. And then, we should
243 investigate the carbon allocation (i.e., induced defense vs. compensatory growth). Moreover, the carbon
244 allocation to belowground for N₂-fixing nodules under eCO₂ is also considered: The present experiments also
245 suggest that among the feeding defenses, CT is particularly in responses to feeding damage as found in induced
246 defense. Furthermore, partitioning of black locust (*Robinia pseudoacacia*) to rhizobia under eCO₂ was
247 isotopically confirmed (Feng et al. 2004). More research should be conducted to acquire information on carbon
248 allocation, including allocation to BVOCs under high CO₂ levels.

249

250 **Acknowledgments**

251 We thank late Mr. K. Ichikawa, Prof. F. Satoh and Prof. K. Sasa for their support in nursery practices, and
252 financial support in part by JSPS research (“Comprehensive studies of plant responses to high CO₂ world by
253 an innovative consortium of ecologists and molecular biologist” led by Prof. I. Terashima). We also thank
254 Mr. T. Agari and Prof. K. Sasa for providing invaluable comments and materials with their interest in the
255 physiological ecology of the alder species, which performs an important function in the field of erosion
256 control.

257

258 **Disclosure statement**

259 The authors report there are no competing interests to declare.

260

261 **References**

- 262 Baur R, Binder S, Benz G. 1991. Nonglandular leaf trichomes as short-term inducible defense of the grey
263 alder, *Alnus incana* (L.), against the chrysomelid beetle, *Agelastica Mni* L. *Oecologia* 87:219–226.
264 Bryant JP, Chapin FS, Klein DR. 1983. Carbon/Nutrient Balance of Boreal Plants in Relation to Vertebrate
265 Herbivory. *Oikos* 40:357–368. <https://doi.org/10.2307/3544308>
266 Douma JC, Ganzeveld LN, Unsicker SB, Boeckler GA, Dicke M. 2019. What makes a volatile organic
267 compound a reliable indicator of insect herbivory? *Plant Cell Environ* 42:3308–3325.
268 <https://doi.org/10.1111/pce.13624>
269 Eguchi N, Morii N, Ueda T, Funada R, Takagi K, Hiura T, Sasa K, Koike T. 2008. Changes in petiole

270 hydraulic properties and leaf water flow in birch and oak saplings in a CO₂-enriched atmosphere. *Tree*
271 *Physiol* 28:287–295. <https://doi.org/10.1093/treephys/28.2.287>

272 Feng Z, Dyckmans J, Flessa H. 2004. Effects of elevated carbon dioxide concentration on growth and N₂
273 fixation of young *Robinia pseudoacacia*. *Tree Physiol* 24:323–330.

274 Fürstenberg-Hägg J, Zagrobelny M, Bak S. 2013. Plant Defense against Insect Herbivores. *Int J Mol Sci*
275 14:10242–10297. <https://doi.org/10.3390/ijms140510242>

276 Gish M, De Moraes CM, Mescher MC. 2015. Herbivore-induced plant volatiles in natural and agricultural
277 ecosystems: Open questions and future prospects. *Curr Opin Insect Sci* 9:1–6.
278 <https://doi.org/10.1016/j.cois.2015.04.001>

279 Hättenschwiler S, Handa IT, Egli L, Asshoff R, Ammann W, Körner C. 2002. Atmospheric CO₂ enrichment
280 of alpine treeline conifers. *New Phytol* 156:363–375. <https://doi.org/10.1046/j.1469-8137.2002.00537.x>

281 Huss-Danell K. 1997. Actinorhizal symbioses and their N₂ fixation. *New Phytol* 136:375–405.
282 <https://doi.org/10.1046/j.1469-8137.1997.00755.x>

283 Jamloki A, Bhattacharyya M, Nautiyal MC, Patni B. 2021. Elucidating the relevance of high temperature and
284 elevated CO₂ in plant secondary metabolites (PSMs) production. *Heliyon* 7:e07709.
285 <https://doi.org/10.1016/j.heliyon.2021.e07709>

286 Kikuzawa K. 1980. Why do alder leaves fall in summer? *Jpn J Ecol*, 30:359–368 (In Japanese with English
287 summary) <https://www.cabdirect.org/cabdirect/abstract/19810670395>

288 Kitahashi Y. 2005. Physiological ecology in water relations of broad-leaved trees with special reference to
289 essential role in their leaf surface structure. PhD dissertation of Grad School Agri, Hokkaido
290 University. 93pp. (in Japanese)

291 Koike T. 1995. Effects of CO₂ in interaction with temperature and soil fertility on the foliar phenology of
292 alder, birch, and maple seedlings. *Can J Bot*, 73:149-157. <https://doi.org/10.1139/b95-018>

293 Koike T, Mastuki S, Matsumoto T, Yazaki K, Funada R, Tobita H, Kitao M, Maruyama Y. 2001. Plant
294 defense chemicals in tree leaves grown under elevated CO₂ and shade. *Tran Hokkaido Br Jpn For Soc*,
295 49 : 27-29 (in Japanese)

296 Koike T, Matsuki S, Choi D, Matsumoto T, Watanabe Y, Maruyama Y. 2006a. Photosynthesis, Leaf
297 longevity and Defense Characteristics in Trees of Betulaceae Planted in Northern Japan. *Eurasian J For*
298 *Res* 9:69–78. <http://hdl.handle.net/2115/24478>

299 Koike T, Tobita H, Shibata T, Matsuki S, Konno K, Kitao M, Yamashita N, Maruyama Y. 2006b. Defense
300 characteristics of serai deciduous broad-leaved tree seedlings grown under differing levels of CO₂ and
301 nitrogen. *Popul Ecol* 48:23–29. <https://doi.org/10.1007/s10144-005-0236-x>

302 Koike T, Watanabe M, Watanabe Y, Agathokleous E, Eguchi N, Takagi K, Satoh F, Kitaoka S, Funada R.
303 2015. Ecophysiology of deciduous trees native to Northeast Asia grown under FACE (Free Air CO₂
304 Enrichment). *J Agric Meteorol* 71:174–184. <https://doi.org/10.2480/agrmet.D-14-00020>

305 Lindroth RL. 2010. Impacts of elevated atmospheric CO₂ and O₃ on forests: Phytochemistry, trophic
306 interactions, and ecosystem dynamics. *J Chem Ecol* 36:2–21. [https://doi.org/10.1007/s10886-009-9731-](https://doi.org/10.1007/s10886-009-9731-4)
307 4

308 Lefrançois E, Quoreshi A, Khasa D, Fung M, Whyte L, Roy S, Greer C, W, 2010. Field Performance of
309 alder-Frankia Symbionts for the Reclamation of Oil Sands Sites. *Applied Soil Ecology*, 46: 183-191

310 Lupitu A, Moisa C, Gavrilas S, Dochia M, Chambre D, Ciutină V, Copolovici DM, Copolovici L. 2022. The
311 Influence of Elevated CO₂ on Volatile Emissions, Photosynthetic Characteristics, and Pigment Content
312 in Brassicaceae Plants Species and Varieties. *Plants* 11:. <https://doi.org/10.3390/plants11070973>

313 Masui N, Agathokleous E, Tani A, Matsuura H, Koike T. 2022. Plant-insect communication in urban forests:
314 Similarities of plant volatile compositions among tree species (host vs. non-host trees) for alder leaf
315 beetle *Agelastica coerulea*. *Environ Res* 204:111996. <https://doi.org/10.1016/j.envres.2021.111996>

316 Masui N, Inoue S, Agathokleous E, Matsuura H, Koike T. 2023a. Elevated ozone alters long-chain fatty
317 acids in leaves of Japanese white birch saplings. *Environ Sci Pollut Res*.
318 <https://doi.org/10.1007/s11356-023-28056-0>

319 Masui N, Shiojiri K, Agathokleous E, Tani A, Koike T. 2023b. Elevated O₃ threatens biological
320 communications mediated by plant volatiles: A review focusing on the urban environment. *Crit Rev*
321 *Environ Sci Technol* 0:1–20. <https://doi.org/10.1080/10643389.2023.2202105>

322 Matsuki S, Koike T. 2006. Comparison of leaf life span, photosynthesis and defensive traits across seven
323 species of deciduous broad-leaf tree seedlings. *Ann Bot* 97:813–817.
324 <https://doi.org/10.1093/aob/mcl041>

325 Matsuki S, Sano Y, Koike T. 2004. Chemical and physical defence in early and late leaves in three

326 heterophyllous birch species native to northern Japan. *Ann Bot* 93:141–147.
327 <https://doi.org/10.1093/aob/mch022>

328 Mizumachi E, Osawa N, Akiyama R, Tokuchi N. 2004. The effects of herbivory and soil fertility on the
329 growth patterns of *Quercus serrata* and *Q. crispula* saplings at the shoot and individual levels. *Popul*
330 *Ecol* 46:203–211. <https://doi.org/10.1007/s10144-004-0188-6>

331 Mostafa S, Wang Y, Zeng W, Jin B. 2022. Plant responses to herbivory, wounding, and infection. *Int J Mol*
332 *Sci* 23:. <https://doi.org/10.3390/ijms23137031>

333 Oikawa M, Matsuki S, Saitoo H, Shibuya M, Koike T. 2012. Survivalship Of insect herbivores with different
334 food habit on deciduous broadleaved trees. *Boreal For Res* 60: 111-112

335 Rosenkranz M, Schnitzler J-P. 2013. Genetic Engineering of BVOC Emissions from Trees. In: Niinemets Ü,
336 Monson RK (eds) 1st edn. Springer Netherlands, Dordrecht, pp 95–118,
337 https://link.springer.com/chapter/10.1007/978-94-007-6606-8_4

338 Sakikawa T, Shi C, Nakamura M, Watanabe M, Oikawa M, Satoh F, Koike T. 2016. Leaf phenology and
339 insect grazing of Japanese white birch saplings grown under free-air ozone exposure. *J Agric Meteorol*
340 72:80–84. <https://doi.org/10.2480/agrmet.D-14-00031>

341 Schoonhoven LM, van Loon JJA, Dicke M. 2005. *Insect-plant biology*. 421, ISBN: 9780198525950

342 Staudt M, Joffre R, Rambal S, Kesselmeier J. 2001. Effect of elevated CO₂ on monoterpene emission of
343 young *Quercus ilex* trees and its relation to structural and ecophysiological parameters. *Tree Physiol*
344 21:437–445. <https://doi.org/10.1093/treephys/21.7.437>

345 Sun Y, Guo J, Li Y, Luo G, Li L, Yuan H, Mur LAJ, Guo S. 2020. Negative effects of the simulated nitrogen
346 deposition on plant phenolic metabolism: A meta-analysis. *Sci Total Environ* 719:137442.
347 <https://doi.org/10.1016/j.scitotenv.2020.137442>

348 Tanahashi I, Shimizu H. 2007. Plantation for greening at quarry pit. *Tech. Rep Civil Eng Res inst Cold*
349 *Region H19*, 1-8

350 Temperton VM, Grayston SJ, Jackson G, Barton CVM, Millard P, Jarvis PG. 2003. Effects of elevated
351 carbon dioxide concentration on growth and nitrogen fixation in *Alnus glutinosa* in a long-term field
352 experiment. *Tree Physiol* 23:1051–1059. <https://doi.org/10.1093/treephys/23.15.1051>

353 Tobita H, Hasegawa SF, Yazaki K, Komatsu M, Kitao M. 2013a. Growth and N₂ fixation in an *Alnus hirsuta*
354 (Turcz.) var. *sibirica* stand in Japan. *J Biosci* 38:761–776. <https://doi.org/10.1007/s12038-013-9369-9>

355 Tobita H, Kitao M, Koike T, Maruyama Y. 2005. Effects of elevated CO₂ and nitrogen availability on
356 nodulation of *Alnus hirsuta* Turcz. *Phyton* 45:125–131

357 Tobita H, Kucho K, Yamanaka T. 2013b. Abiotic Factors Influencing Nitrogen-Fixing Actinorhizal
358 Symbioses. 37:. <https://doi.org/10.1007/978-3-642-39317-4>

359 Tobita H, Uemura A, Kitao M, Komatsu M, Kitao M. 2011. Effects of elevated atmospheric carbon dioxide,
360 soil nutrients and water conditions on photosynthetic and growth responses of *Alnus hirsuta*. *Funct*
361 *Plant Biol* 38:702–710. <https://doi.org/10.1071/FP11024>

362 Tobita H, Uemura A, Kitao M, Kitaoka S, Utsugi H. 2010. Interactive effects of elevated CO₂, phosphorus
363 deficiency, and soil drought on nodulation and nitrogenase activity in *Alnus hirsuta* and *Alnus*
364 *maximowiczii*. *Symbiosis* 50:59–69. <https://doi.org/10.1007/s13199-009-0037-7>

365 Tschardt T, Thiessen S, Dolch R, Boland W. 2001. Herbivory, induced resistance, and interplant signal
366 transfer in *Alnus glutinosa*. *Biochem Syst Ecol* 29:1025–1047. [https://doi.org/10.1016/S0305-1978\(01\)00048-5](https://doi.org/10.1016/S0305-1978(01)00048-5)

367

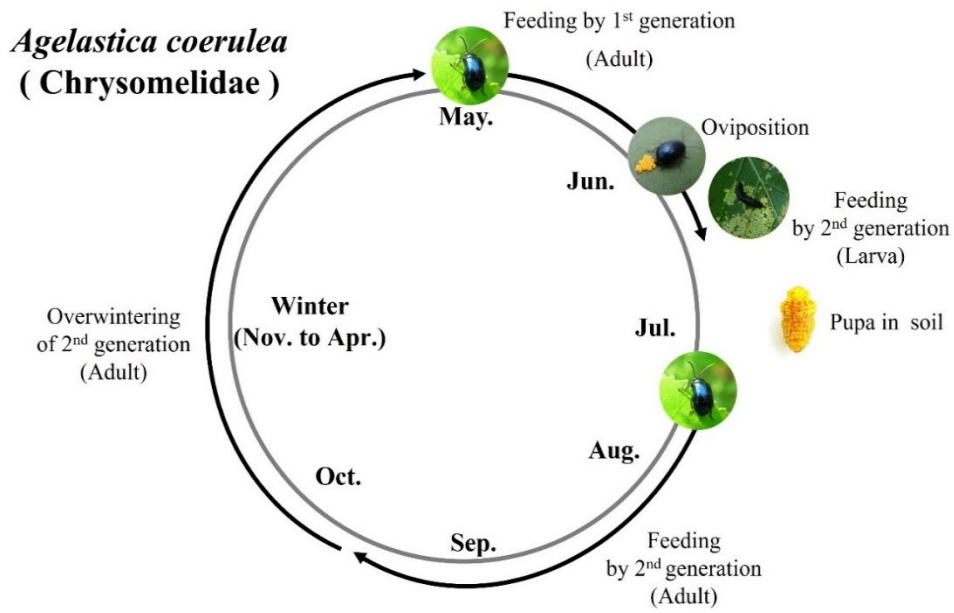
368 Yamanaka T, Okabe H. 2007. Actinorhizal plants and *Frankia* in Japan. *Bull For Forest Prod Res Inst*, 406:
369 67-80, <http://dl.ndl.go.jp/info:ndljp/pid/9366429>

370 Züst T, Agrawal AA. 2017. Trade-Offs Between Plant Growth and Defense Against Insect Herbivory: An
371 Emerging Mechanistic Synthesis. *Annu Rev Plant Biol* 68:513–534. <https://doi.org/10.1146/annurev-arplant-042916-040856>

372

373

374



377 Figure 1. Life cycle of alder leaf beetle (*Agelastica coerulea*) at central Hokkaido Island, Japan
378 Courtesy of Dr. Marion Friedrich for photo of Pupa.
379

380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408

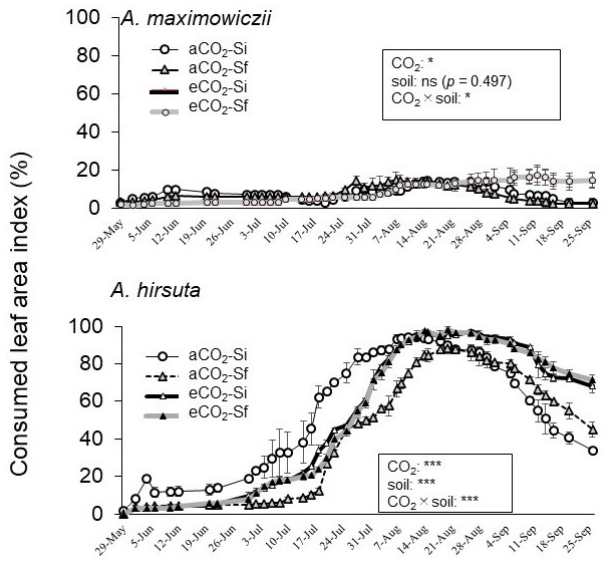


Figure 2. Seasonal changes in the consumed leaf area index (%) in two alder species treated by soil fertility (Si: infertile vs. Sf: fertile) and CO₂ levels (aCO₂: ambient 370 ppm vs. eCO₂: 500 ppm in FACE). The extent to which the ability of alder leaves to defend themselves against feeding varies with Treatments, the feeding damage by alder was assessed. For this, *A. hirsuta*, which showed the largest feeding damage in August, was used as a relative value of 100. Vertical bars indicate standard error. Linear mixed model was conducted with fixed factors (CO₂ level and soil fertility) and a random factor (observation day; 29-May to 26-Sep); *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ns: not significant.

409
 410
 411
 412
 413
 414
 415
 416
 417
 418
 419
 420
 421
 422
 423
 424
 425
 426
 427
 428
 429
 430
 431
 432
 433
 434
 435
 436
 437
 438
 439
 440
 441
 442
 443
 444
 445
 446
 447
 448
 449
 450
 451
 452
 453
 454
 455
 456

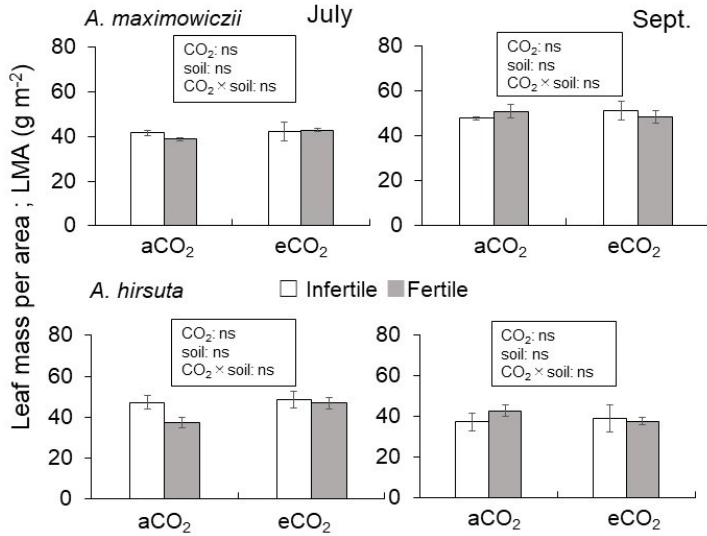


Figure 3. Leaf mass per area (LMA) in two species of alder (*Alnus maximowiczii*, *A. hirsuta*) grown under different CO₂ (aCO₂: ambient 370 ppm; eCO₂: 500 ppm in FACE) and soil fertilities (infertile vs. fertile). Vertical bars indicate standard error. No statistical significance was found in all values ($P > 0.10$; two-way ANOVA).

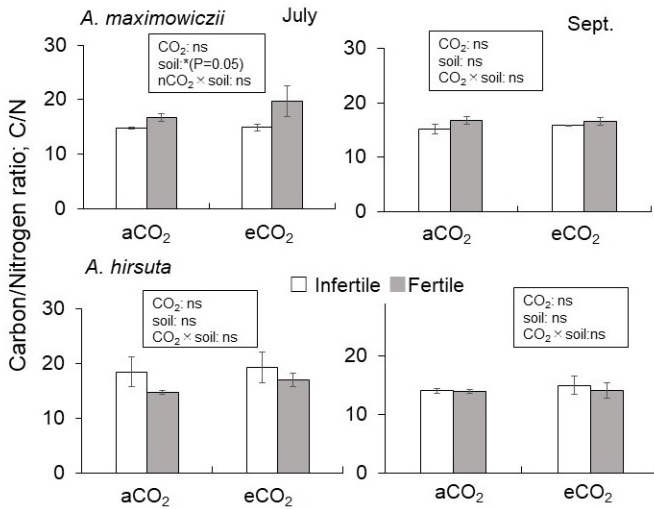


Figure 4. Ratio of carbon to nitrogen (C/N) in leaves of *Alnus maximowiczii* and *A. hirsuta* grown under different CO₂ (aCO₂: ambient 370 ppm; eCO₂: 500 ppm in FACE) and soil fertilities (infertile vs. fertile). Vertical bars indicate standard error. In *A. maximowiczii* grown under fertile soil in July, C/N ratio was increased (*: $P < 0.10$; two-way ANOVA). The rests showed no statistical significance difference ($P > 0.10$).

457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514

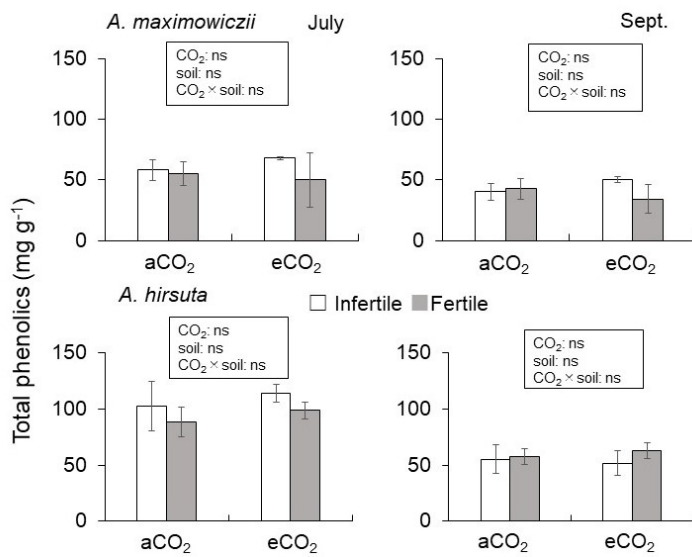


Figure 5. Total phenolics in leaves of *Alnus maximowiczii* and *A. hirsuta* grown under different CO₂ (aCO₂: ambient 370 ppm; eCO₂: 500 ppm in FACE) and soil fertilities (infertile vs. fertile). Vertical bars indicate standard error. No statistical difference was found in all data ($P > 0.10$; two-way ANOVA).

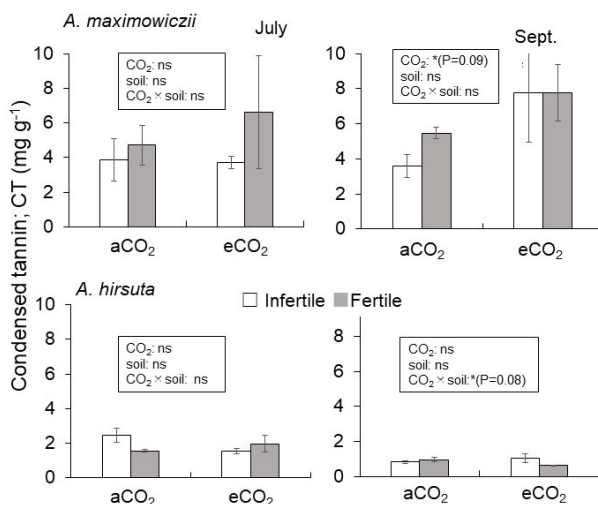


Figure 6. Condensed tannin (CT) in leaves of *Alnus maximowiczii* and *A. hirsuta* grown under different CO₂ (aCO₂: ambient 370 ppm; eCO₂: 500 ppm in FACE) and soil fertilities (infertile vs. fertile). Vertical bars indicate standard error. In *A. maximowiczii* grown under elevated CO₂ in September, CT was increased. The rests showed no statistical difference in all conditions ($P > 0.10$). An interaction effect of CO₂ x soil fertility in *A. hirsuta* was found in July ($P > 0.10$; two-way ANOVA). The rests showed no statistical difference in soil fertility and CO₂ levels.

515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532

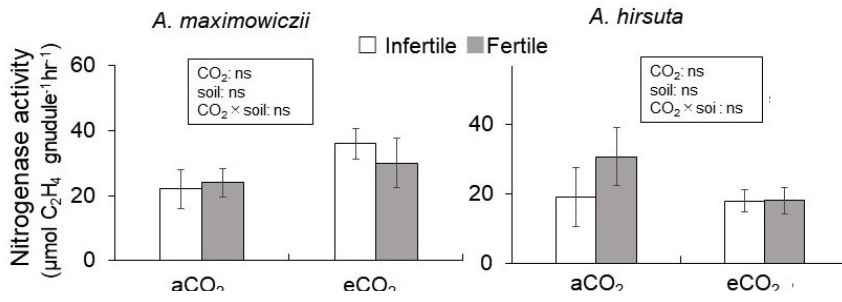


Figure 7. Nitrogenase activity of nodules of *Frankia* sp. in late September of *Alnus maximowiczii* and *A. hirsuta* grown under different CO₂ (aCO₂: ambient 370 ppm; eCO₂: 500 ppm in FACE) and soil fertilities (infertile vs. fertile). Vertical bars indicate standard error. No statistical difference was found in all data ($P > 0.10$; two-way ANOVA).