Eurasian J. For. Res. 22: 63-68, 2022 DOI:10.14943/EJFR.22.63

Elevated ozone disrupts the plant-insect communication; Changes of attractiveness of Japanese white birch leaves to *Agelastica coerulea* via Biogenic Volatile Organic Compounds (BVOCs)

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Abstract

Elevated ground-level ozone (O_3) reduced C-based defense chemicals; however, severe grazing damages were found in leaves grown in the low O_3 condition of a free air O_3-concentration enrichment system. To explain this phenomenon, this study investigates the role of BVOCs (Biogenic Volatile Organic Compounds) as signaling compounds for insect herbivores. BVOCs act as scents for herbivore insects to locate host plants, while some BVOCs show high reactivity to O_3, inducing changes in the composition of BVOCs in atmospheres with elevated O_3. In this study, profiles of BVOCs emitted from birch (*Betula platyphylla* var. *japonica*) leaves were analyzed, and Y-tube insect preference tests were conducted to study the insect olfactory response. The assays were conducted in June and August or September, according to the life cycle of the adult alder leaf beetle *Agelastica coerulea*. The Y-tube tests revealed that the leaf beetles were attracted to BVOCs, and O_3 per se had neither an attractant nor a repellent effect. BVOCs became less attractant when mixed with highly concentrated O_3 (>80 ppb). About 20% of the total BVOCs emissions were highly O_3-reactive compounds, such as β-ocimene. The results suggest that BVOCs emitted from the birch leaves can be altered by elevated O_3, and, thus, potentially reducing the attractiveness of leaves to herbivorous insects searching for food.

Key words: atmospheric lifetime, biogenic volatile organic compounds (BVOCs), herbivorous insects, olfactory response, ozone,

Introduction

Ground-level ozone (O_3) has been elevating in the last decades around the world (Koike et al., 2013, Feng et al., 2019, Sicard et al., 2020), and can damage photosynthesis, growth and development of plants (Watanabe et al., 2017, Grulke and Heath, 2020, Feng et al., 2021). Most carbon-based defense chemicals are synthesized from photosynthates; thus, plant defense capacity can be decreased by elevated O_3. Generally, it has been assumed that insect behavior is linked to the defensive properties of leaves, this is because O_3 can enhance the susceptibility of plants to insect herbivores (Matyssek et al., 2012, Agathokleous et al., 2017). However, in the field at elevated O_3 concentration, herbivorous activities can be altered from theoretical expectations based on foliage quality. For example, in a plant (birch: *Betula platyphylla* var. *japonica*) - insect (alder leaf beetle: *Agelastica coerulea*) communication, grazing damage of leaves were found more in elevated O_3 plots (around 60 ppb) than in ambient plots (around 30 ppb) of a free-air O_3-concentration enrichment (FACE) system, even if in that situation the leaves in the elevated O_3 condition had lower chemical defense capacities (Agathokleous et al. 2017). Furthermore, leaves from the elevated O_3 conditions were often preferred by insects in choice laboratory assays. The results of this array of studies suggest that the field observations for reduced herbivory in the elevated O_3 plots were not due to changes in leaf chemical defense capacity, hinting to a potential role of Biogenic Volatile Organic Compounds (BVOCs) which attract insects as scent chemical signals (Masui et al. 2020).

BVOCs decay through reaction with O_3 after emitted from plants into the atmosphere (Atkinson and Arey 2003, Fuentes et al. 2016). The reactivity is dependent on the volatile compound, but some compounds with high reactivity have very shorten lifetimes in elevated O_3, which means some insect cannot reach at target plants because there are not enough attractants or the blends to detect (Masui et al. 2021). In communication between

(Received; Oct. 2, 2021: Accepted; Oct. 11, 2021)
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the birch and the leaf beetle, there is a question whether O_3-induced disruption on attractiveness of BVOCs occurred. To assess this question, Y-tube preference tests were conducted to investigate the attractiveness of BVOCs emitted from birch under artificially controlled elevated O_3 condition. We hypothesized that the adult leaf beetles would visit less frequently birch leaves when BVOCs are mixed with elevated O_3. To identify specific BVOCs emitted from the birch and understand the insect preference results, we also assessed the profile of BVOCs emitted from the birch leaves by GC-MS analyses.

Material and methods

Japanese white birch (*Betula platyphylla* var. *japonica*), a main species in forests of Hokkaido was used; branches of the birch were collected just before each test from ambient plots of the FACE system. The birch has heterophyllous leaves; early leaves from the beginning of May, and late leaves from around mid-July. The herbivorous insect; alder leaf beetle (*Agelastica coerulea*) is a major oligophagous pest of Betulaceae trees. The adults of 1st generation, which are overwintered-individuals, appears in late May to June and oviposit eggs on leaves in late June to early July. The larvae of 2nd generation, i.e., offspring of 1st generation, are mainly active in July. The adults of 2nd generation appear from mid-August to mid-September. The lifecycle of the adults corresponds to the period of the early leaves (for 1st generation), the period of the late leaves of white birch (for 2nd generation). Compared to feeding by adults (chewer), grazing damage by the larvae (skeletonizer) is more severe as shown in Figure 1. However, because mobility of the larvae is low and thus the grazing damage by larvae depends on where the adults oviposit (Sakikawa et al. 2016), attractiveness to adults is firstly important for the grazing by *A. coerulea*. Thus, the experiments were conducted focusing on adults, not on larvae.

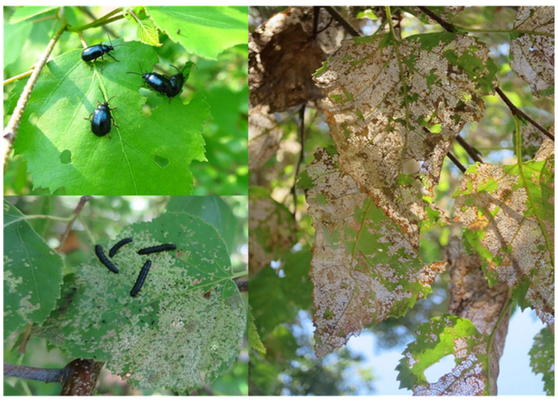

Figure 1. Grazing activity of alder leaf beetle (*Agelastica coerulea*) on Japanese white birch (*Betula platyphylla* var. *japonica*).
Adults as chewer (upper left in this figure) and larvae as skeletonizer (lower left in this figure) graze, grazing damages are more severe by the larvae than by the adults. After grazing by larvae, damaged leaves are colored brown and the aesthetic value also decrease as well as photosynthetic productivity.

Two-choice olfactory response tests (Fig. 2) were conducted to evaluate the attractiveness of leaves in each atmospheric treatment with a Y-shaped glass olfactometer (Masui et al. 2021).

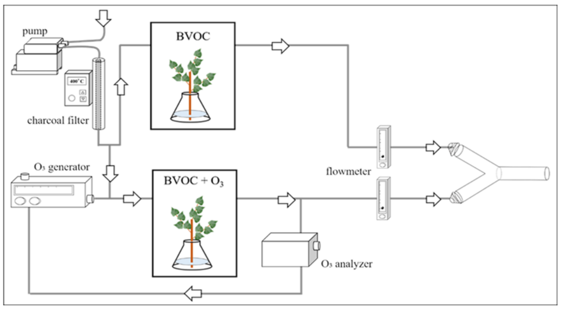

Figure 2. Olfactory response test (Y-tube preference test). Each Arrow indicates the aerial direction.

In this study, three groups of tests were conducted to reveal the air that insects prefer: 1) O_3 vs. Cleaned Air created through charcoal filter (CA), 2) CA vs. BVOCs (emitted from birch leaves) and 3) BVOCs vs. BVOCs mixed with O_3 (BVOC + O_3). In test 1) and test 3), O_3 concentration was set to 40, 80 or 120 ppb. The leaf beetles were subjected to starving for at least two hours before the Y-tube tests begin. Insects were observed for five minutes in each trial, or if insects continuously stayed at one side for one minute within the trial time. In each test, trials were conducted 30 times in total with randomly selected different individuals of the leaf beetle, excluding trials that were discarded due to no choice.

BVOCs emitted from birch leaves, early leaves in June and late leaves in September 2018, were sampled with a branch chamber method (Tani and Kawawata 2008, Masui e al. 2021). In this method, BVOCs emitted in the bag were sampled into sampling tube with inlet air pumps for 30 minutes and two samples were collected per individual (n=5). The sampling BVOCs was analyzed by a gas chromatograph mass spectrometer with an automated two-stages thermal desorber. Each compound was identified with NIST (2011)-database in electron-impact ionization method of scan-analysis. Emission rate (E; nmol m^{-2} s^{-1}) was calculated based on the equation:

$$E = \frac{C_S * V_{out}}{S} \risingdotseq \frac{C_S * V_{in}}{S},$$

where Cs is the concentration (nmol mol^{-1}) of each compound in the sampling bag, and S is the total leaf area (m^2) of the sample branches. V_{out} is the outlet flow rate (mol s^{-1}), and strictly the value of V_{out} is higher than that of the inlet flow rate (V_{in}; mol s^{-1}) depending on the

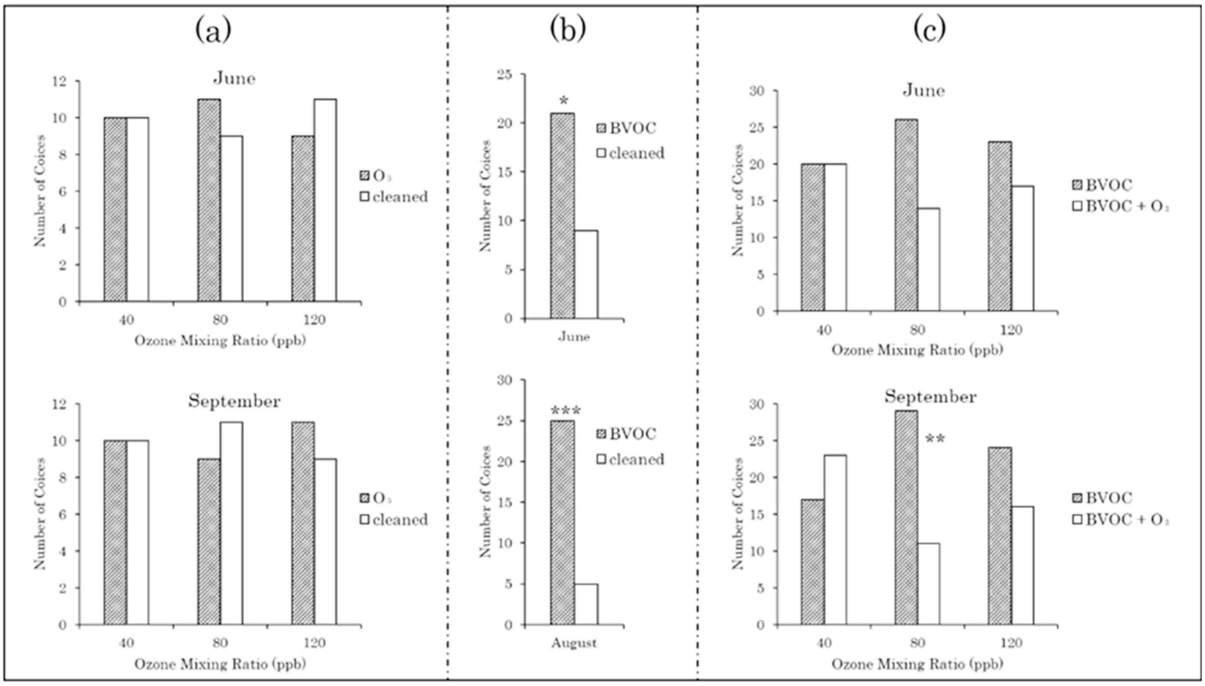

Figure 3. Y-tube preference test for adults of alder leaf beetle (*Agelastica coerulea*) to plant volatiles of Japanese white birch (*Betula platyphylla* var. *japonica*).
(a) O_3 vs. cleaned air (through active charcoal filter), (b) biogenic volatile organic compounds (BVOCs) of Japanese white birch vs. Cleaned air, (c) BVOCs vs. BVOCs + O_3. Asterisks stand for p-value; p > 0.05 *; p > 0.01 **; and p > 0.001 *** in the binominal test.

increase in water vapor concentration by evaporation of the sample leaves. However, because the deference between V_{in} and V_{out} can be ignored because increase ratio of V_{out} compared to V_{in} is up to 2 % (Tani et al. 2010), V_{out} is approximated to V_{in}. Then, relative emission rates (%) of each compound or each chemical group were calculated as specific emission rate divided by the total emission rate and multiplied by 100.

Results

The adult leaf beetles showed no preference/ avoidance against O_3 per se at any concentrations in test 1) (Fig. 3a) and preferred BVOCs emitted from leaves of the birch in test 2) (Fig. 3b). In test 3) (Fig. 3c), the leaf beetles of both 1st and 2nd generation adults had no preference between BVOCs and BVOCs + O_3 at 40 ppb. At 80 ppb, 1st generation as well as 2nd generation tended to prefer BVOCs over BVOCs + O_3, which means the attractiveness of BVOCs was apparently degraded by higher O_3 exposure. However, at 120 ppb, insects preferred more frequently BVOCs by comparison with that at 40 ppb O_3, although the differences were not statistically significant.

It was found that BVOCs emitted from Japanese white birch had phenological differences corresponding to lifecycle of alder leaf beetles both in quantity and quality (Table 1). Total emission rate of late leaves (in September) was significantly higher than that of early leaves (in June). Monoterpene (MT) was a dominant group both in early and late leaves; the dominance was much more pronounced in late leaves. The compositions were significantly different between June (early leaves) and September (late leaves). However, sabinene, a major MT compounds showed highest emission ratio in both leaves. Furthermore, in this study, we detected eight compounds as O_3-reactive compounds (*cis-/trans*-β-ocimene, limonene, 2-carene, γ-terpinene, β-linalool, α-copaene and β-caryophyllene); O_3-reactive compounds means ones of which lifetimes are assumed to be under 1 hour at 80ppb. The total dominances of O_3-reactive compounds were over 20 % in both seasons.

Discussion

In Y-tube experiments, adults of alder leaf beetle showed preference to BVOCs of Japanese white birch. However, the attractiveness of BVOCs decreased under artificially elevated O_3 levels. It was noted that O_3-reactive compounds were found at high ratios over 20 % in total. We can provide two possible mechanisms explaining that alder leaf beetles visited less frequently ozonated birch leaves in the field. First, O_3-reactive compounds functioned as attractant to the leaf beetles in usual; decay of O_3-reactive compounds can lead that host-plants can become less detectable to the leaf beetle in an O_3-enriched atmosphere (due to high reactivity with O_3). The other is the collapse of the BVOCs composition including O_3-reactive compounds, which result in less attractiveness of the BVOCs. In the 2nd

Table 1. Composition ratios of BVOCs emitted from Japanese white birch (*Betula platyphylla* var. *japonica*) in June and September.

The value of each compound in each season in this table means average composition ratio (%) ± SE (n=5). MT: Monoterpene, Oxy-MT: Oxygenated-Monoterpene, NT: Nitrogenous Oa-est: Organic acid-ester terpenoid, SQT: Sesquiterpene, GLV: Green leaf volatile; In individual t-test, Asterisk stands for p-value < 0.05 *; p < 0.01 ** in individual t-tests; horizontal line "–" means "Not found". F-test at each compound was conducted as two-sided test (α = 0.05); "S "and "W" in this table means Student's and Welch's t-test conducted at each compound, respectively.

Number of compounds			June 30	September 27	F-value	t-test type	t-test p-value
Total Emission rate (μmol m⁻²s⁻¹)			0.983 ± 0.182	2.115 ± 0.134	1.85	S	**
Name of compounds	**RT**	**Group**					
α-thujene	21.72	MT	1.485 ± 0.133	1.566 ± 0.188	1.99	S	n.s
α-pinene	22.13	MT	7.495 ± 0.259	7.786 ± 0.229	1.27	S	n.s
camphene	22.84	MT	0.243 ± 0.027	0.297 ± 0.032	1.40	S	n.s
sabinene	23.56	MT	40.980 ± 2.212	50.107 ± 1.804	1.50	S	*
β-pinene	23.88	MT	3.299 ± 0.088	3.459 ± 0.143	2.64	S	n.s
2-carene	25.13	MT	0.600 ± 0.081	0.610 ± 0.125	2.41	S	n.s
o-cymene	25.39	MT	1.789 ± 0.635	1.623 ± 0.440	2.08	S	n.s
trans-β-ocimene	25.48	MT	0.992 ± 0.174	4.334 ± 0.325	3.47	S	**
limonene	25.58	MT	0.454 ± 0.059	0.669 ± 0.041	2.08	S	*
β-phellandrene	25.71	MT	1.006 ± 0.098	1.022 ± 0.144	2.16	S	n.s
cis-β-ocimene	25.89	MT	4.249 ± 0.797	17.920 ± 1.349	2.87	S	**
γ-terpinene	26.53	MT	1.474 ± 0.203	1.467 ± 0.322	2.52	S	n.s
neo-allo-ocimene	28.65	MT	1.185 ± 0.215	5.709 ± 0.426	3.93	S	**
p-Cineole	25.78	Oxy-MT	1.170 ± 0.441	0.238 ± 0.011	1552.13	W	n.s
trans-sabinene-hydrate	27.01	Oxy-MT	0.911 ± 0.117	1.437 ± 0.049	5.60	S	**
linalool oxide	27.48	Oxy-MT	4.706 ± 0.770	0.510 ± 0.044	307.73	W	**
β-linalool	27.71	Oxy-MT	10.333 ± 1.929	0.181 ± 0.036	2829.78	W	**
geranyl nitrile	28.11	N	3.272 ± 0.558	0.173 ± 0.045	156.94	W	**
methyl salicylate	31.14	Oa-est	—	0.062 ± 0.024	—	—	—
ylangene	36.40	SQT	0.900 ± 0.427	0.035 ± 0.010	1666.55	W	n.s
α-copaene	36.57	SQT	0.731 ± 0.336	0.022 ± 0.006	3104.84	W	n.s
β-bourbonene	36.88	SQT	1.470 ± 0.622	0.074 ± 0.016	1451.45	W	n.s
β-caryophyllene	37.93	SQT	0.743 ± 0.317	0.034 ± 0.008	1409.37	W	n.s
β-copaene	38.13	SQT	0.547 ± 0.276	—	—	—	—
aristolene	38.29	SQT	2.461 ± 1.448	—	—	—	—
α-farnesene	39.35	SQT	—	0.030 ± 0.005	—	—	—
germacrene	39.48	SQT	0.807 ± 0.437	—	—	—	—
α-guaiene	39.73	SQT	0.312 ± 0.138	0.023 ± 0.006	482.87	W	n.s
hexanal	16.53	GLV	0.724 ± 0.195	0.151 ± 0.044	20.10	W	*
cis-3-hexen-1-ol	18.82	GLV	1.059 ± 0.111	0.462 ± 0.211	3.64	S	*
trans-3-hexenyl acetate	24.32	GLV	4.009 ± 0.831	—	—	—	—
cis-2-hexenyl acetate	24.54	GLV	0.594 ± 0.134	—	—	—	—

mechanism, O_3-reactive compounds have higher possibility as key attractant compounds, than other compositional compounds. There are some researches that indicate the importance of compositional blends on attractiveness of BVOCs; the attractiveness of a volatile blend is more attracted than when each compositional compound reaches at the target insects individually (Bruce and Pickett 2011, Zhang 2018). By these mechanisms (either or both works), it is estimated that decreasing concentrations of a part of BVOCs (e.g., O_3-reactive compounds) result in the difficulty for alder leaf beetles to find their host Japanese white birch in elevated O_3 levels.

In test [3], preference to the treatment [BVOCs+O_3] by the leaf beetle did not decrease more than theoretically expected from the results at 80 ppb. The change of preference depending on O_3 was found as a biphasic phenomenon. The possibility reason of the biphasic is as below; there are many oxidative products from reactions of BVOCs with O_3 (McFrederick et al. 2008, Mishra and Sihag 2010, Holopainen and Blande 2013), and each oxidative products has an effect (attractive or repellent) on insects. In case the oxidative products are attractive, entire function of BVOCs can decrease at first due to O_3-derived degradation but increase due to existence of oxidative products as reaction with O_3 proceed at higher O_3 levels. In contrast, in case of repellent oxidative products, degradation by O_3 can proceed at an accelerated pace. To clarify how O_3 affects the composition and the attractiveness of BVOCs in detail, comparisons before and after O_3-exposure to volatile blends should be conducted in Y-tube preference tests and GC-MS analysis. In addition, it is indicated that there was a seasonal difference in BVOC compositions between early (June) and late leaves (September), which means each adult leaf beetle (1st or 2nd generation) detect a BVOC composition specific to the active season. Therefore, when evaluating O_3 effect on herbivorous damage by target insects, lifecycle of the insects should be taken into consideration as well as BVOCs profile (e.g., sensitivity with O_3, attractiveness).

Acknowledgments

We thank the financial support by Kuribayashi Educational Scholarship and Academic Foundation (No. PK341563: N. Masui), and by JST (No. JPMJSC18HB: Dr. T. Watanabe and T. Koike). The Start-up Foundation for Introducing Talent of Nanjing University of Information Science & Technology (No. 003080: E. Agathokleous) is also acknowledged.

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