Plant-plant communication via volatile organic compounds in beech

Tomika Hagiwara

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CHAPTER 1

General introduction

1.1 Plant defense strategies against herbivores and pathogens

Plants cannot move like animals because they are rooted in one place. For that reason, they must be sensitive to their surroundings and they protect themselves from natural enemies. When plants are attacked by herbivores and pathogens, they lose their leaves, which are essential for photosynthesis are damaged. Therefore, plants take defensive strategies to protect their leaves. Plant defense strategies can be divided into two categories: (1) constitutive defense, in which plants always defend themselves, and (2) induced defense, in which plants defend themselves in response to feeding damage (Agrawal & Karban 2000). Within each category, they can be further classified into direct and indirect defense. Constitutive direct defense includes physical defenses such as trichomes and spines (Agrawal & Spiller 2004) and chemical defenses such as secondary metabolic products (e.g. tannins, phenols, and alkaloids), which are detested by herbivores (Baldwin 1989; Steppuhn & Baldwin 2007). In constitutive indirect defense, plants support natural enemies of their herbivore by providing alternative food or shelter (Quek *et al.* 2004). In induced direct defense, constitutive direct defense traits are enhanced by feeding damage. Induced indirect defense include the production of volatile organic compounds (VOCs) that attract predators and predatory parasitoids of herbivores (Shiojiri & Takabayashi 2003; Takabayashi & Shiojiri 2019) or inductively defend themselves by increasing the production of alternative food sources for herbivores (Heil & Bueno 2007).

Furthermore, since 2000, new VOC mediated induced defense was discovered and gained attention, in which plants increase their defense by receiving VOCs emitted from the plants themselves or from other neighboring damaged plants (Tscharntke *et al.* 2001; Morrell & Kessler 2017; Karban 2021). Plants sense the VOCs as a signal and then take a variety of defense strategies for survival.

1.2 VOCs emitted by plants

Most of the VOCs in terrestrial ecosystems are plant origin (Guenther et al. 1995). The amount of VOCs emitted by plants is estimated to be up to 10% of the carbon fixed by photosynthesis (Peñuelas & Llusià 2004). Most of VOCs released by plants are mainly terpenes, phenolic aromatics, and low molecular weight compounds derived from lipids. Terpenes have been reported to be the most abundant natural compounds with over 30,000 varieties (Connolly & Hill 2000).

Besides regular emission, plants emit VOCs in response to feeding by herbivores or artificial damage. The main compounds of VOCs induced by artificial damage are called green leaf volatiles (GLVs), which are green-smelling and consist of nine compounds (Matsui 2006; Matsui & Koeduka 2016) (Figure 1-1). These GLVs and terpenes have been reported as signals related to plant defense (Frost et al. 2007; Frost et al. 2008; Heil & Karban 2010; Matsui et al. 2012). VOCs emitted after leaf damage may affect plant itself (within-plant signaling, 1.3) or neighboring plants (VOC mediated plant-plant communication, 1.4).

1.3 VOC induced within-plant signaling

When a part of plant body is damaged by pathogens or herbivores locally, plant transmit this information throughout the plant (Hagiwara & Shiojiri 2020) and the plant will become more resistant to pathogens or herbivores. This phenomenon is called systemic acquired resistance (SAR) or systemic induced resistance (SIR) and has been reported in many plant species (Shiojiri & Karban 2008; Gao *et al.* 2015; Karban 2015; Riedlmeier *et al.* 2017; Wenig *et al.* 2019; Hagiwara & Shiojiri 2020; Lim *et al.* 2020). Previously, these signals are thought to be transmitted to other parts of the same plant by vascular connections such as phloem (Heil & Ton 2008). After one part of a plant is attacked, other parts of the same individual may be at higher risk than other individuals (Karban 2015). Therefore, it is predicted that intra-plant signaling transmits more rapidly and reliably than the signal transmission to other individuals (Heil & Adame-Álvarez 2010). Recently, it has been revealed that damaged leaves of many species release a variety of VOCs into their surroundings that induce SIR in undamaged leaves (Arimura, Huber & Bohlmann 2004; Heil & Ton 2008; Li & Blande 2017). Regarding the progress of signaling for systemic resistance, Heil and Ton (2008) discussed that the signaling by VOCs can be transmitted faster than signaling by the vascular connection when the plant become larger. In sagebrush (*Artemisia tridentata*), VOC signals may be more effective in SIR when the vascular system is limited (Karban *et al.* 2006; Karban & Shiojiri 2009).

1.4 VOC mediated plant-plant communication

Plants emit VOCs, especially GLVs, immediately within a few seconds in response to leaf damage caused by herbivores such as feeding or mechanical damage

(Takabayashi & Shiojiri 2019) (Mochizuki & Matsui, 2018) and continue the emission for three to four days (Arimura *et al.* 2004). When neighboring plants receive such VOCs, they often induce defenses against herbivores. The nearer neighbors from the VOC source showed less damage than the farther trees as early as 7 days and continued until 80 days after exposure (Dolch & Tscharntke 2000). This phenomenon is called plant-plant communication (Shiojiri, Karban & Ishizaki 2009; Heil & Karban 2010; Karban, Yang & Edwards 2014). It has gained attention as a new defense strategy for plants (Heil & Karban 2010). The first study was reported in 1983 showing that defense substances (a type of polyphenol) accumulated when healthy poplar and sugar maple trees were placed in airtight containers with damaged conspecifics (Baldwin & Schultz 1983). In the same year, Rhoades (1983) also reported plant-plant communication in willow trees. However, these studies had drawbacks because they did not separate the “treatment effects” of the VOCs from the "area effects" (e.g. light condition) in each experiment (Fowler & Lawton 1985). Fowler and Lawton (1985) published a paper titled "Rapidly induced defense and talking trees: The devil’s advocate position" criticizing plant-plant communication, and this research put a damper on the research field. In 2000, three studies with more rigorous experimental designs considering the number of replications were reported (Arimura *et al.* 2000; Dolch & Tscharntke 2000; Karban *et al.* 2000), demonstrating the existence of plant-plant communication. In particular, the expression of defense genes after receiving VOCs was demonstrated in lima bean (Arimura *et al.* 2000), and research using similar molecular biological techniques has enabled rapid progress in this field.

When plants are damaged by herbivores and pathogens, events such as expression of defense genes, accumulation of phytohormones, induced production and release of GLVs and terpenoids, and enhancement of direct and indirect defense against herbivores will occur consecutively, resulting in the reduction of leaf damage. Previously, plant-plant communication studies have focused on only a part of this process. However, for a comprehensive understanding of plant-plant communication, the multiscale understanding of these events is essential.

1.5 The importance of plant-plant communication studies in natural forests

Most of plant-plant communication studies were conducted in laboratory, green house or plantation and plant-plant communication in natural forests has not been focused. Few studies were conducted in natural forests such as sagebrush (*Artemisia tridentata*), alder (*Alnus glutinosa*) and birch (*Betula* spp.) (Dolch & Tscharntke 2000; Himanen *et al.* 2010; Karban *et al.* 2014). Since these tree species are bushy trees or early-successional trees, there is no study showing plant-plant communication in late-successional tree species which dominate for a long time in natural forests.

Studies in natural forests are important to understand the adaptive significance of plant defense, plant-insect interactions and population dynamics of these organisms (Karban 2020). Furthermore, knowledge of the relationship between trees and insects and pathogens is essential for future prediction, conservation and pest control in forest ecosystems. Climate change associated with global warming are expected to cause the decline of cool-temperate forests such as beech forests (Matsui et al. 2004) and the insect outbreaks. In agricultural crops, pest control methods that improve plant defenses by exposing plants to injury-induced scents have already been tested (Abe et al. 2020; Uefune et al. 2020). Such methods of pest control are expected to be environmentally friendly since soil may not be contaminated by chemicals sprayed. Therefore, studies about the plant-plant communication in natural forests are essential for the environmentally friendly pest control and the insect biodiversity conservation in natural forests.

Compared to laboratories, forests are heterogeneous systems and various factors may influence the presence and the absence, the strength and the detectability of plant-plant communication. Abiotic heterogeneity may include factors such as wind direction and forest structure. Biotic heterogeneity such as the variation between individuals may also affect plant-plant communication. Natural forests consist of trees of different genetic relatedness and recent studies suggested that plant-plant communication occurred between genetically more related plants (Shiojiri, Ishizaki & Ando 2021). Therefore, it is important to consider the variation of responses within populations due to abiotic and biotic heterogeneity to understand the plant-plant communication in natural forests.

1.6 Purpose and structure of this thesis

The objective of this study is to clarify the plant-plant communication in Japanese beech (*Fagus crenata*) focusing on the micro-level (within-plant signaling via VOCs, identification of VOC emission after leaf damage and the accumulation of plant hormones) to the macro-level (leaf damage after VOC exposure, inter-plant differences in leaf damage due to abiotic and biotic heterogeneities). Beech are late-successional trees dominating cool temperate forests.

In Chapter 2, I identified the predominant pathway for within-plant signaling: the internal transmission system by the vascular connection or the external transmission system using VOCs. In Chapter 3, I conducted manipulation experiments in plantations to clarify VOCs that specifically emitted after clipping. I also examined whether beech exhibits plant-plant communication and clarified the effective distance of plant-plant communication. In Chapter 4, I revealed the plant-plant communication in natural beech forests. I investigated at multiple processes such as the accumulation of salicylic acid (SA) and jasmonic acid (JA), phytohormones related to defenses against pathogens and herbivores, respectively, and leaf damage by them. I focused on abiotic environmental heterogeneity such as wind direction and forest stand structure and biotic heterogeneity such as spatial distribution of damaged trees and genetic relatedness among trees which may affect plant-plant communication in forest ecosystems. I discussed the characteristics of plant-plant communication in forest ecosystems. In Chapter 5, I summarize the results of Chapter 2 to Chapter 4 and discussed the mechanisms of plant-plant communication and their adaptive significance for defense strategy and the characteristics of plant-plant communication in forests and its significance for forest management (Figure 1-2).

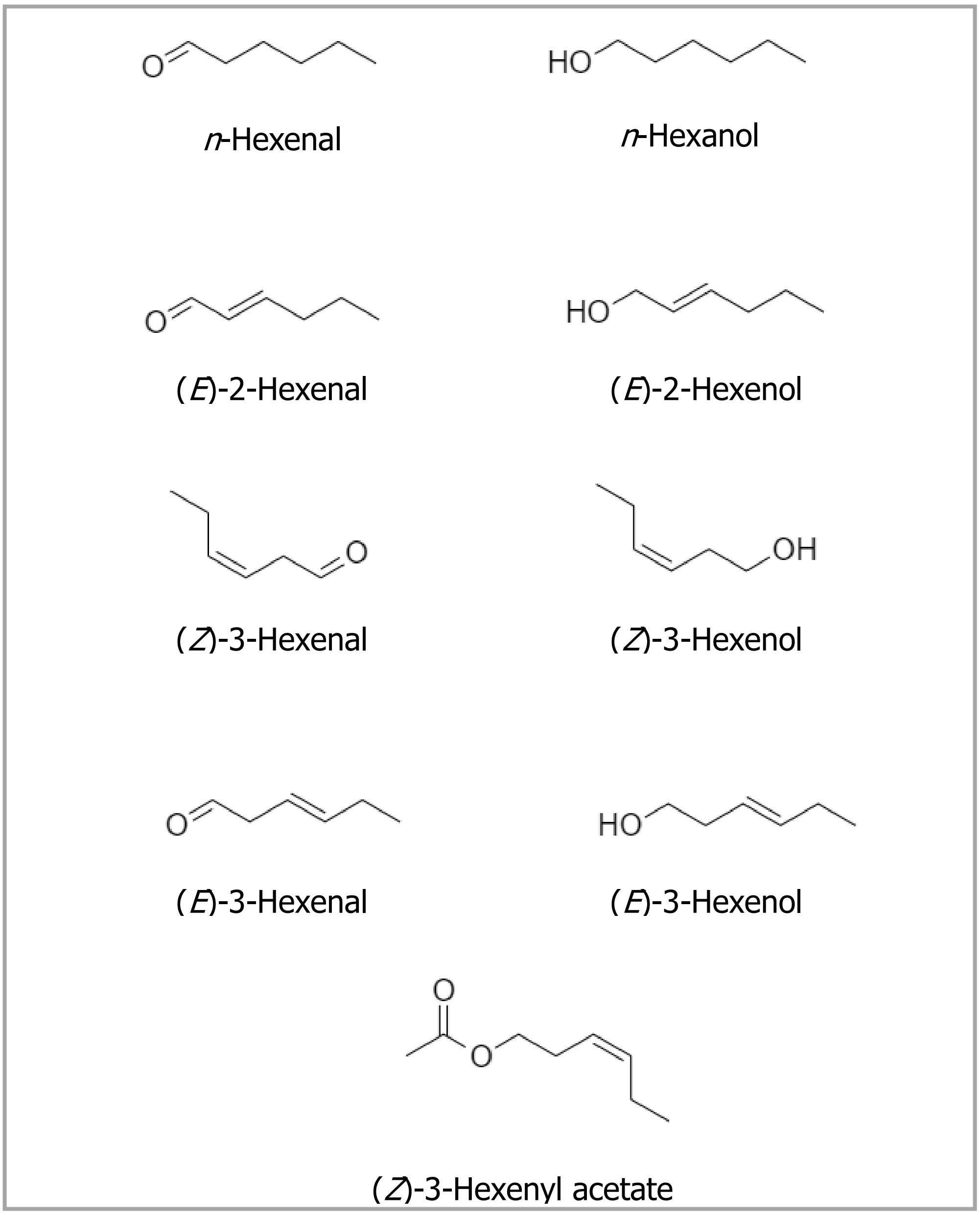

Figure 1-1. Chemical structure of selected green leaf volatiles known to be almost ubiquitously produced by plants.

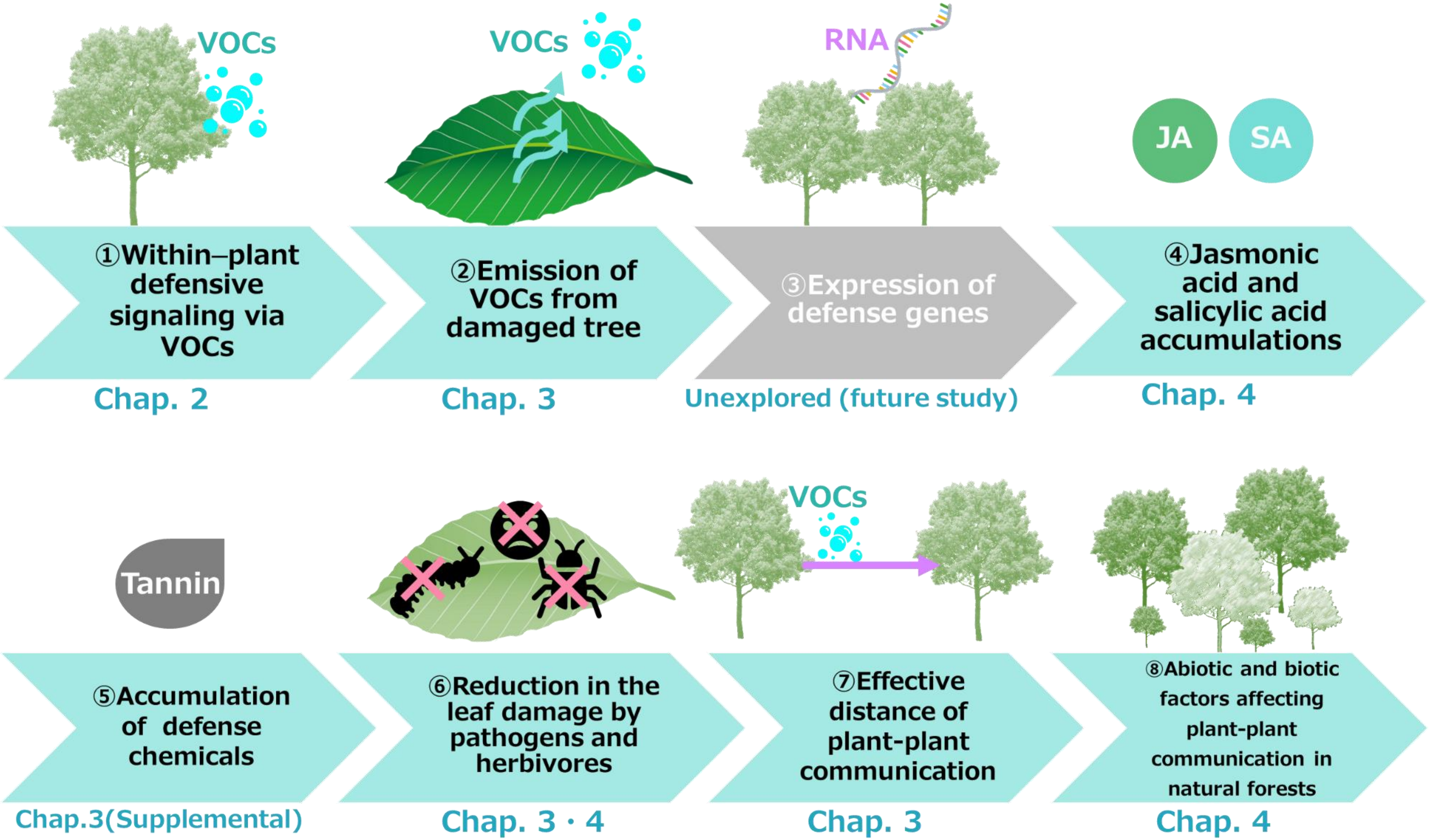

Figure 1-2. Process of plant–plant communication and structure of this study.

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CHAPTER 2

Within-plant signaling via volatiles in beech (*Fagus crenata* Blume)

Abstract

Many plants exhibit systemic induced resistance (SIR) coordinated among numerous branches after damage to a single localized tissue. Recently, it has been revealed that damaged leaves release a variety of volatile organic compounds (VOCs) into their surroundings that induce SIR. The phenomenon has been described for only four species in field experiments. I investigated SIR to herbivory in beech, a dominant tree species in Japan. I examined whether volatile cues were required for SIR by determining natural levels of leaf damage for assay branches that were on the same plant as treatment branches. When a local branch was mechanically clipped, the leaf damage by herbivores was reduced on other assay branches in same tree. However, when a clipped branch was covered with a plastic bag, the leaf damage was the same as assay branches on control treatment. These results suggested that SIR in beech trees are induced by VOCs emitted from the clipped branch within a tree.

2.1 Introduction

It is known that many plants exhibit systemic induced resistance coordinated among numerous branches after damage on a single localized tissue (Karban and Baldwin 1997). The phenomenon is called systemic acquired resistance (SAR) or systemic induced resistance (SIR), and has been reported for various plants such as hybrid poplar (*Populus × euroamericana*), tomato (*Solanum lycopersicum*), arabidopsis (*Arabidopsis thaliana*), and rice (*Oryza sativa*). After one part of a plant is attacked, other parts of the same individual may be at higher risk than other individuals (Karban 2015). Therefore, SIR may be adaptive to plants. Salicylic acid (SA) and jasmonic acid (JA) are known as signals of SIR through an internal pathway (Heil and Ton 2008).

Artificial damage to local leaves resulted in SIR in nonadjacent, orthostichous leaves (vertically aligned on the stem) with direct vascular connections, both up and down the shoot; but no SIR was reported in adjacent, non-orthostichous leaves with less direct vascular connections in eastern cottonwood (*Populus deltoides*) (Jones et al. 1993). For other plants, local damage induced expression of defense genes in non-damaged leaves regardless of the directionality of the phyllotaxis (Arimura et al. 2004).

Recently, it has been revealed that damaged leaves of many species release a variety of volatile organic compounds into their surroundings that induce SIR in undamaged leaves (Arimura et al. 2004; Heil and Ton 2008; Li and Blande 2017b). SIR signal transmission occurs not only through vascular connections but also via volatile organic compounds (VOCs) (Frost et al. 2008). For example, in sagebrush (*Artemisia tridentata*) external volatile cues may be used instead of limited vascular signaling among branches within an individual (Shiojiri and Karban 2008). In several plant species, only those branches that received volatile cues from neighboring clipped branches experienced less damage by herbivores than control branches (Dolch and Tscharntke 2000; Karban et al. 2006; Shiojiri and Karban 2006; Dong et al. 2011; Shiojiri et al. 2017). The phenomenon of intra-plant signaling via VOCs has been described only in eight plant species (Li and Blande 2017b). Only four of these studies were field experiments:

sagebrush (*Artemisia tridentata*) (Karban et al. 2006), lima bean (*Phaseolus lunatus*) (Heil and Bueno 2007), California mugwort (*Artemisia douglasiana*) (Shiojiri and Karban 2008), hybrid aspen (*Populus tremula* × *tremuloides*) (Li and Blande 2017b).

Although volatile signals, unlike vascular signals, move rapidly and can be detected by all leaves that have air contact with the damaged leaves, the transport of volatiles through air is influenced by many environmental factors such as wind, humidity, and temperature (Blande et al. 2014). Therefore, it is very difficult to conduct field experiments in natural condition (Karban et al. 2014). However, field studies provide important insights into whether and how plants detect and respond to VOCs in the natural environment. Beech is a dominant tree in many parts of the world, e.g. *Fagus sylvatica* in Europe, *Fagus orientalis* in East Asia, *Fagus grandifolia* in North America. Japanese beech (*Fagus crenata* Blume) is a dominant tree species throughout cool temperate mesic forests in Japan (Kon et al. 2005) It was reported that herbivores (*Quadricalcartfera puncttell* or *Lymantria dispar*) feeding on beech trees induce not only indirect defense but also increased the carbon/nitrogen ratio and the concentration of leaf tannin and phenolic compounds (Kamata et al. 1996; Aoyama and Koike 2011). Here I asked the following questions: (1) When a branch is mechanically clipped, does beech exhibit SIR and show changes in herbivore damage during the same growing season under natural conditions? (2) Which pathway, volatile, vascular, or both, is responsible for SIR in intra-plant signaling?

2.2 Material and methods

2.2.1 Plant and study site

The study was conducted in the Tomakomai Experimental Forest, Hokkaido

University (42°40’N, 141°36’E, 40 m a.s.l.). The monthly mean temperature ranges from −3.2°C to 19.1°C. Annual rainfall is 1200 mm. Seeds of Japanese beech (*Fagus crenata*) were collected from 5 source sites: Kuromatsunai (42.7°N), Ohirayama (42.6°N), Kikonai (41.6°N), Iwate (38.5°N), and Yabe (32.6°N) in Japan (Osada et al. 2018). The seeds were sown in a common garden in 1991 and trees grown from these seeds were used for the experiment. The other source trees were propagated from cuttings in the Tomakomai Experimental Forest (called Clone). The original scions were collected from a planted tree (height = 15.2 m; DBH = 53.3 cm) within the Hokkaido Forestry Research Institute Donan Branch Station (41°50’N, 140°43’E, 50 m a.s.l.), which has been previously used for temperature manipulation experiments (Kon and Noda 2007). The root stocks were from 5-year-old seedlings collected from Moheji (41°76’N, 140°59’ E) in 1997 (Miyazaki et al. 2014). The grafts were transplanted to the Tomakomai Experimental Forest from the Hokkaido Forestry Research Institute Donan Branch Station in April 2009 (Miyazaki et al. 2014).

All sample trees were grown in a high-light environment under similar soil conditions. Tree height was approximately 10 m (except for Clone trees which were approximately 3 m). Leaf expansion started in early May and leaf abscission started in the middle of October.

2.2.2 Systemic-induced resistance in plants

I examined systemic-induced resistance by surveying natural levels of leaf damage in mechanically clipped and unclipped trees in 2018 (Experiment 1, Figure 2-1a). I chose 2 trees from each of 5 source sites (Iwate, Kuromatsunai, Kikonai, Yabe and Clone) and 8 trees from Ohirayama. A branch was selected from each tree and either treatment (mechanically clipped or unclipped control) was assigned. For branches that were assigned to be clipped, I clipped the distal edge of 20–30 leaves into half with scissors on June 3. Five assay branches were selected from each tree resulting in 45 assay branches for each treatment (Figure 2-1a). To quantify the amount of damage by diverse herbivores and pathogens, I surveyed the leaf damage of each assay branch on September 2 in 2018, 90 days after the clipping, to evaluate the damage during leaf expansion to abscission. The level of leaf damage was evaluated by counting the total number of leaf segments damaged by herbivores and pathogens for all leaves on each assay branch. Each leaf segments was a portion of the leaf blade that was surrounded by parallel leaf veins (Figure 2-2). The mean number of segments per leaf was calculated for each source site by averaging the number of segments of 10 leaves on selected beech trees (n=10 for each source). When a whole leaf was eaten by herbivores, I could not measure the damage. When a leaf remained partially, the number of eaten segments was estimated by subtracting the number of remaining segments from the mean number of segments. Conventional methods for quantifying leaf damage were based on categorical levels of damage (e.g. Nakamura et al. 2014) and determined rather subjectively by researchers. More quantitative method will be based on damaged leaf area. However, it was not possible because I could not identify pathogen damages from the images of scanned leaves. Furthermore, I could not record leaf damage for each leaf because the measurement will take long time and leaves will be wilted during the measurements. Therefore, to be quantitative and practical, I pooled the number of damaged leaf segments for each branch.

2.2.3 Are volatile cues required in systemic induced response?

I examined whether volatile cues are required for systemic induced resistance by determining natural levels of leaf damage for assay branches that were on the same plant as treatment branches in 2019 (Experiment 2, Figure 2-1b:). I chose 4 trees from each of 4 source sites (Iwate, Kuromatsunai, Kikonai and Clone) and 3 trees from Ohirayama. Five assay branches were selected from each tree resulting in 35 assay branches for each treatment (Clipped, Bagged or Control) (Figure 2-1b). For branches that were assigned to be mechanically clipped, I clipped the distal edge of 20–30 leaves into half with scissors on June 1. For branches that were assigned to be mechanically clipped and bagged, I clipped leaves similarly, covered the clipped branch with a plastic bag (500 mm × 350 mm) and then sealed the bag with a wire twist-tie. After 10 days, the plastic bag was removed without giving excessive stress to branch. On August 30, I evaluated the level of damage caused by herbivores and pathogens by counting the total number of segments damaged by herbivores and pathogens in a branch 90 days after the clipping to evaluate the damage during leaf expansion to abscission.

In this study, leaf damage was measured as total damaged segments of each branch but not as that of each leaf. Furthermore, I dealt the damage as an integer and did not deal it as a binomial variable (whether a segment is damaged or not), although damage was evaluated for each segment. This is because herbivores and pathogens may feed on leaves in various patterns and the relationship between sampling units (segments or leaves) may not be consistent among species. For example, chewing larvae feed on a leaf or leaves of a branch in consecutive manner (see Appendix), where neighboring segments within a leaf or neighboring leaves within a branch are more likely to be damaged. This means that damage on segments or leaves is non-random variable and dependency among segments or leaves vary depending on spatial layout and herbivore's feeding patterns. On the other hand, one to several galls may be formed on a leaf resulting in rather uniform distribution of galls within a branch. This means that segments within a leaf may be dependent each other but leaves within a branch may be assumed to be an independent random variable. Pathogens also showed various infection patterns. Such variation of damage occurrence among herbivores and pathogens may be less apparent when the number of damaged segments is pooled for a branch. Therefore, I assumed that leaf damage of a branch as an independent random variable and that may better follow the assumption of the statistical analyses such as generalized linear models than damages at lower sampling unit such as leaves or segments. Of course, branches are nested within a tree and the leaf damage of a branch may depend on tree's factors such as seed origin or resource availability (see 2.2.4 for how I dealt with this dependency). However, the independency/dependency among branches within a tree may be rather uniform among trees (Supplemental Figure 2-1).

2.2.4 Statistical analyses

To test whether the proportion of damaged leaf segments differ between treatments (control or clipped) in experiment 1, I used generalized linear mixed effect model (GLMM) with the maximum likelihood via the lmer function in the lme4 package of R 4.1.2 (R Core Team 2021). All statistical tests were performed using R software. Since the response variable (i.e., the total number of damaged leaf segments of a branch) was a discrete variable, I fitted GLMMs with a Poisson distribution and a log-link function. Since some branches were sampled from the same tree, I used the mixed effect model to account for non-independent errors due to tree identity (Faraway, 2016). In other words, dependency of leaf damage of a branch to tree identity was accounted for as a random variable. Since the number of segments per leaf differed among trees, the total number of segments of a branch was included in the model as an offset term. The full model included the following: treatment (control or clipped) as fixed effect; and tree as a random effect. To test the effect of treatment, I conducted log-likelihood ratio tests between the focal model and the best model.

In experiment 2, the GLMM model with the treatment (control, bagged and clipped) as a fixed effect and tree as a random effect was applied. To compare the leaf damage between the control, bagged and clipped treatments, I conducted Tukey's method using the emmeas package of R 4.1.2 (R Core Team 2021).

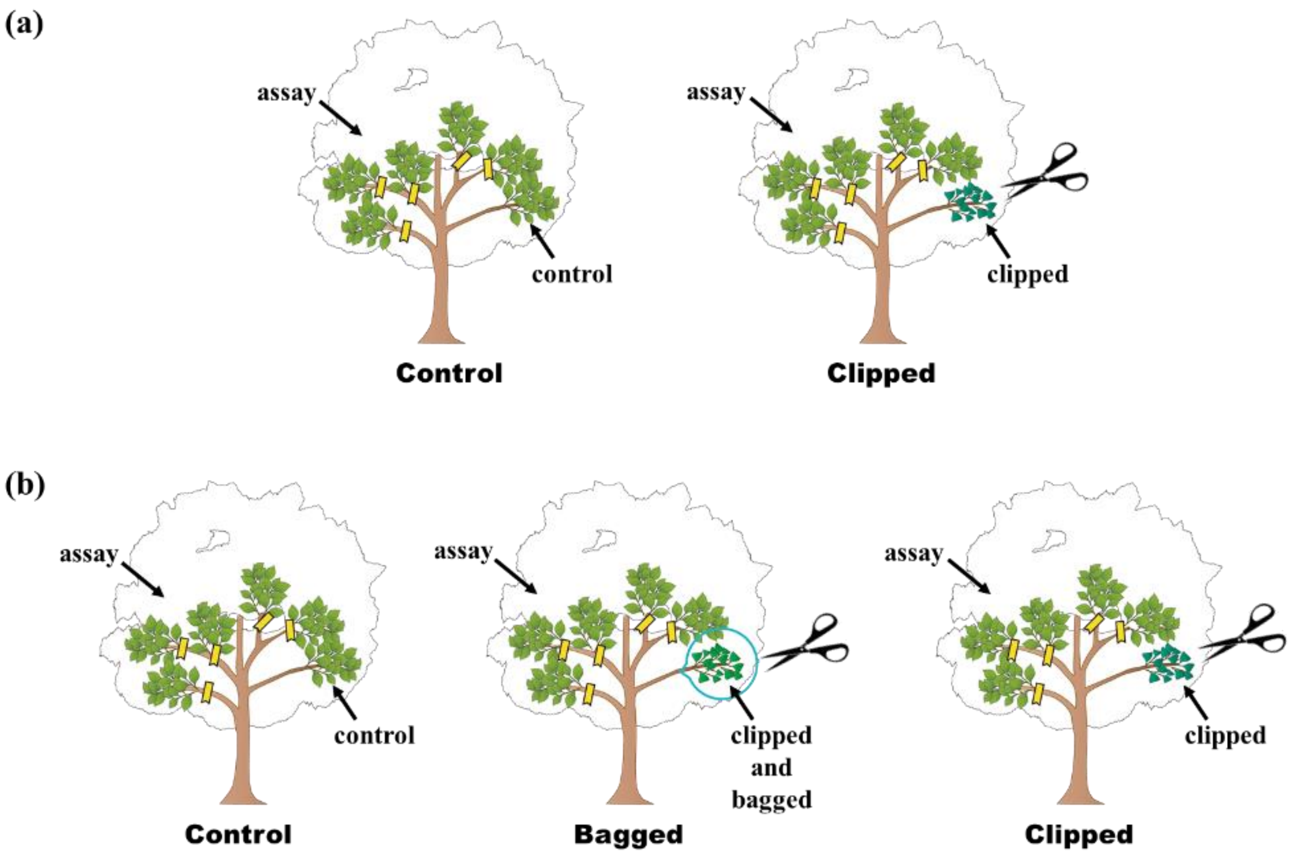


Figure 2-1.

(a) Experiment 1. Control tree (n = 9) with an unclipped branch and five assay branches and clipped tree (n = 9) with a clipped branch and five assay branches. (b)Experiment 2. Control tree (n = 7) with an unclipped control branch and five assay branches, bagged tree (n = 7) with a clipped branch enclosed in a plastic bag and five assay branches, and clipped tree (n = 7) with a clipped branch and assay branches.

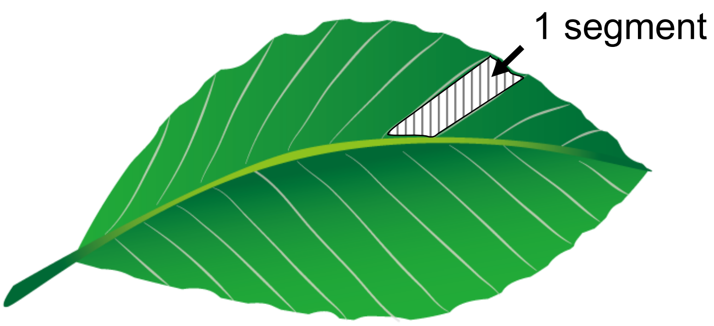


Figure 2-2.

Each leaf segment was a portion of the leaf blade that was surrounded by parallel leaf veins.

2.3 Results

2.3.1 Are volatile cues required in systemic induced response?

In experiment 2, I tested the hypothesis that air contact was necessary for systemic induced response. Fixed effect was treatment (Control, Bagged and Clipped). When air contact was allowed, the proportion of damaged leaf segments was reduced by approximately 50% on assay branches on clipped treatment compared to assay branches on control treatment (Figure 2-3b). When air contact was not allowed by bagging, the proportion of damaged leaf segments was almost the same as controls. Assay branches on clipped treatment showed significantly lower leaf damage than assay branches on control or bagged treatment (Tukey's methods; $p < 0.001$). These results indicate that air contact was required for systemic induced resistance within a single plant.

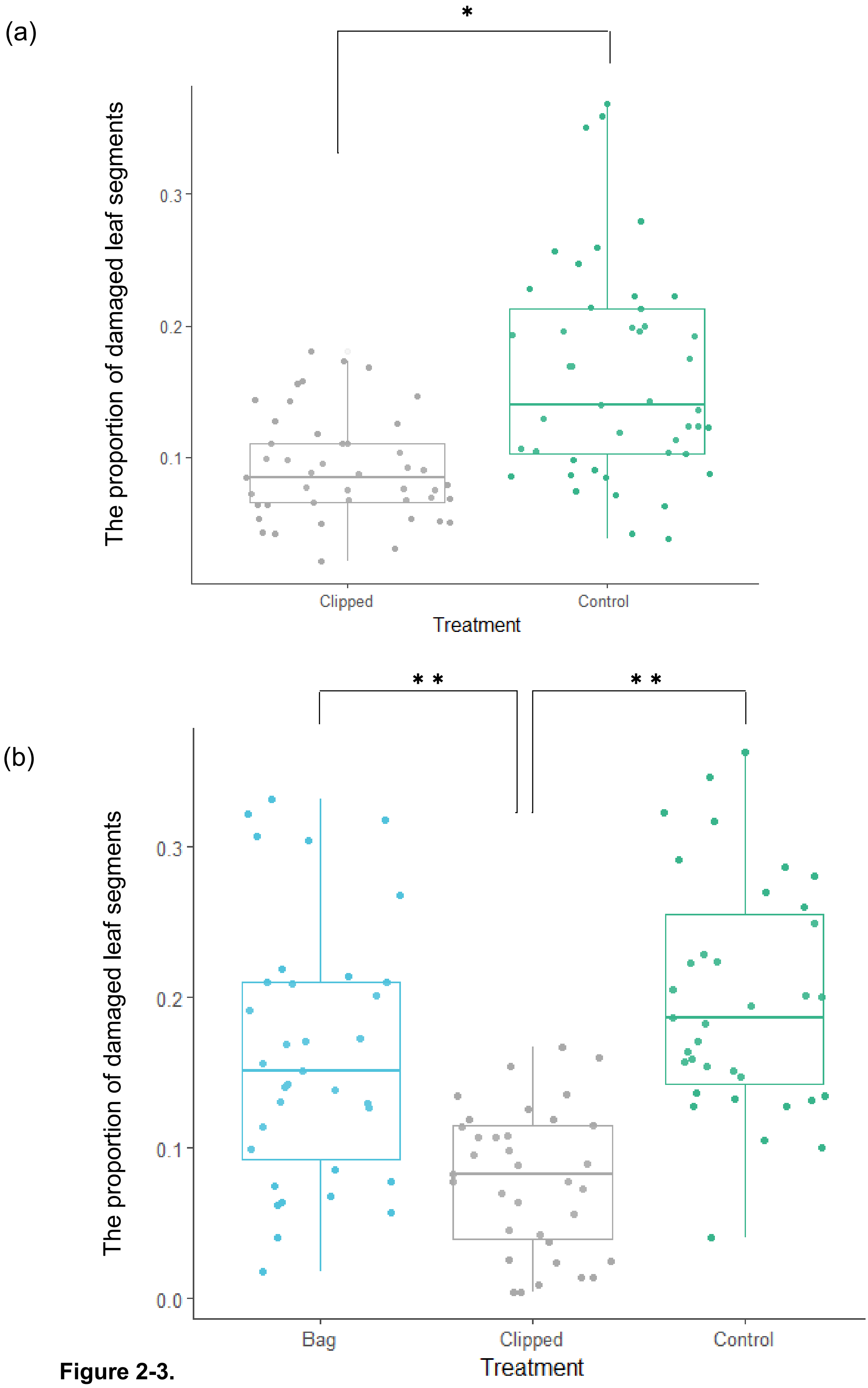


Figure 2-3.
(a) Experiment 1. The proportion of damaged leaf segments for each assay branch (*n* = 45 for each treatment). Letters above the bars indicate statistical differences among means using log-likelihood ratio test. *: $p < 0.01$ (b) Experiment 2. The proportion of damaged segments for each assay branch (*n* = 35 for each treatment). Letters above the bars indicate statistical differences among means using Tukey's method. **: $p < 0.001$. Each branch is shown as a dot.

Table 2-1. Summary of generalized linear mixed effect model (GLMM) for the number of damaged leaf segments of a branch.

Fixed effects of candidate models	Parameter	AIC	LL	Deviance	DF	Chisq	*p*
Experiment 1							
Treatment	**3**	**953.5**	**-473.73**	**947.5**			**<0.01 ***
Null	2	961.6	-478.82	957.7	1	10.2	
Experiment 2							
Treatment	**4**	**1001.4**	**-496.68**	**993.4**			**<0.001 ****
Null	2	1011.8	-503.90	1007.8	1	13.1	

Shown are number of parameters used in the model (Parameter), Akaike's Information Criterion (AIC), log-likelihood (LL), difference in degree of freedom against above model (DF), Chi-squared value (Chisq), *p*-value using log-likelihood ratio test (P). Tree was a random variable. The number of segments were pooled for a branch.

2.3.2 Systemic induced resistance in plants

In experiment 1, assay branches on clipped treatment got approximately 50% less damage than assay branches on control treatment (Figure 2-3). The most-fit model included treatment as a fixed effect (Table 2-1). This result indicates that leaf clipping induced systemic-induced resistance on other branches.

In this experiment, the assay branches and treatment branches (clipped or unclipped) shared vascular connections. I could not determine whether this systemic-induced resistance was induced through shared a vascular connection or through volatile cues.

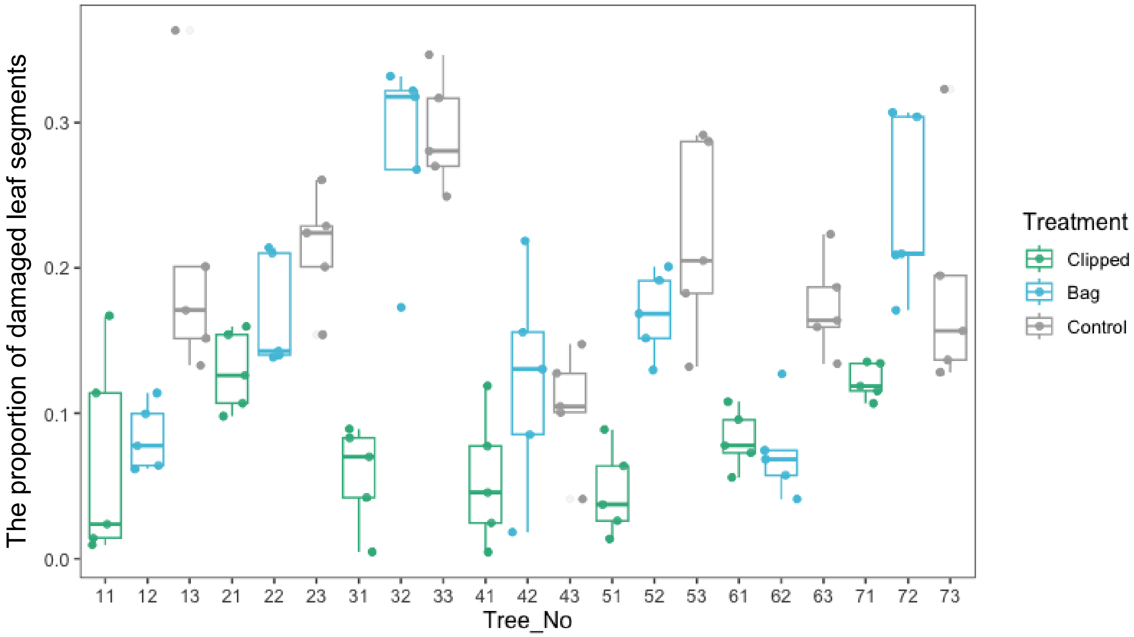
2.4 Discussion

It is known that plants can induce systemic resistance following local damage. My experiments indicate that mechanically clipped beech leaves caused systemic induced resistance throughout the tree (Experiment 1). I found that the clipped treatment decreased damage compared to the control and bagged treatments (Experiment 2). For the bagged treatment, I covered the clipped branch with a plastic bag after clipping. Because the clipped branch may not have continued to emit active cues for more than 7 days, the branches that were bagged for 10 days could not transmit VOCs to neighboring branches. Therefore, the signal for SIR was VOCs from the clipped branches and might not transmitted via vascular connections. In several previous studies, VOCs emitted following actual herbivore damage differed from those emitted following mechanically clipped damage; however, experimental clipping also induced systemic resistance in these plants (Karban et al. 2006; Shiojiri and Karban 2006; Li and Blande 2017a).

Aoyama and Koike (2011) reported that local damage by herbivores (Gypsy moth: *Lymantria dispar*) increased the condensed tannin, total phenolics and the C/N ratio after 10–30 days compared to undamaged beech leaves. Local damage also induced resistance against subsequent herbivores. However, the concentration of plant defense compounds became almost the same as that of the control treatment after 50 days. Although the defense traits was not measured in this study, I found that leaf damage in the clipped treatment was less than half the damage in the control treatment 90 days after the clipping. In brief, the differences in early season defenses may have resulted in the differences throughout the season.

In other studies, early season attacks by chewers and gall makers were strong negative predictors of later season chewing and galling (Cornelissen et al. 2011). Dalin and Bjorkman (2003) determined that *Salix cinerea* L. are usually attacked again later in the season by leaf beetle (*Phratora vulgatissima* L.) larvae; nevertheless, larvae on plants previously exposed to adult grazing consumed less total leaf area and showed more dispersed feeding than larvae on plants protected from previous grazing.

Kessler et al. (2006) found that wild tobacco plants (*Nicotiana attenuata*) with air contact with mechanically clipped sagebrush (*Artemisia tridentata*), were primed for accelerated proteinase inhibitor (PI) activity when subsequently exposed to experimental feeding by *Manduca sexta*. Thus, defense priming by VOC exposure can accelerate responses following attack by herbivores (Kessler et al. 2006). In this study, I clipped leaves in the early season and examined the branches later. These results demonstrate that clipping in the early season may be a strong negative predictor of later season damage. Although I did not examine the activation of defense genes for priming, the trees that got damage on a local branch might be primed systemically for subsequent attack, and the priming response may lead to these results. In this study, I considered artificially clipped leaves as damaged. However, leaves that are damaged by herbivores and pathogens produce different emissions in both timing and quantities. Herbivore damage often causes VOC emissions over a longer time than artificial clipping. Therefore, natural damage by herbivores and pathogens may induce resistance more continuously and more strongly. Future studies should focus on natural damage by herbivores via VOC signaling.



Supplemental Figure2-1.

Experiment 2. The proportion of damaged leaf segments of each assay branch (*n* = 35) of each surveyed tree. Each branch is shown as a dot. The leaf damage differed among trees but less among branches within a tree. Tree 11 – 13 are from same seed origin, and so on (21 - 23, 31 - 33, 41 - 63 and 71 – 73).

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This chapter

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Data availability statement

Data from this manuscript were archived in the publicly accessible repository Dryad （https://doi.org/10.5061/dryad.5mkkwh79n.）.

CHAPTER 3

Effective distance of volatile cues for plant-plant communication in beech

Abstract

In response to volatiles emitted from a plant infested by herbivorous arthropods, neighboring undamaged conspecific plants may become better defended against herbivores; this is referred to as plant–plant communication. Although plant–plant communication occurs in a wide range of plant species, most studies have focused on herbaceous plants. Here, we investigated plant–plant communication in beech trees in two experimental plantations in 2018 and one plantation in 2019. Approximately 20% of the leaves of a beech tree were clipped in half in the spring seasons of 2018 and 2019 (clipped tree). The damage levels to leaves in the neighboring undamaged beech trees were evaluated 90 days after the clipping (assay trees). In both years, the damage levels decreased with a reduction in the distance from the clipped tree. In 2019, I also recorded the damage levels of trees that were not exposed to volatiles (nonexposed trees) as control trees and found that those that were located ≤ 5 m away from clipped trees had significantly less leaf damage than nonexposed trees. By using a gas chromatograph-mass spectrometer, ten and eight volatile compounds were detected in the headspaces of clipped and unclipped leaves, respectively. Among them, the amount of (*Z*)-3- hexenyl acetate in clipped leaves was significantly higher than that in nonclipped leaves. My result

suggests that green leaf volatiles such as (*Z*)-3- hexenol and (*Z*)-3- hexenyl acetate and other volatile organic compounds emitted from clipped trees induced defenses in the neighboring trees within the 5 m radius. The effective distances of plant–plant communication in trees were discussed from the viewpoint of the arthropod community structure in forest ecosystems.

3.1 Introduction

Plants emit volatile organic compounds (VOCs) in response to leaf damage caused by herbivorous arthropods or mechanical damage such as weeding (Takabayashi & Shiojiri, 2019). When neighboring plants receive such VOCs, they often induce defenses against herbivores; this phenomenon is referred to as plant-plant communication (Heil & Bueno, 2007; Heil & Karban, 2010; Karban et al., 2000, 2014; Shiojiri & Karban, 2008b; Tscharntke et al., 2001; Yoneya & Takabayashi, 2014). For example, field-grown sagebrush trees (*Artemisia tridentata*) which were exposed to VOCs from clipped conspecific plants in the early season experienced less herbivore damage in the following seasons (Shiojiri & Karban, 2008a). Lima bean (*Phaseolus lunatus*) plants exposed to VOCs from herbivore-damaged conspecific plants increased the amount of extrafloral nectar that attracts carnivorous arthropods (Choh et al., ,2004, 2006; Choh & Takabayashi, 2006; Heil & Kost, 2006). Plant-plant communication mediated by VOCs has been observed in >40 plant species, most of which are herbaceous (Heil & Karban, 2010; Karban et al., 2014; Li, 2016; Yoneya & Takabayashi, 2014). However, the first evidence of the phenomenon was found in tree species such as sugar maple and poplar (Baldwin & Schultz, 1983).

Besides laboratory or greenhouse conditions (e.g., Arimura et al., 2004; Girón-

Calva et al., 2014), plant-plant communication in trees under field conditions has been reported in only six taxa: poplar (*Populus euramericana*) (Frost, Mescher, Dervinis, et al., 2008), willows (*Salix sitchensis* and S. *eriocalpa*) (Pearse et al., 2013; Yoneya & Takabayashi, 2013), black alder (*Alnus glutinosa*) (Dolch & Tscharntke, 2000; Tscharntke et al., 2001), birch (*Betula* spp.) (Himanen et al., 2010), and sagebrush (*Artemisia tridentata*) (Karban et al., 2006); these taxa are either early-successional tree species or shrub species. Late-successional species dominate forests for a long time and provide important ecological and socioeconomic value. However, to the best of my knowledge, there has been no plant-plant communication studies in late-successional tree species.

Determining the effective distance of VOCs may be important for understanding arthropod communities because the quality of leaves affects arthropod distribution. The effective distance has only been studied in two species (black alder and sagebrush) under field conditions. Karban et al. (2006) revealed that the effective distance of sagebrush (*Artemisia tridentata*) was 0.6 m by comparing leaf damage among control and exposed plants. In black alder, the number of damaged leaves increased with increasing distance (up to 10 m) from a manually defoliated conspecific tree (Dolch & Tscharntke, 2000). The number of specialist herbivores of black alder (i.e., leaf beetles: *Agelastica alni*) was higher on the farthest tree (10.6 m from the defoliated tree) than on the nearest tree (1.3 m from the defoliated tree) (Tscharntke et al., 2001). Furthermore, leaf beetles avoided leaves from the nearest alder tree and preferred those from the farthest tree for both feeding and oviposition in laboratory experimental assays. Although these authors focused on the distance of plant-plant communication in alder trees, they did not compare the damage of exposed trees to that of nonexposed trees.

Therefore, the effective distance for plant–plant communication in forests remains unknown.

Japanese beech (*Fagus crenata* Blume) (hereafter "beech") is a late-successional tree species that often dominates cool-temperate mesic forests in Japan (Hiura, 1995). The dominant herbivorous arthropods are beech caterpillars (*Quadricalcarifera punctatella*) and gypsy moth (*Lymantria dispar*) larvae (Nakamura et al., 2014). Damage caused by these herbivores increases the C:N ratio and the tannin and phenolic compound contents in beech leaves (Aoyama & Koike, 2011; Kamata, 1996). Manual leaf-clipping reduces the leaf nitrogen content (Kamata et al., 1996). These studies showed that beech can induce direct defenses against herbivory. Beech also exhibits an induced defense via VOCs, whereby undamaged beech leaves respond to VOCs from clipped leaves within the same tree (intraplant signaling), causing systemically induced resistance (Hagiwara & Shiojiri, 2020). However, to the best of our knowledge, volatiles of damaged beech have not yet been identified. Since plant– plant communication via volatiles from damaged plants is hypothesized to originate from intraplant signaling (Heil & Bueno, 2007), we hypothesize that undamaged beech trees can eavesdrop on damage-induced volatiles from damaged trees (interplant signaling).

The objective of the present study was to clarify whether beech trees exhibit plant–plant communication under field conditions and to determine the effective distance. We also compared the VOCs and tannins between damaged and undamaged beech. Finally, we discuss the effect of plant–plant communication in beech on the arthropod community in a forest ecosystem.

3.2 Material and methods

3.2.1 Beech plantations

Field experiments were conducted in beech plantations in the Tomakomai Experimental Forest (42°7’N, 141°6’E, 220 m a.s.l.), Hokkaido University (hereafter called Tomakomai), in 2018 and 2019, and in the Hiruzen Experimental Forest (35°3’N, 133°6’E, 510 m a.s.l.), Tottori University (hereafter called Hiruzen), in Japan in 2018. These two plantations were both established in 1991 by planting beech seedlings that were approximately 4 years old. The seeds were obtained from a variety of sources (Osada et al., 2018). The beech seedlings were planted on flat land at the Tomakomai and on a slope facing west in the Hiruzen. In both plantations, beech trees reached heights of 10–13 m. Leaf expansion began in early May and leaf abscission began in mid-October.

3.2.2 Experimental design

I selected three trees in the Tomakomai and two trees in the Hiruzen as volatile-emitting trees in 2018. I clipped approximately 20% of the leaves of each tree into halves (hereafter called clipped trees). I conducted the clipping on May 22 in the Tomakomai and June 12 in the Hiruzen when herbivore species were active. The neighboring undamaged beech trees were assigned as volatile-exposed trees (assay trees). For each clipped tree, ten branches on neighboring trees with 8–12 leaves each that were located at each distance (3, 5, 7, 9, or 11 m from the clipped tree) were marked. When a tree covered two distance levels (e.g., 5 m and 7 m; Figure 3-2), I selected 10 branches for each distance. After 90 days, the number of leaf segments damaged by chewing herbivores and pathogens was recorded for each branch. As a beech leaf consists of segments separated by secondary veins (Figure 2-1), I evaluated the level of damage by counting the total number of segments damaged by herbivores and pathogens in a branch. The mean number of segments per leaf for each site was calculated by averaging the number of segments of a leaf randomly selected from each of the 10 beech trees (21.6 to 24.4 per leaf) Beech leaves were damaged by *Actias aliena*, *Sphrageidus similis*, and other herbivores as well as yellow leaf spot (Wei & Harada, 1998) and other leaf spots caused by pathogens.

To evaluate the effective distance of the plant-plant communication, I compared the damage levels of unexposed beech (control trees) with those of 13 assay trees in 2019 at the Tomakomai. The experimental conditions in 2019 were the same as those in 2018. Clipping was performed on May 31 in 2019. From the 2018 experiment, I found that leaf damage increased as the distance from the clipped tree up to 7–11 m. Among 7-, 9-, and 11-m branches, the difference were not significantly different. This result meant that the control treatment needs to be >11 m away. I assayed 15 trees which were planted 13.0–66.5 m from clipped trees as controls and selected 93 sample branches from these trees on May 31. After 90 days, the number of leaf segments damaged by herbivores and pathogens was recorded for each branch, as in 2018.

3.2.3 Chemical analysis of headspace VOCs from beech leaves

I collected VOCs under field conditions in Tomakomai. The sampling field was different from the field in which the exposure experiments were conducted. Beech seeds were collected in the area of the Hakodate Forestry Office in 1973. To reveal the differences in VOCs between the clipped and control treatments, I randomly selected eight beech trees. On 10 and 11 June 2019 I tightly enclosed a branch with approximately 30 undamaged leaves in a PTS bag (500 mm × 350 mm; Mitsubishi Gas Chemical Co.

Tokyo, Japan). Using Tenax TA as an adsorbent, the VOCs in the bag were collected for 60 min at an airflow rate of 100 ml/min. Airflow was generated using an air pump (Sibata Scientific Technology Ltd., Saitama, Japan). After collecting VOCs from the undamaged leaves, I clipped approximately 50% of the leaves of the branch in half using scissors. After clipping, I re-enclosed the branch to collect VOCs from clipped leaves using the same procedure. The VOCs trapped on Tenax TA were eluted with 2 ml of diethyl ether (FujifilmWako Pure Chemical, Osaka, Japan). I injected a 1 μl aliquot of the eluate into the injection port (250°C) of a gas chromatograph-mass spectrometer (GC–MS: QP2010SE, Shimadzu, Kyoto, Japan) equipped with HP-5MS capillary column (0.25 mm i.d., length 30 m, film thickness 0.25 μm; Agilent Technologies, Santa Clara, CA, USA). The oven temperature of the GC–MS was programmed to increase from 40°C (5 min hold) to 280°C at a rate of 10°C min^{-1}. Detected compounds were tentatively identified by comparison with mass spectra in the Wiley 7 N database. VOCs were further identified by comparing their mass spectra with those of the authentic compounds. The total ion intensities were used to compare the amount of each compound between the undamaged and clipped conditions.

3.2.4 Statistical analyses

To test whether the leaf damage of assay trees changed along the distance from clipped trees in the 2018 experiment, I used a generalized linear mixed effect model (GLMM) which applied the Laplace approximation and maximum likelihood via the lmer function in the lme4 package of R 4.0 (R Core Team, 2020). All statistical tests were performed using R software. Since the response variable (i.e., the total number of damaged leaf segments of a branch) was a discrete variable, I fitted GLMMs with a Poisson distribution and a log-link function. Since some branches were sampled from the same tree, I used the mixed effect model to account for non-independent errors due to tree identity (Faraway, 2016). Since the number of segments per leaf differed among trees, the total number of segments of a branch was included in the model as an offset term. The full model included the following: distance, plantation, and the interaction term between distance and plantation as fixed effects; and tree as a random effect. Tree was a random variable. The number of segments were pooled for a branch. To test the effect of distance and plantation (Tomakomai or Hiruzen), I conducted log-likelihood ratio tests between the full and reduced models.

To estimate the effective distance of plant–plant communication, we applied the GLMM for the 2019 experiment. The full model included distance as a fixed effect and tree as a random effect. Tree was a random variable. The number of segments were pooled for a branch. This model was tested by log-likelihood ratio test against the null model without distance. To further estimate the effective distance, data of branches at a 3-m distance from neighboring trees were combined with those of control trees. The GLMM model with the treatment (control and exposed) as a fixed effect and tree as a random effect was applied. Tree was a random variable. The number of segments were pooled for a branch. This model was tested by the log-likelihood ratio test against the model without treatment assuming no difference between the control and 3-m distance neighboring trees. This process was sequentially repeated for 5-, 7-, 9-, and 11-m distances. Some trees were used for clipping experiments in both 2018 and 2019. The result and conclusion did not change even if 2-year data were analyzed together incorporating the repeated measurements of trees by GLMM (result not shown). To compare VOCs between the control and clipped treatments, I conducted a paired t-test for each compound.

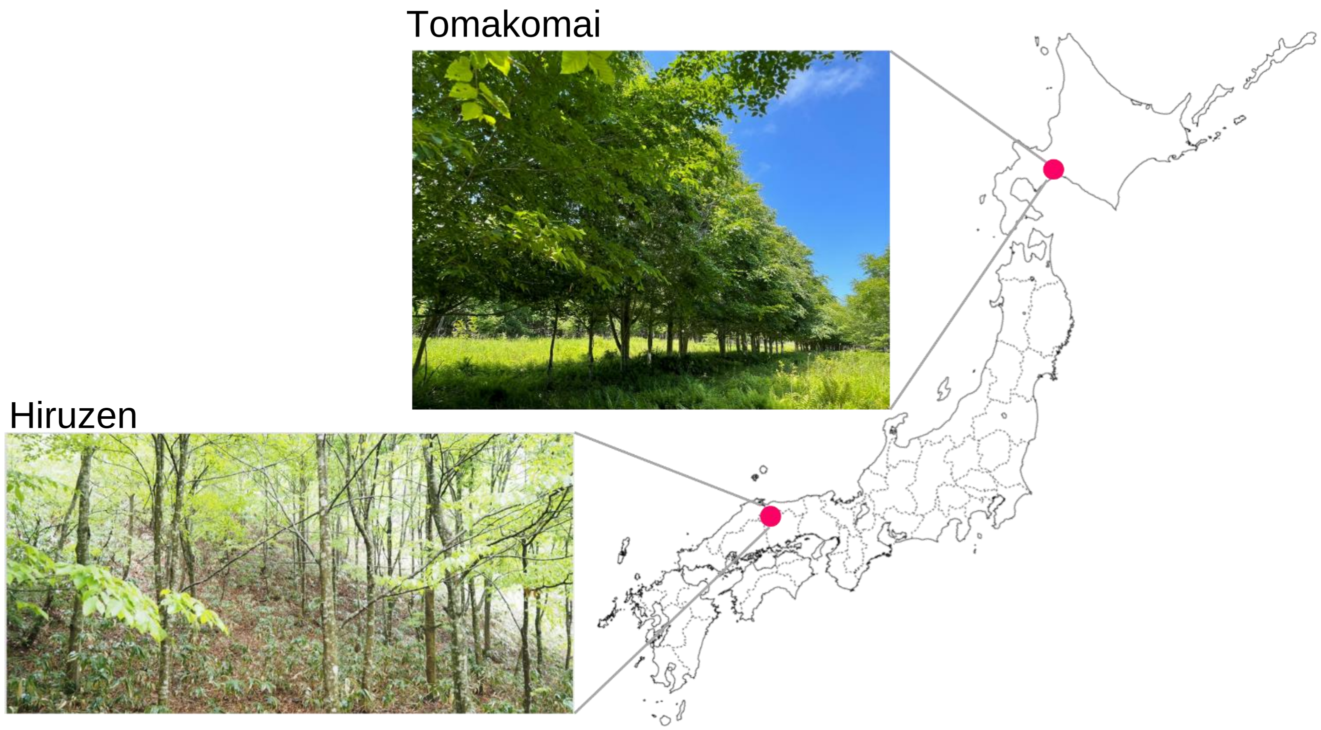


Figure 3-1.

Field experiments were conducted in beech plantations in the Tomakomai Experimental Forest, Hokkaido University, in 2018 and 2019, and in the Hiruzen Experimental Forest, Tottori University, in Japan in 2018.

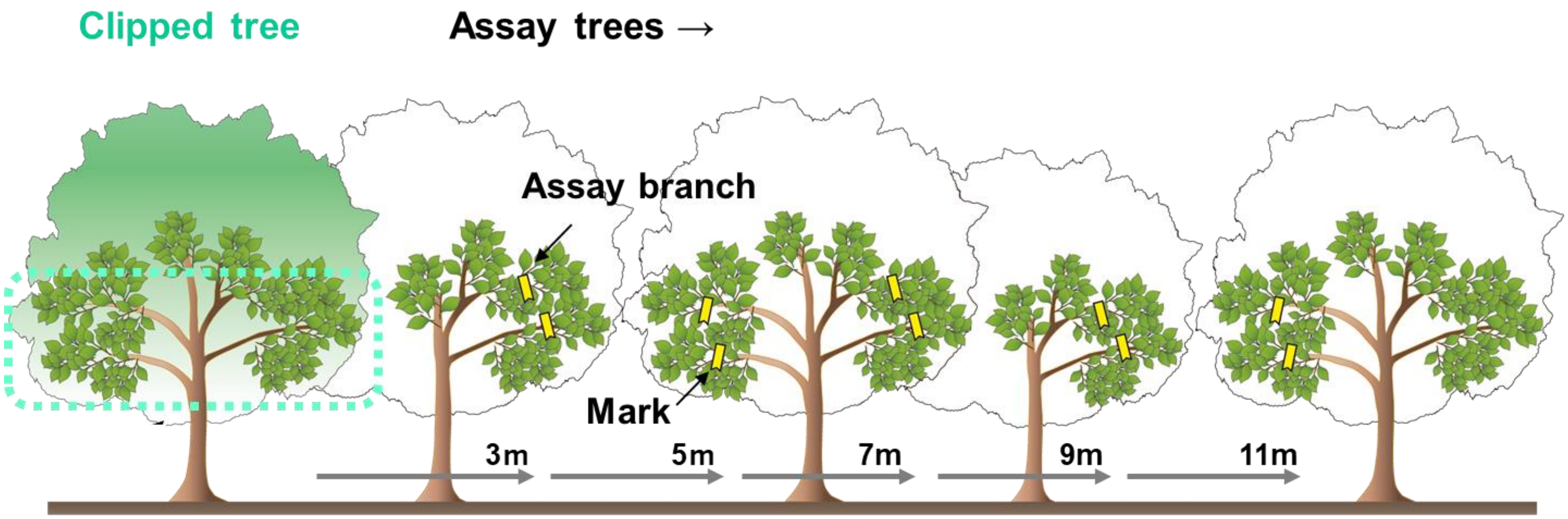


Figure 3-2.
Schematic diagram of experimental designs. Twenty percent of leaves were cut into half by scissors in each clipped tree. The assay branches were selected and marked on neighboring trees (assay trees).

3.3 Results

3.3.1 Effective distance of plant-plant communication

For the 2018 data, the full model with distance, plantation, and the interaction between distance and plantation (Distance × Plantation) as fixed effects was compared with reduced models. Reduced models were the models assuming the effect of distance and plantation (Distance + Plantation), effect of distance alone (Distance), and difference between plantations (Plantation), and the null model assuming no difference between plantation and distance (Null). The most-fit model included distance and plantation as fixed effects in 2018 (Table 3-1). Damaged leaf segments increased with distance from clipped trees. In other words, trees neighboring clipped trees showed less damage than those located at long distances from the clipped trees (Figure 3-3). The leaf damage level in Hiruzen was almost twice as high as that in Tomakomai at each distance in 2018. For the 2019 data, the full model with distance as a fixed effect was compared to the null model assuming no effect of distance (Null). Similar to 2018, the effect of distance was significant, and the nearer neighbors of the clipped tree showed less leaf damage than the farther trees (Table 3-1; Figure 3-4). Assay trees located up to a 5-m distance from clipped trees showed significantly lower leaf damage than control trees (Table 3-2; $p = 0.0030$). Assay trees at a 7-m distance showed a trend (Table 3-2; $p = 0.1080$). The difference between assay and control trees gradually decreased as the distance from the clipped trees increased. Assay trees at distances of 9 and 11 m from clipped trees showed leaf damage similar to those in control trees.

Table 3-1. Summary of generalized linear mixed effect model (GLMM) for the number of damaged leaf segments.

Fixed effects of candidate models	Parameter	AIC	LL	Deviance	DF	Chisq	P
2018							
Distance+Plantation+Distance×Plantation	5	2616.5	-1303.3	2606.5			
Distance+Plantation	**4**	**2616.7**	**-1304.4**	**2608.7**	**1**	**2.2**	**0.1386**
Distance	3	2629.0	-1311.5	2623.0	1	14.3	0.0002
Plantation	3	2680.8	-1337.4	2674.8	1	66.1	< 0.0001 *
Null	2	2687.2	-1341.6	2683.2	1	8.4	0.0037
2019							
Distance	**3**	**1416.0**	**-704.98**	**1410.0**			
Null	2	1436.4	-716.19	1432.4	1	22.4	< 0.0001

Shown are number of parameters used in the model (Parameter), Akaike's Information Criterion (AIC), log-likelihood (LL), difference in degree of freedom against above model (DF), Chi-squared value (Chisq), *p*-value (P).

Tree was a random variable. The number of segments were pooled for a branch. * Log-likelihood ratio test against the model with Distance + Plantation.

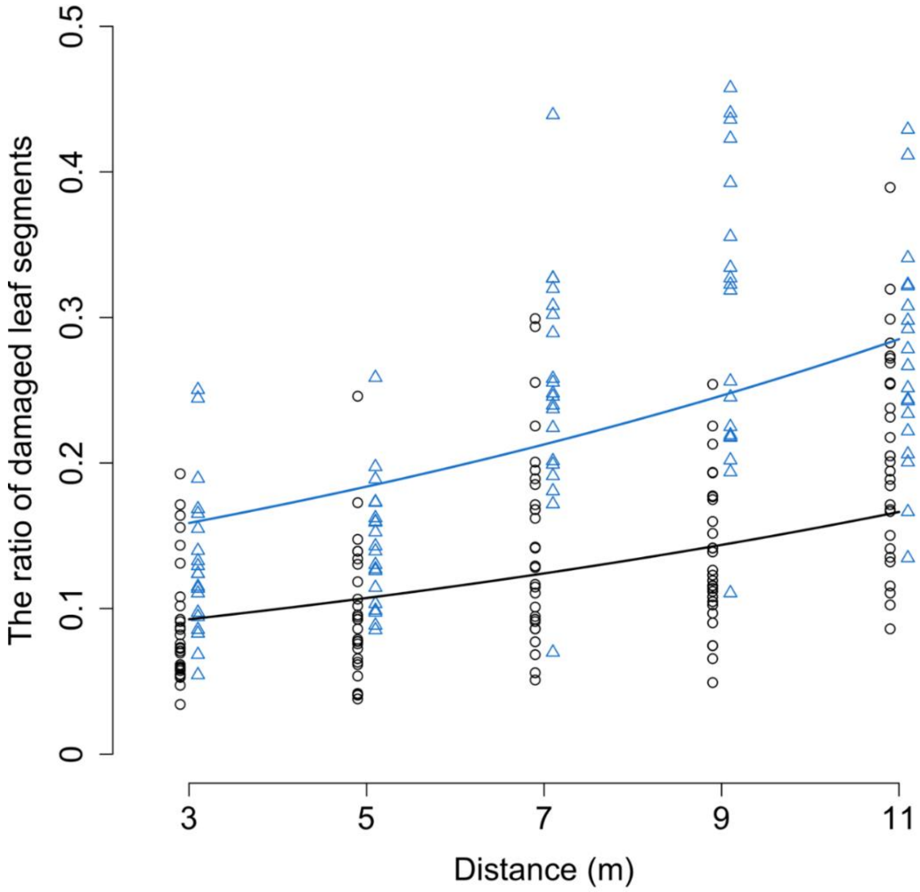


Figure 3-3.
The changes in proportion of the damaged leaf segments along the distance from clipped trees. The circles and triangles are the proportion of the damaged leaf segments to total leaf segments of each sampled branch in Tomakomai (black circle) and in Hiruzen (blue triangle). To avoid overlapping, these circles and triangles were jittered by region. Two curves show the estimated values by GLMM for Tomakomai (black line) and Hiruzen (blue line), respectively. Tree was a random variable. The number of segments were pooled for a branch.

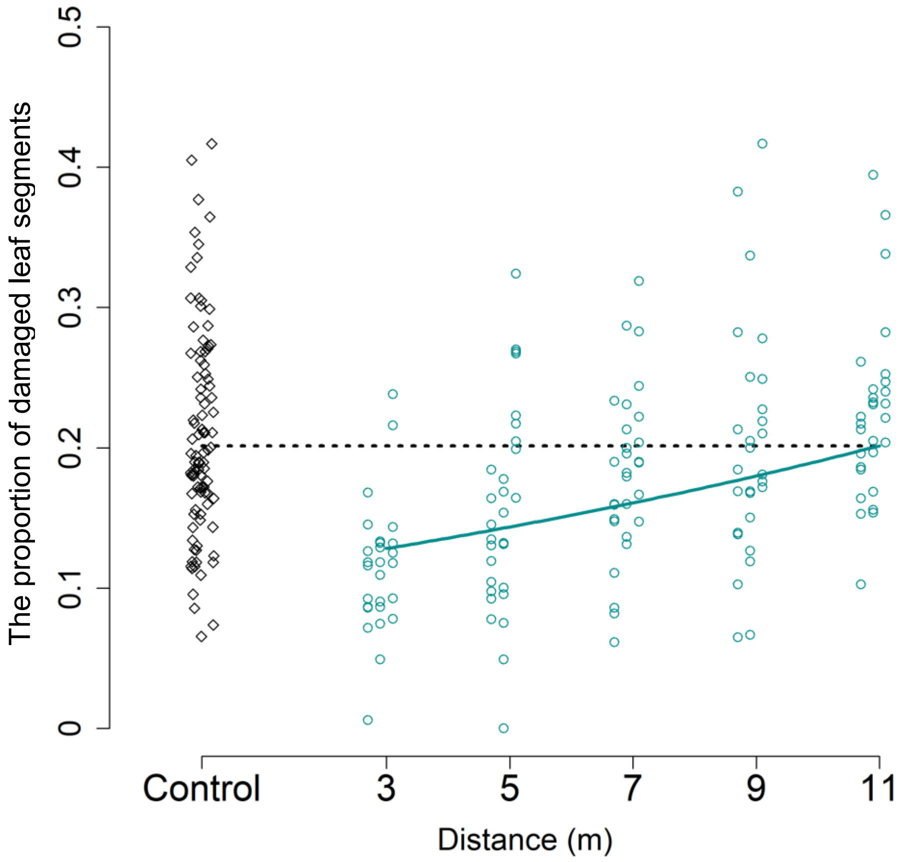


Figure 3-4.

Comparison of the assay and control treatment in the proportion of the damaged leaf segments. The circles are the proportion of the damaged leaf segments to total leaf segments of each assay branch. The diamonds show the proportion of the damaged leaf segments to total leaf segments in the control. Continuous curve and dotted line show the estimated proportion by GLMM for the clipped and control treatments, respectively. Tree was a random variable. The number of segments were pooled for a branch.

Table 3-2. Summary of Likelihood ratio test in comparison with control and exposed trees.

2019

Distance from the volatile source trees	DF	Chisq	P
3m	1	20.17	<0.0001
5m	1	8.83	0.0030
7m	1	2.58	0.1080
9m	1	1.01	0.3150
11m	1	0.29	0.5909

Summary of Likelihood ratio test for the difference between the control and assay trees at each distance in Tomakomai in 2019. For each distance, the model with the treatment (assay and control) as a fixed effect was compared to the model without treatment assuming no difference between assay and control trees (null model). Shown are the difference in the degree of freedom between the model with and without treatment (DF), Chi-squared value (Chisq) and p-value (P). Tree was a random variable. The number of segments were pooled for a branch.

3.3.2 Chemical analysis of headspace VOCs of beech leaves

I detected 10 principal compounds in the control and clipped leaves (Table 3-3). The amounts of released (*Z*)-3-hexenol and (*Z*)-3- hexenyl acetate were significantly different between the control and clipped leaves ($t = -3.2345$, df = 7, and $p = 0.014$; $t = -2.8892$, df = 7, and $p = 0.023$, respectively) (Figure 3-5). These two compounds represented >50% of the total VOCs obtained from the clipped leaves. Caryophyllene alcohol was not detected in the clipped leaves but not in the control leaves; although statistically not significant ($t = -0.88352$, df = 7, and $p = 0.406$). The variance was large for (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (+)-δ-Cadinene, α-Calacorene, Caryophyllenyl alcohol, and α-Cadinol. Sabinene, the principal monoterpene found in European beech (*Fagus sylvatica* L.) (Dindorf et al., 2006) was not detected in Japanese beech.

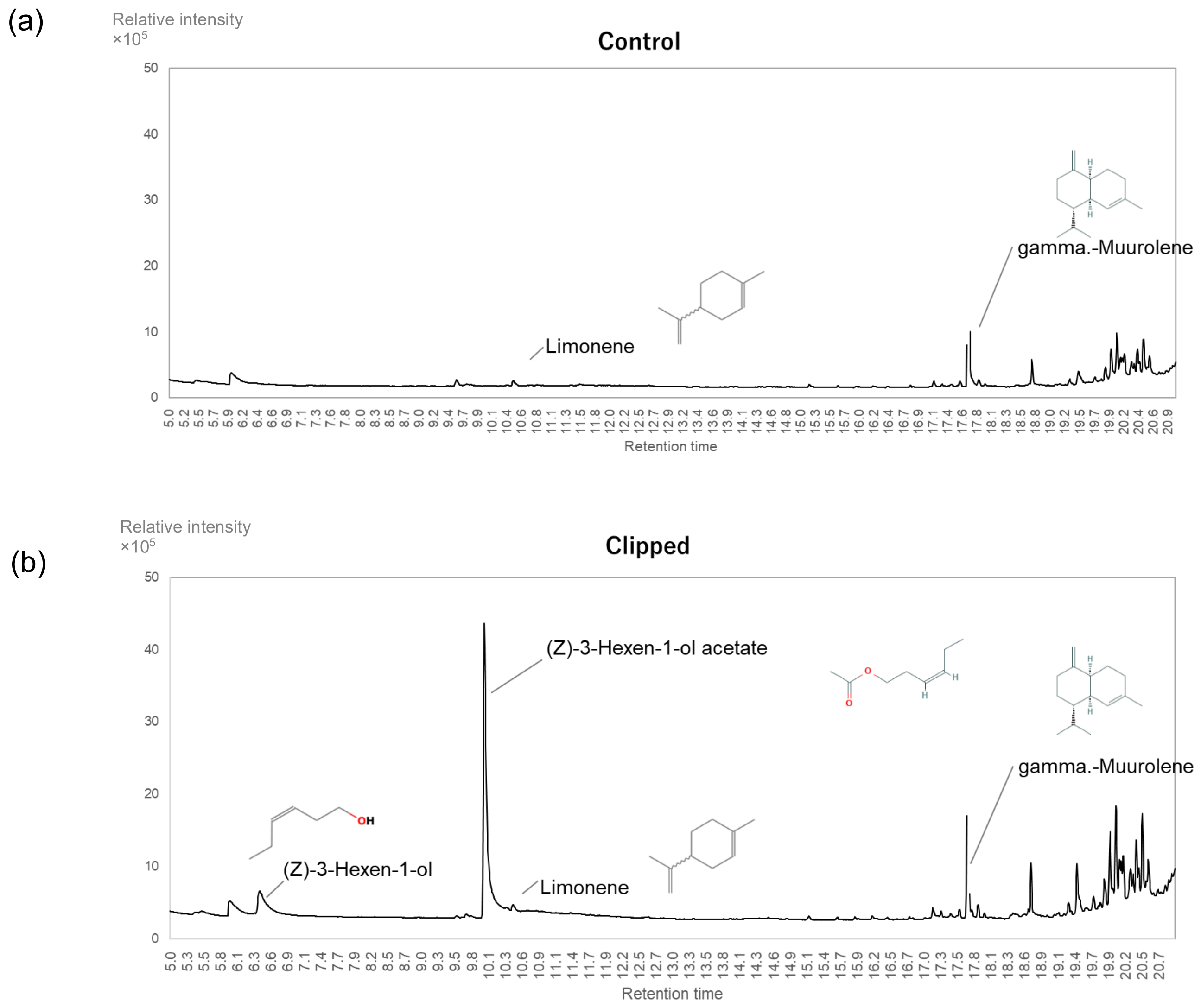


Figure 3-5.

This chromatogram shown for headspace volatiles emitted from beech leaves. Samples were detected from one branch before clipped (Control) and after clipped

Table 3-3. Amounts of volatile compounds from control beech and clipped beech.

	Amout (peak areas (× 10^4) / g)						
Compound name	Control			Clipped			
(*Z*)-3-hexenol	n.d			911.3	±	281.7	*
(*Z*)-3-hexenyl acetate	532.3	±	277.4	3962.3	±	1069.5	*
Limonene	85.2	±	30.2	80.7	±	19.5	
γ- Muurolene	112.1	±	21.3	176.2	±	46.6	
α-Muurolene	120.3	±	14.9	121.9	±	38.7	
(+)-*δ*-Cadinene	221.2	±	44.5	317.7	±	104.8	
α-Calacorene	26.3	±	17.2	16.8	±	11.5	
Caryophyllenyl alcohol	n.d			73.8	±	37.3	
α-Cubeben	54.5	±	20.6	83.4	±	30.1	
α-Cadinol	181.5	±	44.5	364.8	±	110.4	

Data are the peak areas (× 10^4) of total ion chromatograms per dry leaf weight (g) (mean ± SE). n. d. indicates that the compound was not detected. Asterisks indicate significant differences by paired *t*-test at $P < 0.05$.

3.4 Discussion

In both plantations, leaf damage decreased in unclipped trees in close proximity to the clipped trees compared to far-neighboring unclipped trees (Figure 3-3; Table 3-1) or control trees (Figure 3-4; Tables 3-2). The damage level differed between plantations (Figure 3-3), which may be because Hiruzen was in the natural range of beech, while Tomakomai was outside its natural range (Matsui et al., 2004), and because the herbivore communities may have differed between the two sites (Nakamura et al., 2014). I also found considerable variation in the damage level among samples within the same plantation, which may be due to variations in the seed origin or simply heterogeneous distribution of herbivores among trees. Notably, even though my experiment may have included such variations in herbivore communities and beech individuals as in natural ecosystems, I found a significant decrease in leaf damage in nearby neighboring trees.

I preliminary examined the concentration of condensed tannin was not significantly different between exposed and control leaves (Supplemental Figure 3-1; Supplemental Table 3-1). Previous study showed that the concentration of condensed tannin in beech leaves damaged by herbivores was higher than in control leaves from 5 days to 30 days after damage (Aoyama & Koike, 2011). One reason of this contradicting result may be that leaves respond more sensitively when damaged by herbivores than the exposure to VOCs. The exposure treatment may take a long time to accumulate some defense chemicals in leaves. Another reason was the large variation in the concentration of condensed tannin due to seed sources. The variation in the concentration of condensed tannin became higher as time passed (Supplemental Figure 3-1). The reason is the variation of the light condition by each leaf. Further study is needed with larger sample sizes to determine when defense chemicals will accumulate in exposed leaves.

After herbivore damage or manual clipping, plants release VOCs, such as monoterpenes, sesquiterpenes, and green leaf volatiles (GLVs) (Li, 2016). I found considerable variation in the volatiles emitted by beech trees. Among these compounds, (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate (which are categorized as GLVs) were the two most common volatile compounds emitted by the clipped leaves (Table 3-3). Shiojiri et al. (2006) demonstrated that one of the ecological functions of GLVs is defense against herbivores and pathogens in *Arabidopsis thaliana*. GLVs are also known to activate defense in various plant species and are a highly conserved type of signaling molecules (Li, 2016; Yamauchi et al., 2015). After exposure to (*Z*)-3-hexenyl acetate (i.e., a GLV), poplar began priming their defenses prior to experiencing any physical damage (Frost et al., 2008). GLVs are synthesized within a few seconds (Mochizuki & Matsui, 2018) and emitted upon herbivory or pathogen infection by almost every green plant, playing a crucial role in plant defense (Scala et al., 2013). These studies showed that GLVs induced the defense of intact plants. My results show that GLVs and other VOCs emitted from clipped beech tree induced the defense of neighboring beech trees against herbivores and/or pathogens, resulting in reduced damage in neighboring beech. Intra-plant signaling for plant defense against herbivore has previously been reported in late-successional beech species (Chapter 2, Hagiwara & Shiojiri, 2020). However, to the best of my knowledge, this study is the first to show conspecific plant-plant communication in late-successional tree species in plantations.

Assay trees located up to 5 m from clipped trees showed significantly less leaf damage than control trees. As the distance from the clipped trees increased, the leaf damage between the assay and control trees gradually became similar. Therefore, I suggest that the effective distance of plant communication in beech is 5 m under the experimental conditions of this study. However, the effective distance may be farther if the tree that received VOCs starts to emit VOCs (Arimura et al., 2004). Previous studies have shown effective distances of 0.6 m in sagebrush (*Artemisia tridentata*) (Karban et al., 2006) and ≤10 m in black alder (Dolch &Tscharntke, 2000). Evidently, the exact value of the effective distance may vary depending on the tree size, damage intensity, and tree species. Furthermore, effective distance may be affected by herbivore abundance and their spatial distribution. If herbivore density is low and the distribution is highly localized, plants that received the VOCs may not respond easily because the response itself entails certain costs. Plants may have evolved so that only near neighboring trees respond, and the effective distance may be short. On the contrary, if herbivores outbreak at long distances, the effective distance may be far. Karban et al. (2013) suggested that whether or not plants respond after received the VOCs depends on the kin recognition of trees; if so, the effective distance may differ depending on whether the emitter is genetically related. VOC composition may also differ between conspecific trees and may relate to the genetic relatedness of trees (Ishizaki et al., 2011; Karban et al., 2013; Hiura et al., 2021; Shiojiri et al., 2021). Further studies are needed regarding the effective distance of VOCs.

Plant defenses induced via plant-plant communication may affect arthropod community composition, abundance, and spatial distribution in forest ecosystems. Tscharntke et al. (2001) showed that the number of phytophagous generalists and specialists did not differ prior to the experiment in early May. However, 81 days after the treatment, the generalists did not show a significant pattern, whereas the specialists exhibited a clear difference depending on the distance from the volatile source alder tree. In the present study, I evaluated the damage caused by either folivorous insects, leaf mining insects, sucking insects, or pathogens. Future studies to record the damage for each feeding guild are required to clarify the effective distance for each feeding guild.

I found that the effective distance of plant signaling in beech was 5–7 m under the experimental conditions. These beech heights were 10–13 m, that is, half the height of trees in the natural beech forest (Tateishi et al., 2010). The effective distance may farther in the natural beech forest than present study.

Although plant-plant communication via volatiles has been mostly studied in herbaceous species, it is also necessary to study such signaling in tree species. Since trees (especially late-successional species) dominate large areas for long time periods with large biomass, plant-plant communication in trees may have a significant effect on herbivory and pathogen abundance in large forest landscapes. VOC emissions from trees contribute largely to atmospheric VOCs (Matsunaga et al., 2009; Mentel et al., 2013; Šimpraga et al., 2019); therefore, VOC emissions due to herbivory damage may affect atmospheric VOCs. Consideration of the effective distance of damage-related VOCs may be important for further understanding the spatial distribution of herbivore communities and forest ecosystem functions.

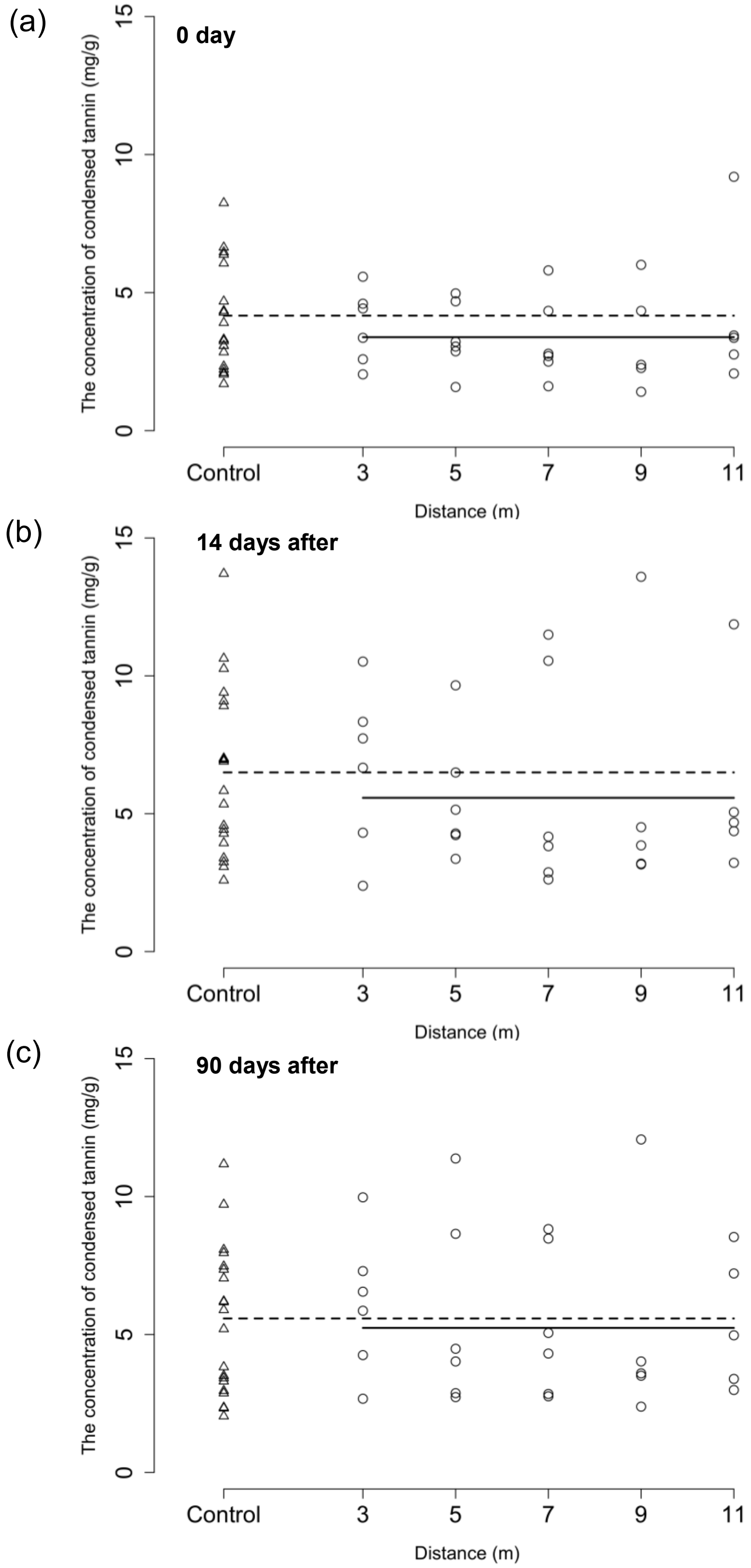
3.5 Supplementary

3.5.1 Material and methods

To prepare samples for analysis, milled leaves (20 to 50 mg) were extracted with 50 ml of 50% methanol and put in ultrasonic washer at 40℃ for one hours, and the extractives were recovered by centrifugation. 1 ml of the supernatant was collected into two centrifuge tubes and I injected 4 ml of butanol-HCl (95: 5) and mixed. One centrifuge tube was kept in the dark and the other tube was set in boiling water for 2 hours and then kept in the dark for 15 minutes. The supernatant was analyzed using an UV-VIS Spectrophotometer (UV-2700, Shimadzu, Kyoto, Japan).

3.5.2 Statistical analyses

To test whether the concentration of condensed tannin of assay trees changed along the distance from clipped trees in the 2019 experiment, I used a linear mixed effect model (LMM) which applied the Laplace approximation and maximum likelihood via the lmer function in the lme4 package of R 4.1.2 (R Core Team 2021). Since some branches were sampled from the same tree, I used the mixed effect model to account for non-independent errors due to nested measurements (Faraway, 2016). The full model included the following: distance as a fixed effect, and tree as a random effect. I conducted log-likelihood ratio tests between the distance model and the null model (Supplemental Table 3-1).



Supplemental Figure 3-1.

Comparison of assay branches of clipped treatment and assay branches of control treatment in the concentration of condensed tannin (mg/g). The circles are the concentration of condensed tannin of each assay branch. The triangles shown the concentration of condensed tannin of each control branches. Continuous and dotted lines show the estimated proportion by LMM for the assay and control branches, respectively. Tree was a random variable. The number of segments were pooled for a branch. On 0 day, I conducted the clipped treatment.

Supplemental Table 3-1. Summary of linear mixed effect model (LMM) for the concentration of condensed tannins.

Fixed effects of candidate models	Parameter	AIC	LL	Deviance	DF	Chisq	P
Before clipped treatment (0 day)							
Distance	4	109.41	-50.70	101.51			
null	**3**	**107.51**	**-50.76**	**101.41**	**1**	**0.11**	**0.7448**
After clipped treatment (14 day)							
Distance	4	148.69	-70.35	140.69			
null	**3**	**146.69**	**-70.35**	**140.69**	**1**	**0.00**	**0.9996**
After clipped treatment (90 day)							
Distance	4	141.39	-66.69	133.39			
Null	**3**	**139.46**	**-66.73**	**133.46**	**1**	**0.08**	**0.7836**

Shown are number of parameters used in the model (Parameter), Akaike's Information Criterion (AIC), log-likelihood (LL), difference in degree of freedom against above model (DF), Chi-squared value (Chisq), p-value using log-likelihood ratio test (P). Tree was a random variable. The number of segments were pooled for a branch.

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Data availability statement

Data from this manuscript were archived in the publicly accessible repository Dryad (https://doi.org/10.5061/dryad.9w0vt4bf2).

CHAPTER 4

Volatile mediated plant-plant communication in natural beech forest

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Abstract

Plants live in heterogeneous and severe natural environments. When plants are damaged by herbivorous arthropods, they emit volatile organic compounds (VOCs). Neighboring intact plants receive VOCs as signals and increase their defense against herbivores. This phenomenon is called as plant-plant communication and has been observed in more than 40 plant species. Previous studies were conducted in laboratories, greenhouses, or plantations, but only few studies have been conducted in natural forests. Studies in natural forests are important for understanding the mechanisms and adaptation of plant defenses, plant-insect interactions, and population dynamics of these organisms. In this study, I investigated plant-plant communication in beech trees in natural forests. As a VOC source, approximately 90% of the leaves of a beech tree were clipped in half in a high-emission experiment, and clipped leaves of approximately 2 kg weight were prepared in a low-emission experiment in the spring season. In the high-emission experiment, I divided the trees into upwind or downwind from the VOC source tree and control. The damage levels of neighboring undamaged beech trees were evaluated four months after clipping. Beech trees neighboring the VOC source tree showed less damage due to herbivores or pathogens than the farther trees in the downwind of the high-emission level and in the low-emission experiment. The plant hormones jasmonic acid (JA) and salicylic acid (SA) were quantified in the sampled leaves

after three days of exposure. The nearer neighbours of the VOC source showed higher SA concentration than the farther trees in the downwind of the high-emission experiment. Genetic relatedness did not explain the difference in leaf damage or plant hormone concentrations among trees. This study is the first to show that late-successional tree species exhibit plant-plant communication via VOCs in natural forests. However, this is not always detectable because of the abiotic and biotic heterogeneity of forest ecosystems. A multiscale approach, from phytohormones to individuals and populations, as in this study, would be important in clarifying the significance of VOCs on forest ecosystems.

4.1 Introduction

Plants live in heterogeneous and severe natural environments. When plants are damaged by herbivorous arthropods, they emit volatile organic compounds (VOCs) (Karban 2021). Neighboring intact plants receive VOCs as signals and increase their defense against herbivores (Farmer & Ryan 1990). This phenomenon is called as plant-plant communication and has been observed in > 40 plant species, mostly herbaceous plants (Heil & Karban 2010; Karban 2021). Recently, tree species such as beech (*Fagus crenata*), alder (*Alnus glutinosa*), and birch (*Betula* spp.) have been found to exhibit plant-plant communication (Dolch & Tscharntke 2000; Himanen et al. 2010; Hagiwara & Shiojiri 2020, Chapter 2; Hagiwara et al. 2021, Chapter 3). These studies were conducted in laboratories, greenhouses, or plantations, but only few studies have been conducted in natural forests (but see Dolch & Tscharntke 2000; Himanen et al. 2010; Karban et al. 2014). However, studies in natural forests are important for understanding the mechanisms and adaptation of plant defenses, plant-insect interactions, and population dynamics of these organisms (Karban 2020). Such studies are necessary for the management of insect and pathogen outbreaks in forest ecosystems.

Plant-plant communication in natural forests may differ from that in artificial settings, such as laboratories, green houses, and plantations. As a first step in plant-plant communication, VOCs must reach target trees. Whether the VOCs reach or not is affected only by physical distance but also by various factors in natural forests. One of these factors is the abiotic environmental heterogeneity. Even if the same amount of VOCs is emitted, VOC diffusion is affected by the surrounding topography and wind direction (Yeo et al. 2020). Another factor is the complexity of the inhabiting organisms. Natural forests are composed of various tree species with different tree heights, leaf shapes, leaf angles, and branching structures that create complex and large forest canopy structures (Osada, Nabeshima & Hiura 2015; Toda et al. 2022). These canopy structures disrupt VOC diffusion (Kuroyanagi et al. 2012). In addition, when other tree species are damaged by herbivores, these trees may also emit VOCs, which may disrupt the plant-plant communication of focal tree species.

Furthermore, in natural forests, the defense response after receiving VOCs may differ between individuals within a population owing to genetic variation between individuals. After exposure to VOCs, sagebrush (*Artemisia tridentata*) for which is genetically closely related to a VOC emitting conspecific tree showed less damage than related trees (Karban et al. 2013). Natural forests consist of trees of different genetic relatedness, and such genetic structures are created through reproductive processes such as pollination, seed dispersal, and regeneration (Shimatani et al. 2007). Therefore, it is important to consider the variation in responses within populations owing to genetic relatedness and biotic and abiotic heterogeneity in natural forests.

When plants receive VOCs, defense genes are expressed, and plant hormones accumulate. As a result, plants become more defensive and can reduce damage (Frost et al. 2008). In plant-plant communication, herbivores do not evade VOCs, but plants increase their defense against herbivores (Karban 2007). To clearly distinguish such repellent effects from plant-plant communication, I must clarify induced defense by focusing on plant hormones. Recent studies have shown that plants express defense genes and accumulate plant hormones related to plant defense after receiving VOCs and fed by herbivores for 24 h (Frost et al. 2008). Tscharntke et al. (2001) quantified plant hormones after herbivory in natural forests. However, no study has examined plant hormones in forests after exposure to VOCs.

Previous studies on plant-plant communication have only focused on herbivory. However, plants have been exposed to pathogens since their birth, and plant-plant communication may also have evolved towards pathogens. Plants accumulate hormones, such as jasmonic acid (JA) and salicylic acid (SA), when damaged by herbivores and pathogens respectively (Yuan & Lin 2008; Thaler, Humphrey & Whiteman 2012). For a better understanding of plant-plant communication, I need to determine which hormones accumulate through VOC reception and what kind of damage will be reduced by plant-plant communication.

The objective of the present study was to clarify whether beech trees exhibit plant-plant communication in natural forests. I conducted manipulative experiments focusing on abiotic and biotic heterogeneity, and genetic relatedness among the trees. The defense response is evaluated by leaf damage by herbivores and pathogens, and by the accumulation of SA and JA. I hypothesized that trees nearer to the VOC source would be more exposed to VOCs, and as a result, these trees will accumulate plant hormones and can reduce leaf damage. In addition, I determined the genetic relatedness of all trees and predicted that trees that were more closely related to the VOC source tree would show less damage. To determine the effect of VOC exposure, I conducted two types of experiments: high and low VOC emissions. In addition, owing to abiotic heterogeneity in natural forests, trees in the high-emission experiment were separated into three categories: control, upwind, or downwind from the VOC source tree.

4.2 Material and methods

4.2.1 Study site

Field experiments were conducted in natural secondary beech forests at the Kawatabi Field Center, Tohoku University (38°7'N, 140°7'E, 555 m a.s.l. hereafter called Kawatabi) and the Ashiu Forest Research Station, Field Science Education and Research Center, Kyoto University (35°2'N, 135°4'E, 730 m a.s.l. hereafter called Ashiu) (Figure 4-1). I set three sites in Kawtabi (K1, K2, and K3) and Ashiu (A1, A2, and A3). At all sites, leaf expansion occurred from April to early May. In this study, I only used beech trees with a stem diameter at breast height (DBH) ≥ 5 cm.

K1 site was a mixed stand composed of beech (n = 126) and other tree species such as *Quercus crispula*, *Acer japonicum* and *A. sieboldianum.* The canopy height was approximately 20 m. The oldest tree age was approximately 100 years, as estimated by tree rings (Yoshihisa Suyama unpublished data). A 20m-height tower was established in 2005, neighboring two beech trees. Six beech trees colud be accessed from the towers. In addition, I set K2 and K3 sites in May 2021, where beech trees were grown at a high density. Almost all trees were beech at K2 (n = 22), whereas the K3 site was a mixed stand of beech (n = 20) and other tree species such as *Quercus crispula*. In Ashiu, A1 (n = 12), A2 (*n* = 16) and A3 (*n* = 25) sites were continuous mixed stands of beech, *Cryptomeria japonica*, and other tree species. The canopy heights of K2, K3, and A1 to A3 were 10–20 m.

4.2.2 Experimental design

I conducted two experiments with different VOC emission levels: a high-level assuming insect outbreak and a low level assuming lower than outbreak damage by herbivores. The beech caterpillar (*Quadricalcarifera punctatella*) feeds on beech leaves and the populations occasionally reach epidemic densities causing total defoliation of trees (Kamata, Igarashi & Ohara 1996). Therefore, I set high-emission level as totally defoliated tree.

4.2.2.1 High-emission experiment

I selected one beech tree in the tower as a VOC source tree at K1 site and clipped approximately 90% of the leaves into half using scissors in May 2021. I measured the wind direction using Davis wind speed/direction smart sensors with a HOBO USD micro station (S-WCF-M003, Onset, MA, USA) at the top of the tower from the clipped day to 1.5 day. The most frequent direction was 300 ° magnetic north (Supplemental Figure 4-1), and I divided these trees into upwind or downwind from the VOC source tree or control: downwind trees were located in the direction of 30 ° to 210 ° (*n* = 30) and upwind trees (*n* = 57) were located in the direction of 210 ° to 360 ° and 0 ° to 30 ° (Figure 4-1). Both downwind and upwind trees were located within ≤ 35m from the source tree. Distance was set to ≤ 35 m because the logging road passed from 35 to 40 m on the downwind side. Downwind trees were assumed to be more exposed to VOCs than upwind trees were. Trees at a distance of 50–100 m and in the direction of 30–210 ° north were set as control trees (n = 39), assuming that they were not exposed to VOCs from the source tree. I could not set the control trees in downwind because of logging roads. I excluded trees as treatment trees that did not determine the accumulation of SA and JA or the genetic relatedness of the VOC source tree (Figure 4-1: grey circles).

4.2.2.2 Low-emission experiment

At each of the K2, K3, A1, A2, and A3 sites, I hung a mesh bag filled with approximately 2 kg of clipped beech leaves as the VOC source (hereafter called VOC bag). Leaves were collected from trees growing over 2 km from these sites to avoid unintended VOC exposure from the sampled trees. Each bag was hung with a pole at a height of approximately 3–6 m. After three days of exposure, the bags were removed.

4.2.3 Damage level

For all sites, I randomly sampled a lower-canopy branch from each neighboring tree of VOC sources in September before leaf abscission and examined the leaf damage occurred during the growing season of leaf expansion April or early May to August or September. I randomly selected 9–12 leaves from each branch and counted the total number of leaf segments damaged by chewing herbivores and pathogens. Each leaf segment is a portion of the leaf blade surrounded by parallel leaf veins (Hagiwara *et al.* 2021). Because the number of segments per leaf differed between Kawatabi and Ashiu, the mean number of segments per leaf for K1 to K3 sites was calculated by averaging the number of segments of a leaf randomly selected from the 18 beech trees in K1 (the number of leaves, n = 18). For sites A1 to A3, the number of segments per branch was calculated for all branches (n = 529).

Beech leaves were damaged by *Fagineura crenativora*, *Actias aliena*, and other herbivores as well as pathogens causing yellow leaf spots (Wei & Harada 1998) and other leaf spots.

4.2.4 Quantification of JA and SA

In the high-emission experiment, I sampled two to three leaves from a lower-canopy branch of each neighboring tree three days after clipping the VOC source tree and quantified the phytohormones (SA and JA). The accumulation of SA (ng/g FW) was highest at three days after clipping on beech saplings (unpublished data).

I analysed SA (ng/g FW) and JA (ng/g FW) accumulation in leaves by liquid chromatography-tandem mass spectrometry (LC/MS/MS) according to Ozawa *et al.* (2017). To prepare samples for analysis, leaves (0.3–0.5 g) were immediately frozen in liquid nitrogen after sampling, homogenised with ethyl acetate (2.5 ml), and spiked with 10 ng of d2-JA (Tokyo Chemical Industries Co., Tokyo, Japan) and 1 ng of d4-SA (C/D/N Isotopes, Pointe-Claire, Quebec, Canada) as internal standards. After centrifugation of the mixture at 2,300 × g for 10 min at 4 °C, 1 ml of the supernatant was transferred to a 1.5 ml tube and then evaporated to dryness under vacuum. The residue was suspended in 50 μl of 70% methanol / water (v/v) and centrifuged to clarify the liquid phase. The supernatant was analysed using an LC/MS/MS system (LCMS-8050, Shimadzu, Kyoto, Japan). Separation by high-performance liquid chromatography was performed using a Mightysil RP-18 GP column (100 × 2.0 mm, 3 μm particle size, Kanto Chemical, Tokyo, Japan) at a flow rate of 200 μl min^{-1} with a linear gradient [0.1% formic acid aq. (A) and methanol (B), 5–95% B / (A + B) for 16 min]. Accumulation of JA, d2-JA, SA, and d4-SA was determined by multiple reaction monitoring (MRM). The monitored mass transitions were m/z 209–59, 211–59 for d2-JA, m/z 137–93, and 141–97 for d4-SA. The conditions for MS were optimised for MRM using authentic d2-JA (Tokyo Chemical Industries), d4-SA (C/D/N Isotopes), JA (Tokyo Chemical Industries), and SA (Wako Pure Chemical Industries, Osaka, Japan).

4.2.5 Genetic relatedness

I measured the degree of genetic relatedness for all neighboring beech trees and the VOC source tree using multiplexed inter-simple sequence repeat genotyping by sequencing (MIG-seq) (Suyama & Matsuki 2015) at the K1 site. Leaves were sampled on each branch per tree at the K1 site in August 2020.

DNA was extracted from the dried leaves using the CTAB method (Murray & Thompson 1980). Library construction to obtain genome-wide sequence data from each tree was performed using the MIG-seq method (Suyama & Matsuki 2015; Suyama et al. 2022). Library validation was adjusted following Illumina's standard protocol (adjusted from 4 nM) and used for sequencing as a 12 pM library containing 1% Phi X. Sequencing was conducted on Illumina MiSeq Sequencer (Illumina) using the MiSeq Regenet Kit v3 150 cycle kit (Illumina) with 80 cycles (pair-end on one side). For quality control, low-quality reads were removed using Trimmomatic 0.39 (Bolger, Lohse & Usadel 2014). After quality control, Stacks 2.41 was used for de novo SNP discovery (Catchen et al. 2013). To calculate genetic relatedness, I used SNPs extracted for loci detected in all samples, one SNP per locus (322 SNPs in total). I calculated the genetic relatedness using the SNPs with vcftools v0.1.13, according to Manichaikul et al. (2010).

4.2.6 Statistical analysis

To test whether the leaf damage of neighboring trees changed with distance from VOC emission sources, I used generalised linear models (GLM) or generalised mixed effect models (GLMM) with the maximum likelihood via the lmer function in the lme4 package of R 4.1.2 (R Core Team 2021). Because the response variable, that is the total number of damaged leaf segments of a branch, was a discrete variable, I fitted GLM with a Negative binomial distribution for K1 and GLMMs with a Poisson distribution for K2 to K3 and A1 to A3 sites. Tree was a random variable. The number of segments were pooled for a branch. The full model for the K1 site included distance and genetic relatedness as the fixed effects. That for the other sites included distance as a fixed effect and the site as a random effect. The total number of segments of a branch was included in the model as an offset term. The total number of segments was calculated by multiplying the mean number of segments of a leaf by the number of surveyed leaves on the branch.

To test whether the accumulation of SA and JA changed along the distance from the VOC source tree at the K1 site, I used linear models (LM). The full model included distance and genetic relatedness as the fixed effects. For all GLM, GLMM, and LM, the best models were selected using Akaike's Information Criterion (AIC).

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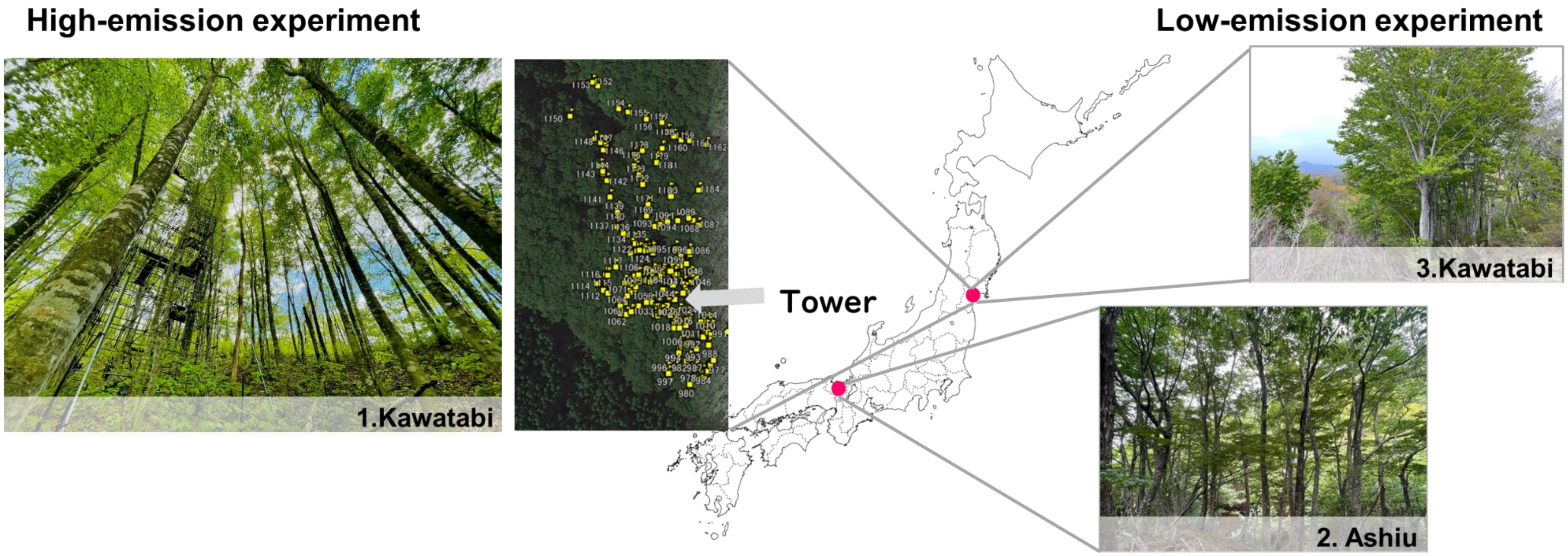


Figure 4-1.

Field experiments were conducted in natural secondary beech forests at the Kawatabi Field Center, Tohoku University and the Ashiu Forest Research Station, Field Science Education and Research Center, Kyoto University. I conducted two experiments with different VOC emission levels: a high-level assuming insect outbreak and a low level assuming lower than usual damage by herbivores.

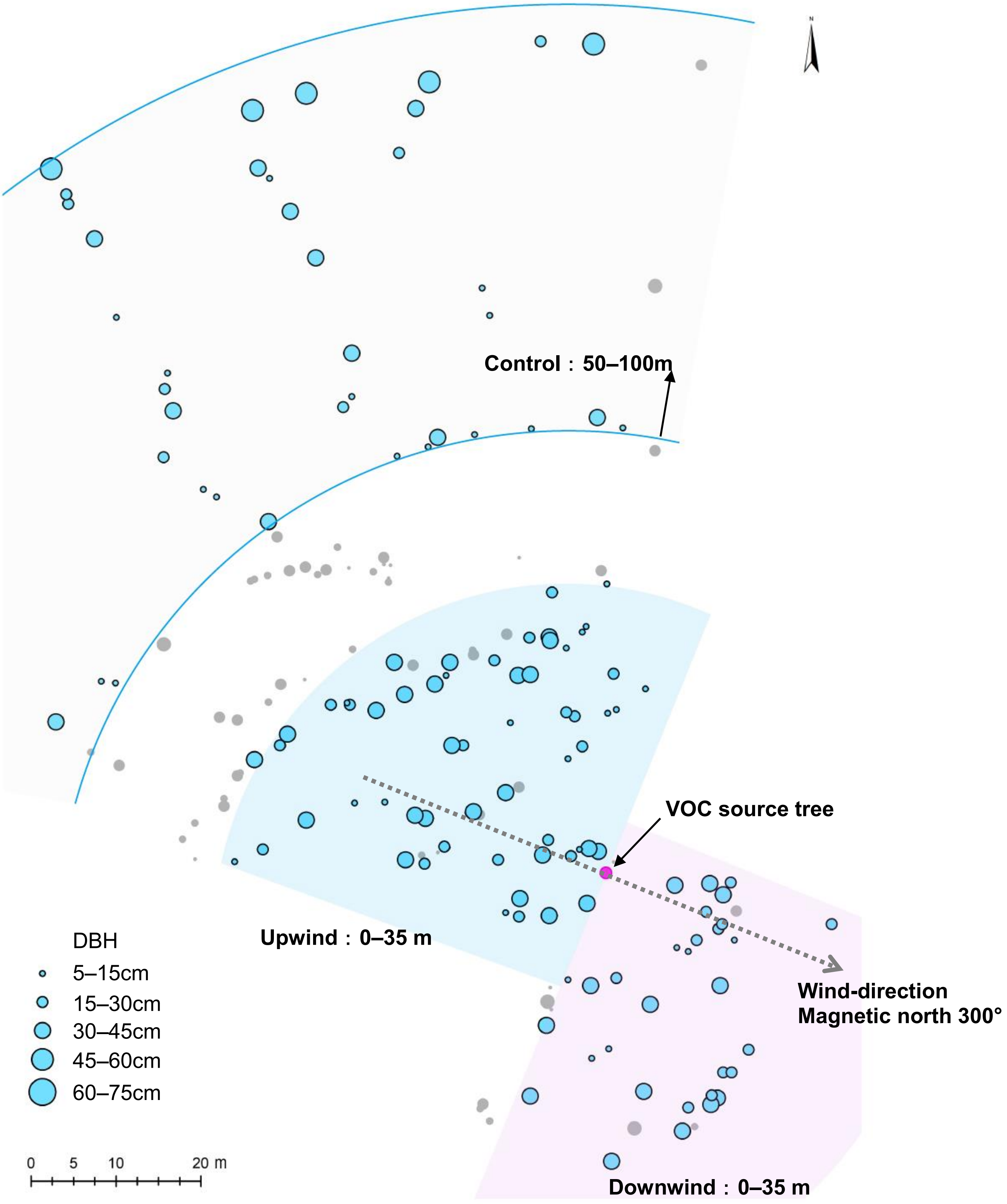


Figure 4-2.
Location of all beech trees and their sizes in diameter at breast height in high-emission experiment K1 site in Kawatabi (blue circles: surveyed trees; gray circles: unsurvey trees). I selected one beech tree surrounded by a tower as a VOC source tree and clipped approximately 90% of the leaves into half using scissors in May 2021. The most frequent wind direction at this site was 300 degrees magnetic north. I divided trees into 3 groups: downwind, upwind and control which were in the distance of 50–100m from the source tree.

4.3 Results

4.3.1 High-emission experiment

The full model included distance and genetic relatedness as fixed effects. Reduced models were the model assuming the effect of distance alone (Distance), that assuming the effect of genetic relatedness alone (Relatedness) and the null model assuming no difference due to distance and genetic relatedness (Null). In the downwind, the best models explaining leaf damage due to pathogen or herbivores included distance from the VOC source as fixed effects. Log-likelihood ratio test between the best model (Distance) and the null model also showed significant difference (Table 4-1; pathogen damage, $p = 0.0165$; herbivore damage, $p = 0.0242$). Leaf damage due to pathogens or herbivores increased with distance from the clipped source tree (Figure 4-3a, b). Since the variance of leaf damage among trees was large even after considering distance from the VOC source tree, I randomized the tree distance from the source tree and conducted GLM and log-likelihood ratio test for the each of 1,000 randomized datasets. The cases which had smaller or equal P value as the GLM of original data (Table 4-1) were 24 and 29 cases out of 1,000 times for pathogen and herbivore damage, respectively (i.e. $p < 0.025$ and $p < 0.030$). Therefore, the probability of obtaining the significant effect of distance by chance was very low and the result was robust even with large variance of damage levels.

In upwind, the best models explaining leaf damage due to pathogens or herbivores were the null model (Table 4-1). Leaf damage due to pathogens or herbivores did not change with distance from the VOC source (Figure 4-3a, b).

For the concentration of SA and JA in leaves, the full model included distance and genetic relatedness as fixed effects. I selected Distance model which included distance from the VOC source as a fixed effect in downwind as a best model (Table 4-2). Although the AIC was smallest in the full model, the difference in AIC between the full model and the Distance model was less than two and therefore I chose more simple Distance model as the best model (Kubo 2012). The accumulation of SA decreased with distance from the VOC source, while that of JA increased with distance from the VOC source (Figure 4-4a, b). The nearer neighbors of the VOC source showed higher SA concentration than the farther trees. In contrast, the farther neighbors of the VOC source showed higher JA than the nearer trees. In upwind, the best model explaining the accumulation of SA and JA were null model (Table 4-2). SA and JA accumulation did not change with distance from the VOC source (Figure 4-4a, b).

4.3.2 Low-emission experiment

In A1 to A3 sites, the full model included distance as a fixed effect (Distance). Reduced models assumed no difference due to distance (Null). The best model was Distance (Table 4-3). Distance model was significantly different from the null model (Table 4-3). These damages due to pathogens and herbivores increased as the distance from VOC source increased (Figure 4-5). On the other hands, in K2 and K3, the best model explaining the leaf damage due to pathogens or herbivores was null model (Table 4-3). These damages due to pathogens and herbivores did not change along the distance.

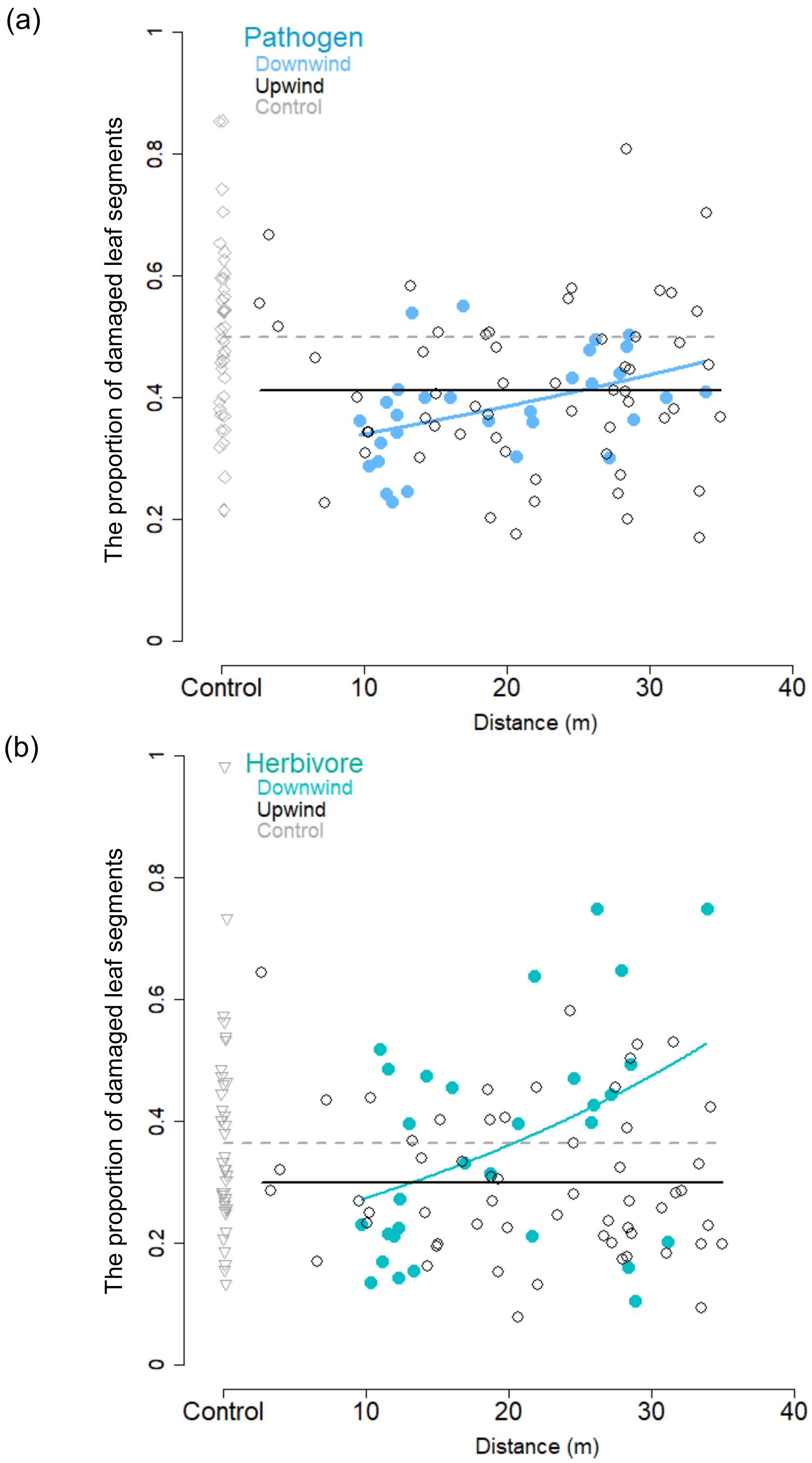


Figure 4-3.
The change in leaf damage by (a) pathogens and (b) herbivores along the distance from the VOC source tree in the high-emission experiment K1 site. The proportion of the number of damaged leaf segments to total number of leaf segments of each sampled branch for trees in downwind of the VOC source tree (blue or green circles), those for trees in upwind (black circles) and those for control trees (grey diamonds and triangles) are shown. Continuous curves show the estimated proportion using GLM for downwind and upwind trees and dotted ones show the proportion for the control trees.

Table 4-1. Summary of generalized linear model (GLM) for the number of damaged leaf segments of a branch in the high-emission experiment K1 site.

Fixed effects of candidate models	**Parameter**	**AIC**	**LL**	**RD**	***df***	***p***
Downwind-Pathogen						
Distance+Relatedness	3	264.99	-256.99	29.80	1	0.5056
Distance	**2**	**263.43**	**-257.43**	**29.74**		
Relatedness	2	266.15	-260.15	29.99	0	
Null	1	267.18	-263.18	29.94	1	0.0165 *
Downwind-Herbivore						
Distance+Relatedness	3	308.09	-300.09	30.96	1	0.9162
Distance	**2**	**306.10**	**-300.10**	**30.96**		
Relatedness	2	309.76	-303.76	31.09	0	
Null	1	309.18	-305.18	31.13	1	0.0242 *
Upwind-Pathogen						
Distance+Relatedness	3	564.27	-556.27	57.89	2	0.9976
Distance	2	562.28	-556.28	57.89	1	0.9717
Relatedness	2	562.27	-556.27	57.89	1	0.9516
Null	**1**	**560.28**	**-556.28**	**57.89**		
Upwind-Herbivore						
Distance+Relatedness	3	548.49	-540.49	58.23	2	0.2973
Distance	2	548.09	-542.09	58.29	1	0.3634
Relatedness	2	547.13	-541.13	58.25	1	0.1805
Null	**1**	**546.92**	**-542.92**	**58.33**		

Shown are number of parameters used in the model (Parameter), Akaike's Information Criterion (AIC), log-likelihood (LL), residual deviance (RD), difference in degree of freedom against the selected model (in bold) (df), p-value (p) for the log-likelihood ratio test between the focal model and the best model. *p<0.05

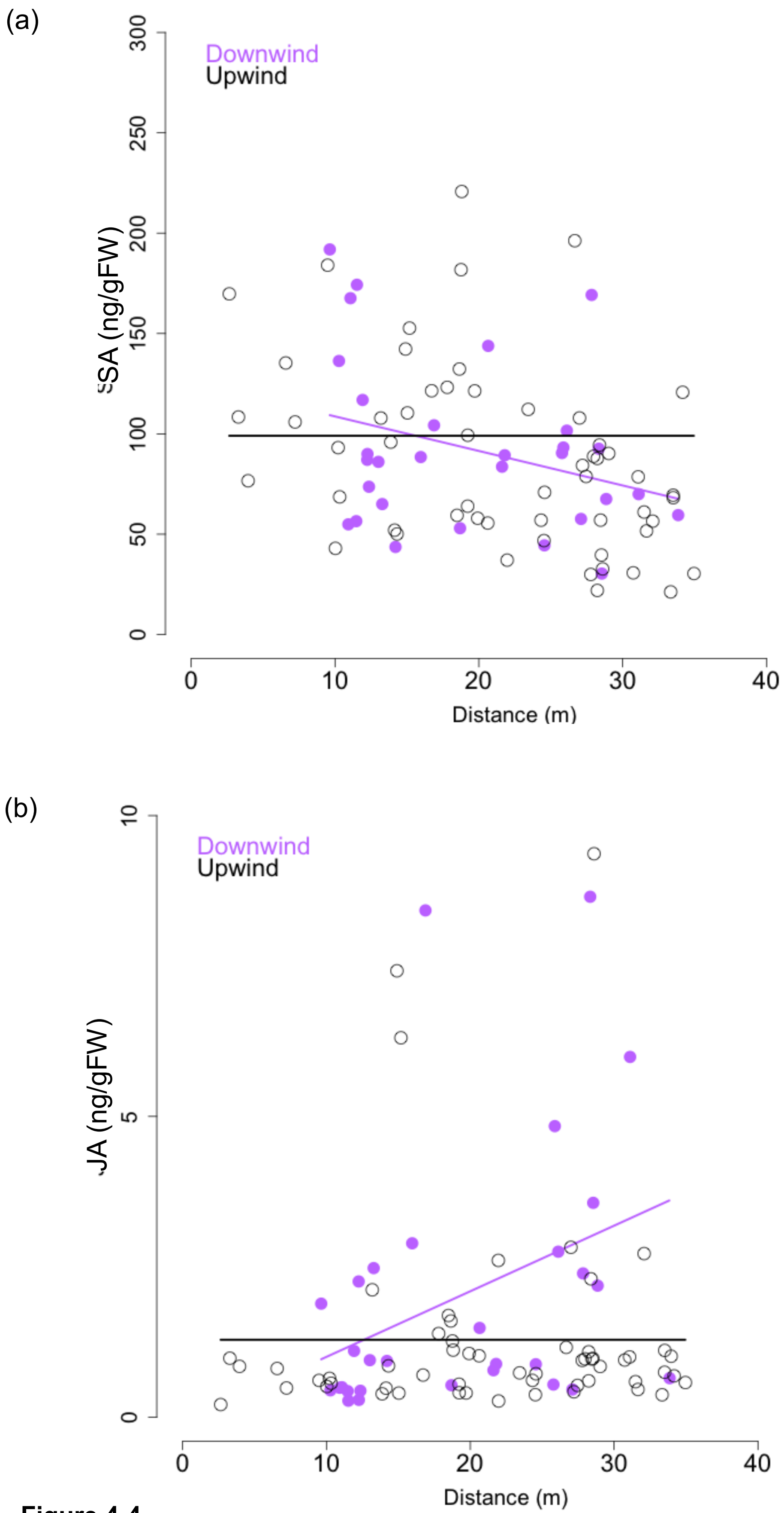


Figure 4-4.
The changes in accumulation of (a) salicylic acid (SA, ng/gFW) and (b) jasmonic acid (JA, ng/gFW) in leaves of downwind (purple circle) and upwind trees (black circle) along the distance from the VOC source tree in the high-emission experiment K1 site. Continuous lines show the estimated accumulation using LM.

Table 4-2. Summary of linear model (LM) for the accumulation of salicylic acid (SA) and jasmonic acid (JA) in the high-emission experiment K1 site.

Fixed effects of candidate models	**Parameter**	**AIC**	**RD**	***df***	***p***
SA					
Downwind					
Distance+Relatedness	4	310.84	27	1	0.1337
Distance	**3**	**311.24**	**28**		
Relatedness	3	314.16	28	0	
Null	2	312.30	29	1	0.0829
Upwind					
Distance+Relatedness	4	657.61	54	1	0.6223
Distance	3	655.61	55	1	0.3260
Relatedness	3	656.60	55	0	0.9994
Null	**2**	**654.60**	**56**		
JA					
Downwind					
Distance+Relatedness	4	136.02	27	1	0.5055
Distance	**3**	**134.51**	**28**		
Relatedness	3	136.25	28	0	
Null	2	136.86	29	1	0.0366 *
Upwind					
Distance+Relatedness	4	226.16	54	1	0.8303
Distance	3	224.50	55	1	0.8205
Relatedness	3	224.22	55	0	0.5743
Null	**2**	**222.55**	**56**		

Shown are number of parameters used in the model (Parameter), Akaike's Information Criterion (AIC), residual deviance (RD), difference in degree of freedom against the selected model (in bold) (df), p-value (p) for the log-likelihood ratio test between the focal model and the best model. *$p<0.05$

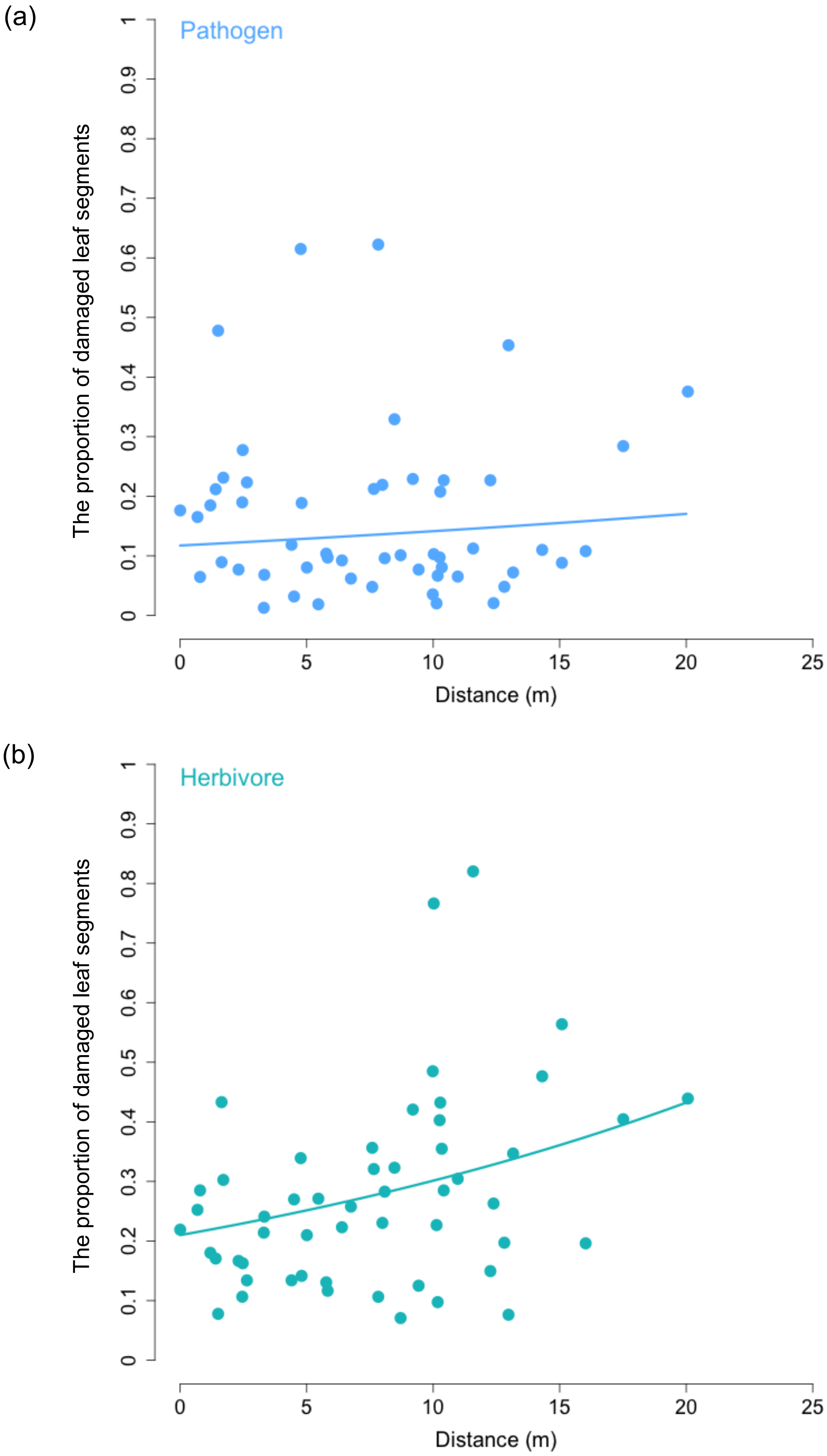


Figure 4-5.
The changes in leaf damage by (a) pathogens and (b) herbivores along the distance from VOC source bags in low-emission experiment A1–3 sites. The circles are the proportion of the number of damaged leaf segments to total number of leaf segments of each sampled branch from trees in various distance from sources. Continuous curves show the estimated proportion using GLMM.

Table 4-3. Summary of generalized linear mixed model (GLMM) for the number of damaged leaf segments in low-emission experiment A1−3 sites of Ashiu and K2−3 sites of Kawatabi.

Fixed effects of candidate models	Parameter	AIC	LL	*df*	*p*
Ashiu					
Pathogen					
Distance	**3**	**1205.69**	**-599.85**	**1**	**0.0002** ***
Null	2	1217.39	-606.69		
Herbivore					
Distance	**3**	**990.57**	**-492.28**	**1**	**<0.0001** ***
Null	2	1069.69	-532.84		
Kawatabi (K2-3 sites）					
Pathogen					
Distance	3	608.86	-301.49		
Null	**2**	**606.99**	**-301.43**	**1**	**0.7189**
Herbivore					
Distance	3	1077.92	-535.96		
Null	**2**	**1076.03**	**-536.01**	**1**	**0.7433**

Shown are number of parameters used in the model (Parameter), Akaike's Information Criterion (AIC), log-likelihood (LL), difference in degree of freedom against the selected model (in bold) (df), p-value (p) for the log-likelihood ratio test between the focal model and the best model. ***p<0.001

4.4 Discussion

4.4.1 VOCs mediated plant-plant communication against herbivore and pathogen damage

Beech trees neighboring near the VOC sources showed less damage than the farther trees in the downwind site of the high-emission experiment of the K1 site and the low-emission experiment A1 to A3 sites (Figure 4-3 and 4-5; Table 4-1 and 4-3). Plant-plant communication in tree species has been rarely studied in the field. In natural forests, leaf damage after VOC exposure was surveyed only in early-successional tree species (ca. alder (Dolch & Tscharntke 2000) and birch (Himanen et al. 2010)) and the trees neighboring near the clipped tree showed less damage. These results were consistent with my results. My study is the first to show plant-plant communication in late-successional beech trees in natural forests. Early-successional tree species tend to invest relatively more resources to reproduction and growth and the late-successional tree species to their defense (Matsuki & Koike 2006). Furthermore, herbivore damage was higher in intermediate and late successional forests than in early successional ones due to higher tree diversity, complexity of forests and higher abundance and diversity of insects (Neves et al. 2014). Late successional forests may be characterized by higher herbivory pressure. Therefore, it is not surprising that the late-successional tree species showed plant-plant communication in natural forests.

Furthermore, my study is also the first to show that the plant-plant communication is effective for reducing damage due to both herbivores and pathogens. Previous studies about plant-plant communication focused on the damage caused by herbivores and few studies focused on the damage caused by pathogens. Huang et al. (2012) showed that (*E*)-b-caryophyllene emission resulted in more resistance against pathogen in leaves, reduced cell damage and higher seed production in *Arabidopsis thaliana*. They showed that (*E*)-b-caryophyllene may directly inhibit bacterial growth. In beech, although the VOCs were emitted after clipping which mimicked the damages by herbivores, they may have induced defense against pathogens as well.

Leaf damage and emission of VOCs after the damage may not only result in plant-plant communication but also the repellent effect of herbivores and pathogens. To distinguish communication from repellant effect, the defense response after VOC reception should be clarified by the accumulation of plant hormone. Beech trees neighboring near the VOC source showed more salicylic acid (SA) than the farther trees in downwind of the source tree (Figure 4-4; Table 4-2). When plants accumulate SA, plants will induce systemic acquired resistance (SAR) against pathogens (Gao *et al.* 2015; Ding & Ding 2020). Neighboring downwind beech trees may have induced SAR against pathogens after accumulating SA and as a result the leave damage by pathogens were reduced.

Plant-plant communication has been focused mostly on the herbivore defense and jasmonic acid (JA) which is a hormone involved in defense against herbivores. Recently, Zhang et al. (2019) showed that whitefly-induced volatiles prime SA-dependent defenses in tomato. To the best of my knowledge, their study is the first to show the SA enhancement after the herbaceous plant was exposed to the VOCs. My study is the first to demonstrate in trees.

When plants accumulate JA and SA, plants produce chemical repellent compounds (e.g., tannins and alkaloids) which have negative effects on herbivores and pathogens(Baldwin 1989; Steppuhn & Baldwin 2007). For example, when beech caterpillar (*Quadricalcarifera punctatella*) were fed on the leaves with higher tannin concentration, the survival rate was low and body size of larvae were small (Kamata, Igarashi & Ohara 1996). The downwind trees neighboring near the VOC source tree tended higher SA but less JA (Figure 4-4; Table 4-2) even though these trees experienced lesser damage by both pathogens and herbivores (Figure 4-2; Table 4-1). The SA-JA antagonism has been clearly presented in many plant species (Thaler, Humphrey & Whiteman 2012 but see Ullah et al. 2022) and JA and SA levels changed inversely with each other (Koornneef et al. 2008; Luo et al. 2011). Furthermore, gene NONEXPRESSOR OF PR GENES1 (NPR1), the redox-sensitive transcriptional regulator of SA responses and mediator of SA-JA cross-talk, regulates several SA-responsive transcriptional factors with a direct or indirect role in suppressing JA-responsive gene expression (Pieterse et al. 2012). I quantified that SA accumulates 3 days after clipping because my preliminary experiment showed that SA accumulated 3 days after VOCs exposed. The downwind trees neighboring near the VOC source may have shown a low level of JA against SA by the SA-JA antagonism. The interaction of herbivores and pathogens and regulation of JA and SA should be further studied to understand the mechanisms of plant-plant communication.

4.4.2 Abiotic and biotic environmental heterogeneity affecting plant-plant communication in natural forests

Leaf damage by pathogens and herbivores did not differ along the distance from the VOC sources in the upwind site of the high-emission experiment and low-emission experiment K2 to K3 sites (Figure 4-5; Table 4-3). These results might be explained by the abiotic and biotic factors. One of the abiotic factors is wind direction. VOCs are known to diffuse according to wind direction and the nearer place from the VOC source showed larger concentration of VOCs than the farther place (Yeo et al. 2020). In sagebrush, the leaf damage was reduced only in the downwind site with a clipped neighbor and the damage experienced by the upwind shoot with clipped neighbors did not differ to that by upwind shoot with unclipped neighbors (Karban et al. 2006). Similarly, beech trees in upwind of the VOC source may have exposed to small amount of VOCs and as a result leaf damage was not reduced in near neighbor of the VOC source tree (Table 4-3). Furthermore, in low-emission experiment K2 to K3 sites, leaf damage was not reduced in near neighbor of the VOC source bags, the amount of VOCs may be heterogenous and the defense response might not be quantified. These results showed that the plant-plant communication is affected by the wind direction in the natural environment.

The second abiotic factor is the environment of a forest stand. Although the damage did not differ along the distance from VOC source in K2 to K3 sites, the nearer neighbors from the VOC sources were less damaged in A1 to A3 sites of Ashiu (Figure 4-5; Table 4-3). Forest structure differed between these sites. In A1 to A3 sites were in a continuous beech forest with no understory vegetation because of deer overgrazing while K2 to 3 sites had more understory vegetation and were composed of various tree species. In A1 to A3 sites, VOCs may have reached more to the neighboring beech trees without physical obstacles due to complex forest stand structure.

Beside these abiotic factors, biotic factors may be also affecting whether I can detect plant-plant communication or not. One of the factors may be the spatial distribution of damaged trees in natural forests. I found considerable variations in the damage and in the SA accumulation among upwind trees (Figure 4-3; Figure 4-4). Furthermore, SA concentration of upwind trees was similar to that of downwind trees. These results may be partly explained by the highly damaged upwind tree approximately 40 m apart from the VOC source tree. The tree showed high herbivore damage such as ca. 70 %. It might have emitted VOCs and neighboring downwind trees may have been exposed to VOCs. Indeed, neighboring downwind trees showed less pathogen damage and marginally lesser herbivore damage than downwind trees located farther from this severely damaged tree (Supplemental Figure 4-2; Supplemental Table 4-1). Because of such unintended VOC sources, plant-plant communication may be difficult to detect in natural forests.

The second biotic factor may be the variability among trees in the duration of defense responses. In alder, the nearer neighbors from the VOC source showed less damage than the farther trees and the damage rate varied less among trees at the same distance from VOC source on 39 days after the clipping. However, the variation in the damage increased with time and on 133 days after the clipping the effect of distance on the damage was not detected due to the high variation among trees (Dolch & Tscharntke 2000). This result suggests that duration of effectiveness of plant-plant communication differ among trees. Therefore, plant defenses via plant-plant communication become more variable and difficult to detect as time passes. I evaluated the leaf damage on 120 days after the exposure treatment started, which may be one of the reasons why the variance in damage was large and why I could not detect plant-plant communication in some cases. Research about temporal changes of plant-plant communication effectiveness such as Dolch & Tscharntke (2000) are needed for further understanding.

The third biotic factor is the regional difference of VOCs. In Japanese cedar (*Cryptomeria japonica*) and sagebrush, the composition of VOCs differed among region (Karban et al. 2016; Hiura et al. 2021). Furthermore, the amount of VOCs which plant will start their defense may also differ among regions. I set low-emission experiment in Kawatabi and Ashiu but I could detect plant-plant communication only in Ashiu (Figure

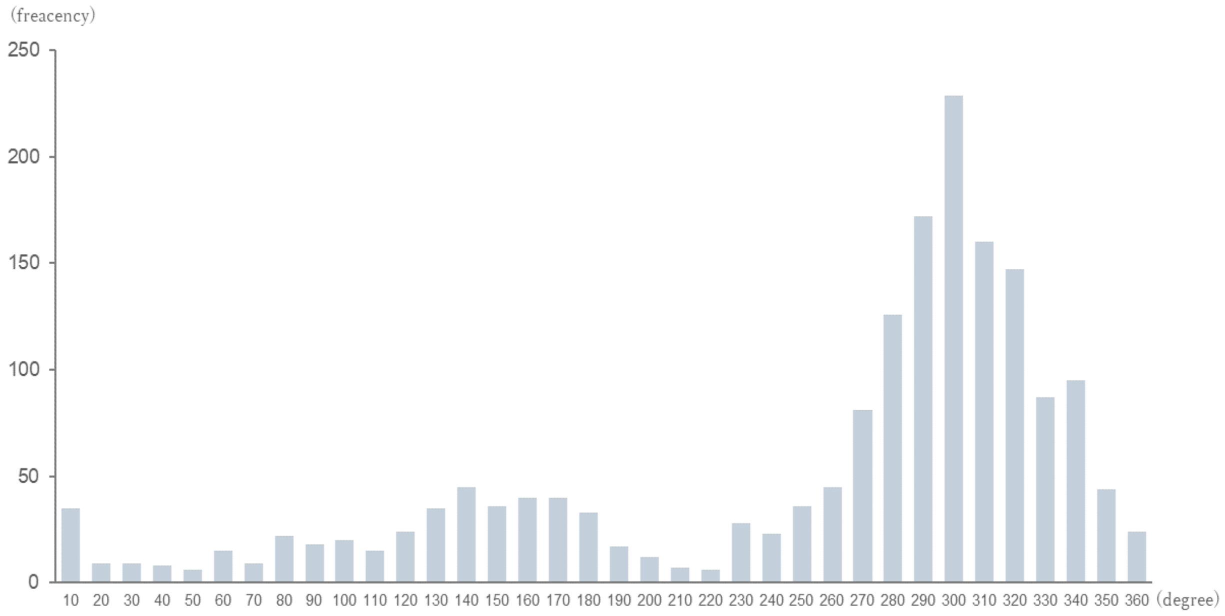
4-3 and 4-5). Consideration of the effective amounts and composition of VOCs may be important for further understanding the plant-plant communication.

The fourth biotic factor is the genetic relatedness. In the present study, I focused on the difference of genetic relatedness among trees, which is one of the characteristics of natural forests. The genetic relatedness did not explain the difference in leaf damage nor the difference in plant hormone concentrations among trees (Table 4-1 and Table 4-2). This result was not consistent with previous results. Sagebrush responded to the cues from plants of the same population more effectively than the cues from plants originally growing 230 km away (Karban et al. 2016). In tall goldenrods (*Solidago altissima* L), VOC profiles diffed profoundly among different genotypes, while that were similar among the same genotype. Tall goldenrod which received volatiles from the same population had less damage than those receiving volatiles from other population originally growing at sites 4.5 to 17.5 km apart. There might have been little or no genetic variation within the same population because plants can propagated from the rhizome (Shiojiri, Ishizaki & Ando 2021). These studies showed that plants respond more defensively when receiving VOCs from genetically identical or close plants than receiving VOCs from genetically distant plants that are originally growing in a different population of 4 to 230 km away. The limitation of these experiments is that they are creating unnatural situations receiving VOCs from far distance. On the other hand, I conducted experiment in natural forests and tested genetic relatedness within a natural population. Hiraoka and Tomaru (2009) reported that within-population genetic diversity is generally high in beech, but decreased from southwest to northeast populations of the Japan Sea lineage. In this study, the Kawatabi site was a northeast site in Japan and the population is the Japan Sea lineage (Suyama unpublished data) so the genetic variation within population may have been low

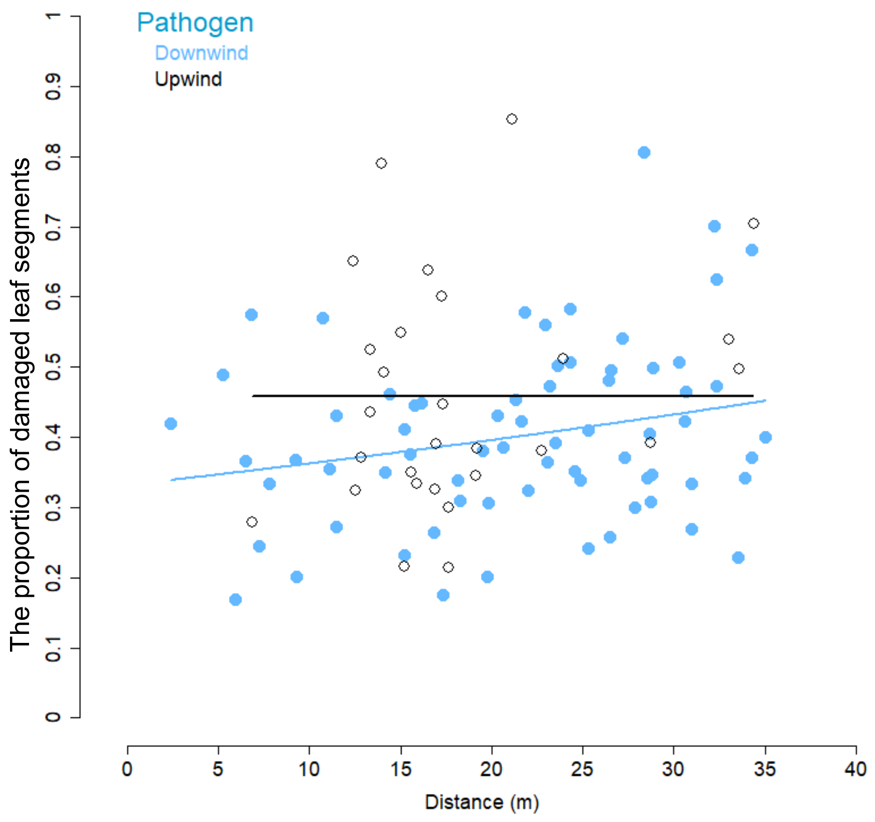
(Tomaru et al. 1997). If I compared the defense response of more genetically distant trees to that of closely related trees, I might have detected the effect of genetic relatedness on leaf damage. However, it is important to compare VOCs mediated defense response within the natural genetic structure in order to understand the actual defense response of plants.

4.5 Conclusions

My result suggests that beech exhibit plant-plant communication via VOCs in natural forests although it is not always detectable due to abiotic and biotic heterogeneity of forest ecosystems. A multiscale approach from phytohormones to individuals and to populations, as in this study, would be important in clarifying the effects of VOCs on forest ecosystems.



Supplemental Figure 4-1.
The frequency distribution of wind direction at the K1 site of Kawatabi. The frequency of the wind direction was detected by weathervane every minute at the top of the tower for 36 hours.



Supplemental Figure 4-2.
The change in leaf damage by pathogens along the distance from the tree no.432 in the high-emission experiment K1 site. The proportion of the number of damaged leaf segments to total number of leaf segments of each sampled branch for trees in downwind of the tree no.432 (blue circles) and those for trees in upwind (black circles) are shown. Continuous curve and line show the estimated proportion using GLM for downwind and upwind trees.

Supplemental Table 4-1. Summary of generalized linear models (GLM) for the number of damaged leaf segments. Distance is from the individual no.432 in upwind of K1 site.

Fixed effects of candidate models	Parameter	AIC	LL	RD	*df*	*p*
From no. 432						
Downwind-Pathogen						
Distance	**2**	**667.36**	**-661.36**	**69.81**	**1**	**0.0434** *
Null	1	669.44	-665.44	69.88		
Downwind-Herbivore						
Distance	2	665.22	-659.22	70.56	1	
Null	**1**	**666.43**	**-662.43**	**70.67**		**0.0729**
Upwind-Pathogen						
Distance	2	285.17	-279.17	28.36	1	
Null	**1**	**284.89**	**-280.89**	**28.41**		**0.1910**
Upwind-Herbivore						
Distance	2	268.37	-262.37	28.63	1	
Null	**1**	**266.39**	**-262.39**	**28.62**		**0.8820**

Shown are number of parameters used in the model (Parameter), Akaike's Information Criterion (AIC), log-likelihood (LL), residual deviance (RD), difference in degree of freedom against the selected model (in bold) (df), p-value (p) for the log-likelihood ratio test between the focal model and the best model. * p <0.05

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CHAPTER 5

General discussion

5.1 Introduction

This study revealed VOCs-mediated plant-plant communication in beech at multiple scales from micro-level (within-plant signaling via VOCs, identification of VOCs emitted after leaf damage and the accumulation of plant hormones) to the macro-level (leaf damage after VOC exposure, inter-plant differences in leaf damage due to abiotic and biotic heterogeneities) (Figure 5-1). Firstly, for plant-plant communication to be effective, it is necessary for VOCs to induce defense response within a plant. In Chapter 2, I revealed that when a beech tree was locally damaged, the released VOCs caused within-plant signaling and subsequently enhance the defense in undamaged part of the tree. After damage, VOCs composition changed with one green leaf volatile (GLV) increased and another GLV and caryophyllenyl alcohol newly detected (Chapter 3). Beech trees near the VOC source tended to show higher concentration ofSalicylic acids (SA), which is a phytohormone related to plant defense, (Chapter 4). I verified whether these changes in the plants lead to a reduction in leaf damage. In plantations, beech trees neighboring near the VOC sources showed less damage than the farther trees. The effective distance for the communication was determined to be ≤ 5 m (Chapter 3). Furthermore, in natural forests, trees neighboring the VOC sources were found to be significantly less damaged by herbivores or pathogens. From these results, beech trees exhibit VOCs mediated plant-plant communication in both plantation and natural forests (Chapter 3 & 4). Furthermore, I examined abiotic environmental factors such as wind direction and forest stand structure and biotic environmental factors such as spatial distribution of damaged trees, variability among trees in the duration of defense responses and the amount and/or composition of VOCs that induce defense, and the degree of genetic relatedness between trees as factors that influence plant-plant communication in natural forests (Chapter 4).

5.2 Mechanisms of plant-plant communication and its adaptive significance for plant defense strategy

5.2.1 VOCs as signal for plant-plant communication

For plant-plant communication to be effective, VOCs must be synthesized in the plant. Furthermore, the VOCs must be received by other plants and as a result plants induce the resistance. Among the VOCs common to terrestrial plants, GLVs are known to be VOCs specifically emitted when leaves were clipped (Matsui 2006). GLVs are also known to be effective VOCs for plant-plant communication (Matsui et al. 2012; Shiojiri et al. 2012; Sugimoto et al. 2014; Matsui & Koeduka 2016). Regarding the evolution of synthesis of GLVs in plants, Tanaka, Koeduka & Matsui (2021) confirmed that monilophytes and lycophytes are able to synthesize GLVs, while bryophytes did not synthesize with a few exceptions. On the other hand, a moss *Ceratodon purpureus* has been reported to synthesize more than 100 VOCs (Rosenstiel et al. 2012). Thus, at least VOCs are widely synthesized in land plants.

In this study, specifically three major VOCs after leaf damage were identified in beech, including GLVs and terpenes (Chapter 3). The VOCs identified and considered in this study are limited to those that have already been reported as plant VOCs by previous studies. Because of the difficulty of identification, unknown VOCs specific to beech were not surveyed in this study. It is possible that VOCs newly gained by plants during evolution may be used for plant-plant communication in some species and further research is needed.

It is not clear whether only one VOC or the blend as a whole is effective in inducing the defense of other trees. One of the GLVs, (*Z*)-3-hexenyl acetate, which was also released by beech, was found to induce a defense response in poplar (Frost et al. 2008). However, Karban et al. (2014) reported that blend of VOCs in sagebrush is region specific and it can be divided into two chemotypes. When chemotypes were the same for VOC source and receiver individuals, they showed plant-plant communication. On the other hand, when the chemotypes were different, they did not show plant-plant communication (Karban et al. 2016).

Even if plants can synthesize VOCs, it is not clear whether all higher plants release VOCs and exhibit plant-plant communication. Taxonomic groups that have been identified to exhibit plant-plant communication include both herbaceous and woody plants of Poales, Fagales, Malpighiales, Sapindales, Brassicales, Malvales, Ericales, Solanales, Apiales, and Asterales (Karban, Yang and Edwards 2014). Therefore, plant-plant communication has been observed in a wide range of species of Angiosperms from the order Poales to Asterales. Beech is a member of the Fagaceae family within the order Fagales, the same order as alder (Dolch & Tscharntke 2000) and birch (Girón-Calva et al. 2014), which have been shown to exhibit plant-plant communication. Although tree species in the Fagaceae family such as *Fagus* and *Quercus* often dominate cool- and warm-temperate forests, the existence of plant-plant communication has remained unexplored (Hagiwara et al. 2021). This study is the first study to show plant-plant communication in Fagaceae. Furthermore, it is the first in a late-successional tree species. Together with previous studies, this study suggests that plant-plant communication may be a common defense strategy of trees, regardless of the successional stages. On the other hand, plant species which lack plant-plant communication were rarely reported (Shiojiri & Karban 2008; Karban, Yang & Edwards 2014). Therefore, further studies are needed to test whether plant-plant communication is species or taxa specific phenomenon or common phenomenon widely observable in plants.

The composition and amounts of VOCs for plant communication may vary within the same species. My preliminary experiments showed that the composition and amounts of VOCs in beech vary among regions. In the plant-plant communication experiments in natural forests, beech showed plant-plant communication at the low-emission experiment in Ashiu but not in Kawatabi (Chapter 4). This result suggests the possibility that the amount of VOC emission at which plant-plant communication is detected may differ between the regions. It is necessary to clarify the differences in the amount and composition of VOCs by region, as well as the within-species variation in plant-plant communication.

5.2.2 Effective distance of plant-plant communication via VOCs

The effective distance of VOCs in plant-plant communication was found to be within 5 m (Chapter 3). The height of the beech trees in the experimental sites was approximately 10 m. In the natural forest, the effective distance was not determined but it may be less than 20 m (Figure 4-3). In sagebrush, the effective distance for VOCs was 60 cm (Karban et al. 2006), which was the same as sagebrush’s height. These results suggest that effective distance will be approximately half or equal to tree height. Why is the effective distance for plant-plant communication relatively short?

One possible reason is that plants may have physiological limitations that prevent them from releasing large amounts of VOCs. For example, (*E*)-2-hexenal, one of aldehyde, is toxic to plants (Tanaka et al. 2018). Therefore, (*E*)-2-hexenal is transformed to and emitted as (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate (Figure 1-1), which were emitted after clipping treatment. It is possible that plants have not evolved to maintain large amounts of aldehyde in the tissues because of the increased risk of causing functional failure in the plant.

The second reason is that plant-plant communication may be by-product. Originally, plants may synthesize, emit and receive VOCs for within-plant signaling and induce the defense. They may not be able to distinguish between VOCs emitted by themselves and those from other plants. They may be “eavesdropping” on the VOCs of other plants, unintentionally resulting in plant-plant communication. In beech, within-plant signaling by VOCs was observed (Chapter 2). Similar within-plant signaling by VOCs has been reported in eight plant species (Li & Blande 2017). The spatial extent of the VOCs to reach for within-plant signaling may be tree height and canopy width. The fact that the effective distance for plant-plant communication was similar to these supports this hypothesis. In previous studies, plant-plant communication was observed among genetically identical or closely related individuals and was not exhibited among geographically distant and genetically distant individuals (Karban et al. 2014).

The third reason is the possibility that plants have evolved not to respond to the VOCs from spatially distant individuals. For plants to receive signals from distant plants, plants may need to increase the amounts of VOCs synthesized and released and/or to be sensitively perceiving VOCs. Both are likely to involve costs. In the case when herbivores are abundant and the herbivory pressure is high, the benefits of increasing effective distance of plant-plant communication may be larger. Neves et al. (2014) shown that herbivores are more abundant in late-successional forests and the late-successional species such as beech may be more likely to perform plant-plant communication. On the other hand, if the herbivore distribution is highly localized, plant-plant communication may be also spatially localized. If the sharing of information about the herbivores and pathogens within a population may prevent outbreaks and increase plant’s fitness, the distance of plant communication may be large even if there is a substantial cost. Furthermore, plant population show genetical spatial structure and it may be adaptive to share the information of leaf damage among plants that are genetically closely related, which are spatially close as well. In the high-emission experiment in the Kawatabi site, genetic-related individuals to the VOC source tree were located within ca. 30 m (Numano & Suyama 2006) (Chapter 4). Therefore, beech may be emitting suitable amount of VOCs to transmit signals only to these closely related trees resulting in short effective distance although the effect of genetic relatedness was not detected on plant-plant communication (Chapter 4). To clarify the adaptive significance and evolution of plant-plant communication, the costs of synthesis and emission of VOCs and the cost of perceiving VOCs as well as the spatial distribution of herbivores (Karban & Yang 2020) should be quantified in natural forests. Studies about cost-benefit based model and comparative studies about effective distances among plant species will be needed.

5.2.3 Phytohormone response after VOC exposure

The results of Chapter 4 showed that exposure to VOCs after beech leaf clipping was effective in reducing damage caused by both herbivores and pathogens. The accumulation of SA, a phytohormone involved in defense, was greater in individuals close to the VOC source. So far, only JA, a hormone involved in defense against herbivores but not for pathogens, has received attention (Arimura, Huber & Bohlmann 2004). The only empirical evidence of SA accumulation after exposure to VOCs is a study of tomato by Zhang et al (2019). Mine is the first study in woody plants. My preliminary experiments showed that exposure to VOCs resulted in significantly more SA accumulation in exposed beech trees than in unexposed trees after 3 days of exposure. Furthermore, genes involved in the synthesis of SA were found to be expressed after 3 days of exposure compared to 0 day. Thus, it is suggested that the expression of genes involved in SA synthesis and subsequent accumulation of SA after exposure to VOCs resulted in the resistance to pathogens in beech.

Despite the reduced damage caused by the herbivores, no accumulation of JA was observed after 3 days of VOC exposure (Figure 4-3). My preliminary experiments with beech saplings indicated that beech also accumulates JA but this accumulation tends to occur earlier than 3 days after exposure to VOCs (Hagiwara unpublished data). It has been reported that JA and SA show antagonistic effect (Thaler, Humphrey & Whiteman 2012 but see Ullah et al. 2022). Therefore, it is possible that JA accumulated in beech trees prior to 3 days after exposure to VOCs. It is also possible that the accumulation of SA alone could have driven systemic acquired resistance and defense against herbivore damage. Time-series quantification of both JA and SA is necessary to understand the mechanisms of plant-plant communication.

5.2.4 Leaf damage after VOC exposure

Researches on plant defense mechanisms against pathogens are limited compared to those against herbivores. Wei and Harada (1998) reported yellow leaf spot as a disease of beech. Few studies have been reported about pathogens. In Chapter 4, I showed for the first time in woody plants that plant-plant communication is also effective against damage caused by pathogens. It remains to be studied whether increased resistance to pathogens through plant-plant communication is a phenomenon found only in beech or in a wide variety of plants.

Herbivores are divided into specialists and generalists. In Chapter 3, I have identified plant-plant communication in beech at two plantations in Tomakomai and Hiruzen. Because Hiruzen is in the natural distribution area of beech, both specialist and generalist herbivores were present. On the other hand, Tomakomai is located beyond the northern limit of beech, so specialist herbivores were not present. Nevertheless, in both plantations, leaf damage after the exposure to VOCs were less in trees neighboring VOC sources. This means that plant-plant communication is effective for generalist herbivores. In Hiruzen, where specialists should be also present, the amount of damage was almost twice that of Tomakomai regardless of distance from source trees (Figure 3-3). This suggests the possibility that plant-plant communication in beech may not be effective against specialists or herbivore and pathogen were more abundant in Hiruzen than in Tomakomai. Opposing to current result, previous studies have shown that plant-plant communication works effectively against specialists. In black alders (*Alnus glutinosa*), when VOCs were artificially emitted as in beech, generalist herbivores were found to be present regardless of their distance from the VOC sources, while specialists were significantly more abundant in trees far from the VOC source (Tscharntke et al. 2001). This result suggests that in the black alders, plant-plant communication is effective to the specialist herbivore (Tscharntke et al. 2001). It is not clear whether this is because plants accumulated chemicals on their leaves and became more defensive and specialists avoided those leaves or because specialists were suppressed by natural enemies attracted by VOCs. It is also unknown whether a generalist was able to feed on leaves that had accumulated defensive chemicals. It is possible that the plant itself has a strategy to specifically enhance its defenses against the specialist.

In this study, beech trees close to the VOC source tree were also damaged to some degree. This suggests that plants are not fully successful to prevent damage caused by herbivores or pathogens but that plants and their herbivores or pathogens may be coevolving in an arms race. In actual natural ecosystems, herbivore community may be composed of herbivores that have acquired traits that allow them to forage regardless of plant defenses and those that have not. In my study, it is not clear whether the damage is caused by one type or multiple types of herbivores. Further study is needed to determine which kinds of herbivores are affected by plant-plant communication. Identification of comprehensive herbivore communities by environmental DNA and the verification of plant-insect-pathogen interactions through field observations, experiments, simulations and modeling are desirable.

Furthermore, previous plant-plant communication studies have been conducted on temperate species, but little is known in the tropics (Glinwood & Blande 2016). Tropical ecosystems have enormous diversity in plants and insects. These insects are characterized by abundant generalist herbivores and more natural enemies of herbivores than in temperate ecosystems (Pareja & Pinto-Zevallos 2016). Therefore, plant-plant communication may also be functioning in tropical ecosystems. On the other hand, it may not be effective in rare tree species because the benefit may be low compared to the costs of plant-plant communication due to the low density of conspecific trees. Whether plant-plant communication is a phenomenon unique to temperate regions or not also remains to be examined.

5.3 Characteristics of plant-plant communication in natural forests and its significance for forest managements

5.3.1 Characteristics of plant-plant communication in natural forests

Plant-plant communication may not be detected in natural forests as it is in the plantations, green houses or laboratories or there may be variation in the effectiveness on plant-plant communication in natural forests. In the current study, I showed plant-plant communication in natural forests under abiotic heterogeneities such as wind direction and the biotic heterogeneities such as relatedness. Natural forests are characterized by spatial variability in distribution of herbivores and the associated multiple sources of VOC emission, the herbivore community composition and/or the variation in responses within plant species. In alder, the nearer neighbors from the VOC source showed less damage than the farther trees and the damage rate varied less among trees at the same distance from VOC source on 39 days after the clipping. However, the variation in the damage increased with time and on 133 days after the clipping the effect of distance on the damage was not detected due to the high variation among trees (Dolch & Tscharntke 2000). This result suggests that duration of effectiveness of plant-plant communication differ among trees. Therefore, plant defenses via plant-plant communication become more variable and difficult to detect as time passes. It is surprising that this study successfully detected plant-plant communication in such heterogeneous natural forests. It is needed to clarify the effects of heterogeneous factors in natural forests and examine how they affect plant-plant communication.

5.3.2 Geographic variation in plant-plant communication in forests

Japanese beech is known to show geographic variation in leaf traits and herbivore communities (Osada et al. 2018). Chewers tended to be more abundant in the north, while gallers tended to be more abundant in the south (Nakamura, Inari & Hiura 2014). In Hiruzen, where both specialists and generalists also inhabit, the damage was about twice as much as in Tomakomai with only generalists (Chapter 3). The effectiveness of plant-plant communication differed between low-emission experiment Kawatabi sites and Ashiu sites (Chapter 4). Furthermore, my preliminary experiments have shown that the composition and abundance of VOCs also vary geographically. Thus, it is suggested that the effectiveness of plant-plant communication may vary depending on geographically varying leaf traits, VOC composition, intra-specific variation in response and the herbivore and pathogen community. Comparative studies or transplanting studies are needed to know the generality and geographical variation in plant-plant communication.

5.3.3 Significance of plant-plant communication in pest control in forests

From the past to present, chemical pest (herbivores and pathogens) control using agrochemicals has been the main method for pest control. Chemical pest control is known to have problems such as soil and river pollution. In addition, pests have been reported to reappear as a result of acquired resistance to agrochemicals. There have also been reports of agrochemicals killing natural enemies of the plants at the same time, resulting in the outbreak of previously non-specialist potential pests (van den Bosch et al. 1971). Facing these problems, biological control methods have been adopted more often in recent years. For example, introducing natural enemies of pests (Waage et al. 1988), using pheromones to attract and trap target pests (Flint, Salter & Walters 1979), and changing plant CN ratio and other properties by using fertilizers and soil amendments (Qian & Schoenau 2002). In addition to these methods, plant-plant communication is also considered as an effective method. This method has already been applied to agricultural crops (Abe et al. 2020; Uefune et al. 2020). For example, rice is known to exhibit plant-plant communication, and it has been tested which types of VOCs can artificially reduce insect damage (Shiojiri unpublished).

Similary, plant-plant communication has a potential for applications in forests as a biological control method. For example, in the past, aerial spraying of chemicals has mostly been used against outbreaks of pests and diseases on a national scale, such as pine wilt disease. My study indicates that VOC exposure can increase tree defenses and reduce leaf damage caused by herbivores and pathogens. (*Z*)-3 hexenyl acetate, the VOC most commonly released after beech leaf clipping (Chapter 3), has also been investigated as an attractant for natural enemies of herbivores (Uefune et al. 2020). In other words, spraying artificial VOCs may be possible to reduce disease and herbivore damage by increasing tree resistance and further attracting the natural enemies of herbivores. This is expected to be an effective method of biological pest control that preserves insect biodiversity and reduces environmental impacts.

In Japan, it has been pointed out that global warming may limit the potential distribution of beech forests to be 1/10 of the current area within this 100 year (Matsui et al. 2004). Most of diminishing populations are located in the western Japan, which is already a relatively warm and dry environment (Matsui et al. 2009). Meanwhile, in recent years, many beech forests in Japan have experienced outbreaks of beech caterpillar (*Sntypistis punctatella*) and beech sawfly (*Fagineura crenativora*) (Kamata 2002; Watanabe, Taniwaki & Kasparyan 2018). The beech caterpillar (*Quadricalcarifera punctatella*) feeds on beech leaves and populations occasionally reach epidemic densities that totally defoliate trees (Kamata, Igarashi & Ohara 1996). The decline of beech forests caused by these outbreaks has been reported since the 1980s (Ishimura et al. 2011). The high temperatures and drought stress caused by climate change and exposure to feeding damage by herbivores may accelerate the decline of beech forests. Therefore, biological pest control via VOCs has potential applications such as controlling outbreaks of pests and diseases in beech forests and preventing the decline of beech forests due to the synergistic effects of pest outbreaks and global warming. The role of VOCs in various tree species as beech needs to be further studied.

5.3.4 Significance of plant-plant communication research under climate change

In recent years, it has gradually become clear how the VOCs released by plants are affected by drought. For example, white spruce (*Picea glauca*) has been found to change the composition of VOCs emitted under drought stress, with the emission of monoterpenes being suppressed (Perreca et al. 2022). It has also been found that monoterpene emission tends to decrease in piñon pine (*Pinus edulis*) (Trowbridge, Bowers & Monson 2016). Thus, under drought stress, the composition and amounts of VOCs in plants may change. My study suggested that plants may not exhibit plant-plant communication when VOC exposure is reduced (low-emission experiment sites in Kawatabi, Chapter 4). Climate change, such as drought, may change the amount and composition of VOCs, which may affect plant-plant communication that have evolved through long history (Trowbridge 2014) and possibly leading to disease and insect outbreaks. However, the indirect global warming effects on plant-plant communication via VOCs remain unknown.

It is concerned that changes in trees due to global warming may cause negative feedback by decreasing CO_2 absorption and storage and further accelerating global warming. Therefore, it is important to clarify the response of trees to global warming. It has been reported in many places of the world that trees are subjected to stresses such as (1) high temperature, (2) drought and (3) pathogen and herbivore damage due to environmental changes associated with global warming (Holopainen et al. 2018; Hamann et al. 2021). Trees have evolved mechanisms to acquire tolerance and resistance to these three stresses (Derksen, Rampitsch & Daayf 2013; Gao et al. 2015). Three phytohormones play key roles in the acquisition of tolerance and resistance: (1) high temperature: salicylic acid, (2) drought: abscisic acid, and (3) herbivore and pathogen damage: jasmonic acid and salicylic acid, respectively. Three plant hormones do not act independently but have antagonistic and synergistic effect (Yasuda et al. 2008; Thaler, Humphrey & Whiteman 2012). In other words, there is a triple-talk between phytohormones, which may regulate the actual stress responses of plants (Derksen, Rampitsch & Daayf 2013) (Figure 5-2).

This study revealed the VOCs-mediated defense, plant-plant communication, in beech. The VOCs-mediated communication among plants may not be limited against herbivores and pathogens but also against drought stress (Derksen, Rampitsch & Daayf 2013) (Figure 5-2). As described above, drought stress may change VOCs, and the antagonistic or synergistic effect of tolerance genes and three phytohormones on different stresses may determine individual plant tolerance or resistance to high temperature, drought and pest and pathogens stress simultaneously. The stress response of trees may spread within the forest through VOCs. Thus, communication among trees via VOCs is a topic that needs to be further studied in order to predict forest responses to climate change and to take adaptation and mitigation measures. In this study, I investigated plant-plant communication in a late-successional tree, that is dominate in cool temperate forests. I believe that this study will be an important first step for the conservation of forest ecosystems under climate change with the perspective on plant-plant communication via VOCs.

5.4 Conclusion

This study detected plant-plant communication in Japanese beech, a late-successional tree species dominating cool temperate forests, from the micro scales such as within-plant signaling, emitted VOCs and phytohormone accumulation to the macro scales such as the insect and pathogen damage, the inter-plant variation and the abiotic and biotic heterogeneity affecting the effectiveness of plant-plant communication in natural forests. Current study of VOCs-mediated plant-plant communication in beech has contributed to the understanding of the adaptive significance of plant defense and the plant-insect interactions as well as for the pest control and the conservation and managements of beech forests under climate change.

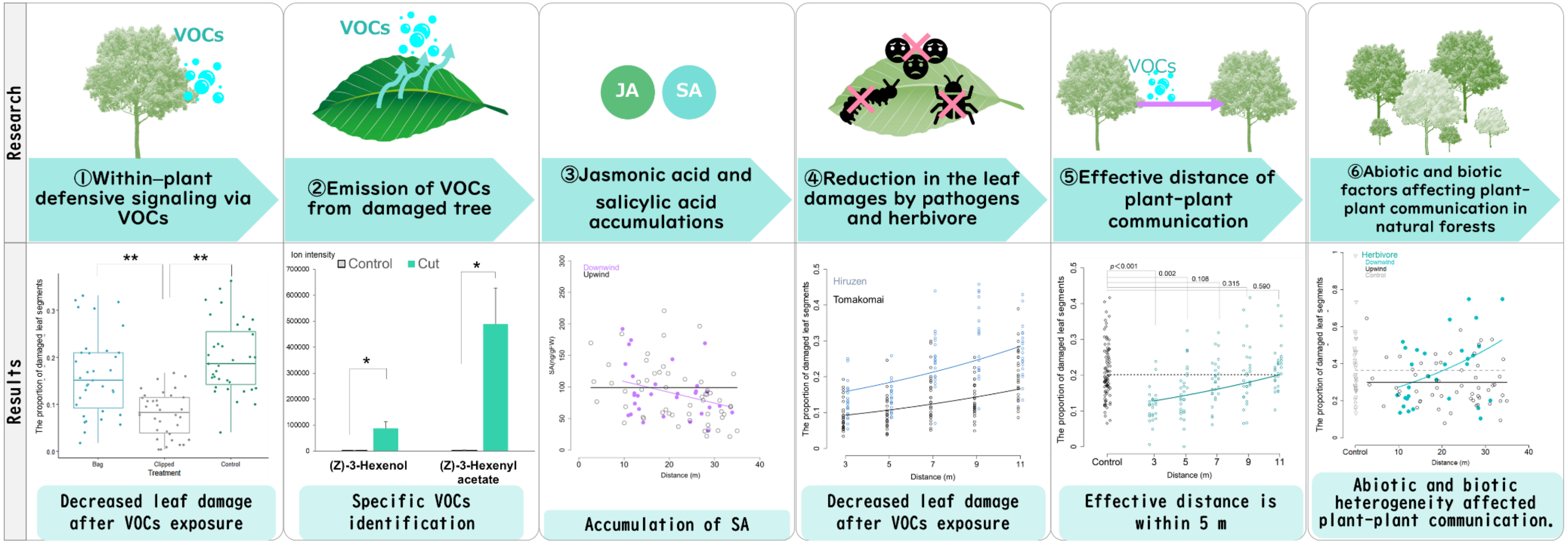

Figure 5-1. Structure and results of this study

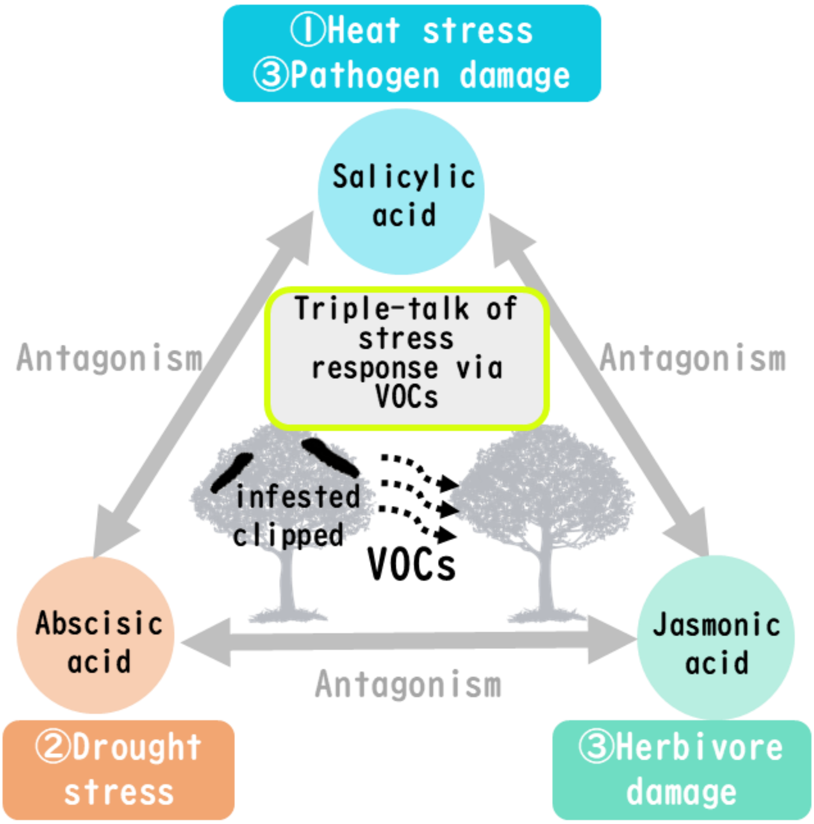

Figure 5-2. Triple-talk between three phytohormones related to stress response in plants.

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Appendix

1.*Operophtera brumata*

ナミスジフユナミシャク

2.*Callitera lunulata*

アカヒゲドクガ

3.*Erannis golda*

チャバネフユエダシャク

4.*Syntypistis punctatella*

ブナアオシャチホコ

5.Gall midge

Diptera: Cecidomyiidae

ブナハマルタマバエ

6. Gall midge

Diptera: Cecidomyiidae

7.*Calliteara pseudabietis*

リンゴドクガ

8.*Actias aliena*

オオミズアオ

9.*Sphrageidus similis*

モンシロドクガ

10.Pathogen damage

11.

Species no. 1 to 5 were found in Kawatabi, Species no. 6 to 10 were found in Tomakomai, species no.1, 3, 8 and 11 were found in Ashiu, no.8 was found in Hiruzen.

Samples of leaves damage in Ashiu.

Sample of clipped leaves.

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