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Effect of aerial spraying of thiacloprid on pine sawyer beetles (*Monochamus alternatus*) and honey bees (*Apis mellifera*) in pine forests

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Abstract

Monochamus alternatus is a very important vector of the pine wood nematode (*Bursaphelenchus xylophilus*) which is the causal agent of pine wilt disease. To reduce population density of *M. alternatus*, thiacloprid has been sprayed by aircrafts on pine forests. Thus, we examined the effect of aerial spraying as contact toxicity and thiacloprid residues of pine branches as ingestion toxicity on the mortality of *M. alternatus* in pine forests in Yangsan, Korea. In addition, the effect of aerial spraying on honey bees, *Apis mellifera*, was tested in the same locality. Thiacloprid was sprayed once a month from June to August in 2016. To test the effect of thiacloprid, eight *M. alternatus* and ten honey bees were put into each small mesh cage, which were hung on pine trees in each study plot. Thiacloprid appeared to be effective for reducing longevity of *M. alternatus*, while mortality and abnormal behaviors were not found for honey bees. In addition, longevity of *M. alternatus* beetles was declined by thiacloprid residues of pine branches compared to the control group. However, we found that dead pine trees infected by pine wood nematodes in the next year similarly occurred in our study area whether thiacloprid-aerial spraying occurred or not. Consequently, thiacloprid sprayed by aircraft may be an effective control agent for *Monochamus* beetle adults without negative effect on honey bees, but aerial application to prevent expansion of pine wilt disease should be reassessed.

Key words: Non-target insects, Pine wilt disease, Pine wood nematode, Toxicity

Introduction

Pine sawyer beetle (*Monochamus alternatus*, PSB), which is a vector insect of pine wood nematode (*Bursaphelenchus xylophilus*, PWN) causing pine wilt disease (PWD), is an important forest pest in pine forests of Korea as well as Japan and China. The PWD was firstly reported from Mt Geumjeong, Busan-si in 1988, and has now expanded throughout South Korea. From 1988 to 2019, over 20 million native pine trees were lost due to PWD in South Korea.

The PWD is caused by the interaction of host plants, PWNs, and vector insects (e.g., PSBs). When the pupae of PSBs emerge to adults in PWN-infected dead pine trees, PWNs move and enter into the trachea of PSB adults. PWNs are transferred to other healthy pines when PSB adults gnaw upon new pine shoots for sexual maturation and acquiring energy for moving. In Korea, *Pinus densiflora*, *P. koraiensis*, and *P. thenbergii* were reported as host species of PWNs, which is transmitted by two vector species, such as *M. alternatus* and *M. saltuarius*, in Korea (Korea Forest Service 2004). Thus, pest management of vector species is a primarily important step for reducing PWD in pine forests.

To reduce expansion of PWD in Korea, several methods are conducted, such as fumigation as chemical methods, chipping and burning woods as physical methods, and silvicultural control, aerial spraying, and trunk injection as preventative methods (Shin 2008). The aims of chemical and physical

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methods are to eliminate PSB larvae and nematodes in dead pine trees, while killing PSB adults is a fundamental goal for the prevention methods. Although an application of insecticides on wide areas has been criticized by non-governmental organizations, aerial spraying is still considered as a primary control method to prevent the expansion of PWD because the population density of PSBs can be directly reduced by sprayed insecticides through contact toxicity (Kwon 2008). In addition, PSB adults can be exposed to residues of insecticides on leaves and branches of pines affecting PSBs behavior and longevity, which called ingestion toxicity.

Since the occurrence of PWD in Korea, fenitrothion 50% emulsion had been used for aerial spraying until 2000s, and there were some studies on changes of the community structure of insects and microbes in fenitrothion-sprayed pine forests (Kwon 2008, 2010; Kwon *et al.* 2003, 2005a, 2005b). Given the high toxicity of fenitrothion for environments (Fawell & Hedgecott 1996; Kevan 1975; Peveling *et al.* 1999), it has been replaced with thiacloprid (10% liquid wettable powder, Calypso, Bayer CropScience, Cambridge, UK), which is the second member of Bayer's chloronicotinyl insecticide family (Jeschke *et al.* 2001) and is known to be less toxic and relatively safe to honey bees (Iwasa *et al.* 2004), since the 2010s. However, it was also reported that thiacloprid as well as other neonicotinoid insecticides are toxic to fish and birds (Tomizawa & Casida 2005), and spraying flowering crops is harmful to honey bees, either by direct contact or ingestion (Tomlin 2003). In addition, studies examining lethal effects of neonicotinoid insecticides on *Monochamus* beetles (i.e., target species) and honey bees (i.e., non-target insects) in pine forests are still limited. To improve pine forests health, therefore, assessing the lethal effects of thiacloprid on target and non-target insects is essential from the perspective of forestry and ecotoxicology.

This study was conducted to investigate the insecticidal effect of aerial spraying as contact toxicity and thiacloprid residues of pine branches as ingestion toxicity on mortality of *M. alternatus* in pine forests in Yangsan, Korea. In addition, we tested the effect of aerial spraying on the mortality of *Apis mellifera*, an important pollinator species in ecosystems.

Material and Methods

Study area

Three aerial sprayed plots (a total of 24 ha) and two unsprayed plots (a total of 24 ha) were selected from two mountainous pine forests, namely Mt. Geumjeong and Mt. Obong in Yangsan, Gyeongsangnam-do, Korea (Fig. 1A, B). The study areas consist of two pine species, i.e., about 70–80% were *P. thunbergii* and the others were *P. densiflora*. The density of pine trees in the study areas was about 750 trees per 1 ha. The pine trees were 25–30 cm in diameter at breast height (D.B.H.) and 10–12 m in height.

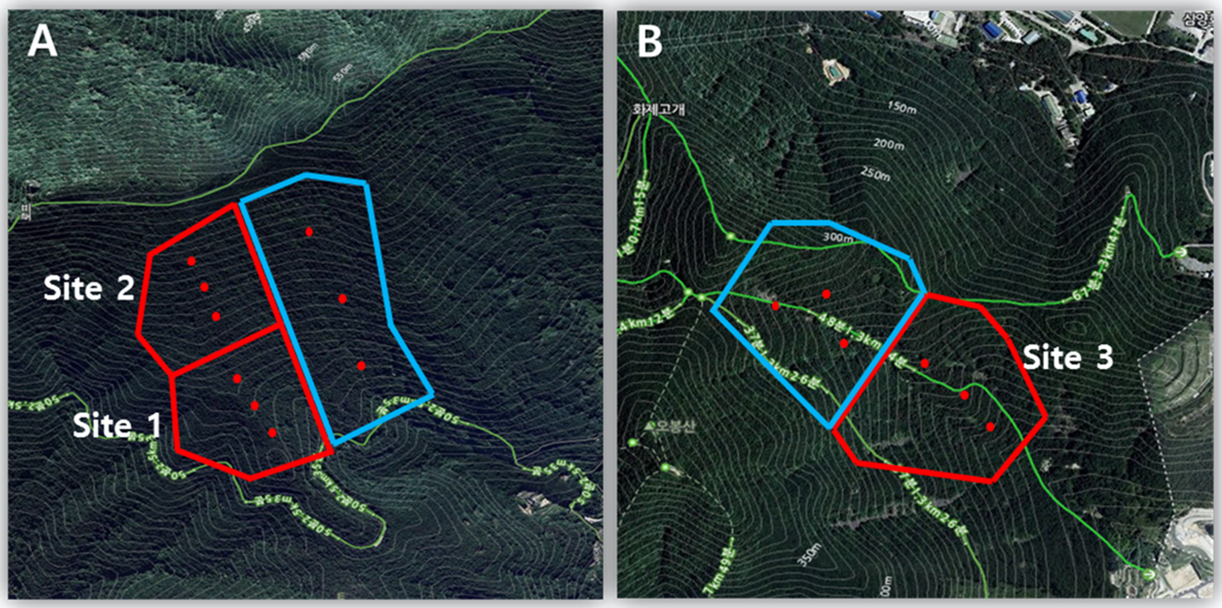

Figure 1 Aerial photographs of study areas in Yangsan, Korea (A, Mt. Geumjeong; B, Mt. Obong). Red-filled circles indicate sampling site, and red and blue lines indicate area of aerial sprayed and unsprayed plots, respectively.

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Aerial spraying and pesticide residue test

Thiacloprid was sprayed once a month from June to August in 2016. 50-fold diluted thiacloprid 10% flowable (FL) was sprayed, and a total of 50 L per 1 ha was sprayed using by a D6 nozzle on a medium-sized helicopter (Bell 206; Bell, Fort worth, USA). Although aerial spraying was conducted at three times (June, July, and August), branches of pine trees in July were not sampled due to heavy rainfalls after spraying. Thiacloprid residues branches in June and August were analyzed as follows. We sampled branches from nine pine trees (high, 10–12 m; low, 6–8 m) after aerial application in each study plot, and moved them to the laboratory in the National Institute of Forest Science, Seoul.

Thiacloprid residues were analyzed by the ABsolution Co., Ltd. (Suwon, Korea) according to the following steps: (i) 10 g of the samples was added to 20 mL of distilled water, and extracted with 100 mL of acetonitrile using a homogenizer at 3500 rpm for 1 minute; (ii) The extracts were filtered under reduced pressure, and acetonitrile was distilled off under reduced pressure using a rotary vacuum evaporator; (iii) The aqueous solution was transferred to a 500 mL separatory funnel, and 50 mL of saturated NaCl solution and 100 mL of distilled water were added; (iv) The mixture in (iii) was dispensed twice with 50 mL, 100 mL of dichloromethane; (v) The water was removed through an anhydrous sodium sulfate layer; (vi) The solution was concentrated under reduced pressure using a rotary vacuum evaporator and the dry solid was then dissolved in 10 mL of dichloromethane; (vii) 1 mL of 6 mL of dichloromethane solution was added to SPE (NH_2, 1 g) tube activated 6 mL of dichloromethane; (viii) The solution was washed with 5 mL of dichloromethane and eluted with 6 mL of mixtures (dichloromethane: acetone = 95:5); (ix) The eluate was concentrated using a nitrogen concentrator and dissolved in 1 mL of acetonitrile; (x) A certain amount (1 uL) was injected into LCMS/MS; and (xi) The peak area on the chromatogram was measured and the concentration was calculated by the standard calibration curve. The detection limit for thiacloprid residues was 0.01 mg/kg.

Contact toxicity of thiacloprid on PSB adults and honey bees

Reared PSB adults (Kinsect Co., Ltd., Cheonan, Korea) and honey bees (Kyungnong Co., Ltd., Seoul, Korea) were used for the insecticidal effect test of thiacloprid-sprayed pine forests. To see whether thiacloprid could affect PSB and honey bees in pine forests, two experiments were conducted.

The first experiment comprised direct exposure of thiacloprid on PSBs and honey bees. Before aerial spraying, three trees were randomly selected in each study plot, and the small mesh cages (for PSB, 20 cm in diameter, 45 cm in length; for honey bees, 5 cm in diameter, 15 cm in length) containing either 8 PSBs or 10 honey bees were placed at two different heights (top, 10–12 m; middle, 6–8 m) of pine trees. After aerial spraying (about 2 hours), the small mesh cages were taken back from the trees, and PSBs were transferred to the insect jar (8 cm in diameter, 12 cm in length, SPL Life Sciences Co., Ltd., Pocheon, Korea) individually with thiacloprid-free branches as feeds for the survivorship of PSBs during transportation to laboratory. The number of dead PSBs and honey bees were counted every day for 10 days and 2 days, respectively.

Ingestion toxicity of thiacloprid on PSB adults

Reared PSB adults, which were maintained under laboratory condition (i.e., not from pine forests), were fed on branches with thiacloprid residues sampled from top and middle branches of three pine trees in our study plots (3 aerial sprayed and 2 unsprayed plots). The branches were cut into several parts (size of each part of branches are: 1–1.5 cm in diameter, 7 cm in length), and a fragment of branches was provided as feeds for PSB adults until whether died or ten days. Ten PSB adults for each branch were tested, and number of dead PSB adults was counted every day for 10 days. In total, 135 PSB adults were tested.

Investigation of the infested wood caused by pine wilt disease

The number of wilted or dead trees caused by PWD was counted, and their spatial location (i.e., latitude and longitude) was individually measured using global positioning system (GPS) from May to October 2016. The number of wilted or dead trees in 2016 was compared between thiacloprid sprayed and unsprayed plots. In addition, the location map of wilt or dead trees was drawn using by ArcGIS (Arc Geographic Information System, http://gportal.nifos.go.kr.arcgis/home).

Data analysis

To test the mortality of PSBs and honey bees between thiacloprid-sprayed and unsprayed plots, generalized linear mixed models (GLMMs) were used given the fact that our sampling design was unbalanced, and mortality data of PSBs and honey bees did not meet the assumptions of normality. In addition, linear mixed models (LMMs) were also used to analyze the differences of thiacloprid deposition between heights (low and high) and between month (June and August), which fulfill the assumptions of normality. Since the treatment group (thiacloprid-sprayed and unsprayed plots) were nested in the mountains (Mt. Geumjeong and Mt. Obong) (Fig. 1), we considered the treatment group as a fixed effect, and location of the study plot as a random effect in the models.

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In addition, we considered the time interval in laboratory as a random effect in the models. Arcsine-transformed mortality data were used. Variables with statistical significance were compared to assess pairwise comparisons (α = 0.05) of least-squares means.

All statistical analyses were performed with R version 3.3.2 (R Core Team 2016). We performed the GLMM using the 'lme4' package. We also used the 'lsmeans' and the 'multcompView' packages to assess all pairwise comparisons of least-squares means.

Results

A comparison of thiacloprid residues

The average amount of thiacloprid residues in the sprayed plots was 0.331 mg/kg (ranging between 0.00 and 2.86 mg/kg), but it was varied according to study plots, treatment times and sampling positions. In particular, treatment times were more affected by the amount of thiacloprid residues, which was significantly higher in June compared to that in August (Wald's χ^2 = 13.19, $P < 0.001$) (Fig. 2). In the sampling positions, the average amount of thiacloprid residues was similar between heights at same treatment time (Wald's χ^2 = 0.16, P = 0.692), although it was varied according to the location of sampled branches.

Contact toxicity of thiacloprid on PSB adults and honey bees

The mortality of *M. alternatus* in small mesh cages hung on high heights in thiacloprid-sprayed plots was significantly higher than unsprayed plots (Wald's χ^2 = 45.77, $P < 0.001$) (Fig. 3a). However, a lethal effect of thiacloprid on honey bees was not found whether thiacloprid-sprayed or not (Wald's χ^2 = 3.78, P = 0.286) (Fig. 3b).

Ingestion toxicity of thiacloprid on PSB adults

The mortality of PSB adults in treatment groups that fed on branches with thiacloprid residues was significantly higher compared to PSB adults that fed on branches sampled from unsprayed plots (Wald's χ^2 = 22.03, $P < 0.001$), but the mortality of PSBs was not different between two different heights (Fig. 4).

Comparison of number of dead woods by PWN

The number of dead woods caused by PWN at the end of the field study was compared using aerial photos between thiacloprid-sprayed and unsprayed plots (Fig. 5). In Mt. Geumjeong, 234 and 109 trees were infested by PWN in thiacloprid-sprayed and unsprayed plots, respectively. In Mt. Obong, 28 and 46 trees were infested by PWN in thiacloprid-sprayed and unsprayed plots, respectively.

Discussion

Effect of aerial spraying on PSBs and suppression of PWD expansion

Our study showed that the survival of pine sawyer beetles (PSBs) in pine forests can be significantly influenced by aerial spraying of thiacloprid. The aerial spraying of thiacloprid could affect the survival of PSBs by contact toxicity, although the effect was varied depending on where the PSBs were

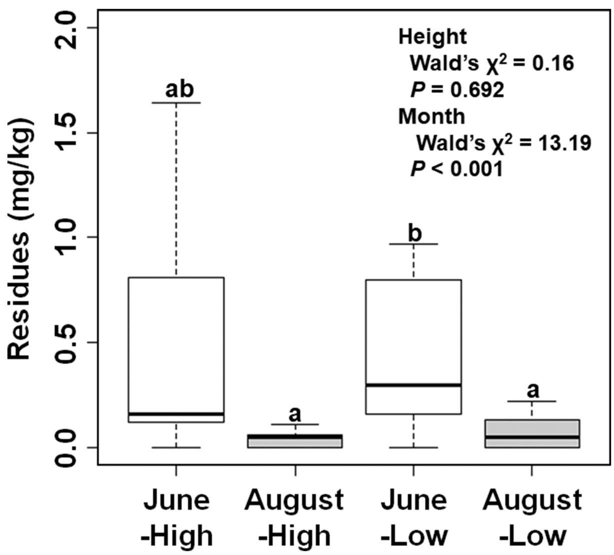

Figure 2 Deposition of thiacloprid by aerial spraying in *Pinus densiflora* forest in Yangsan, Korea.

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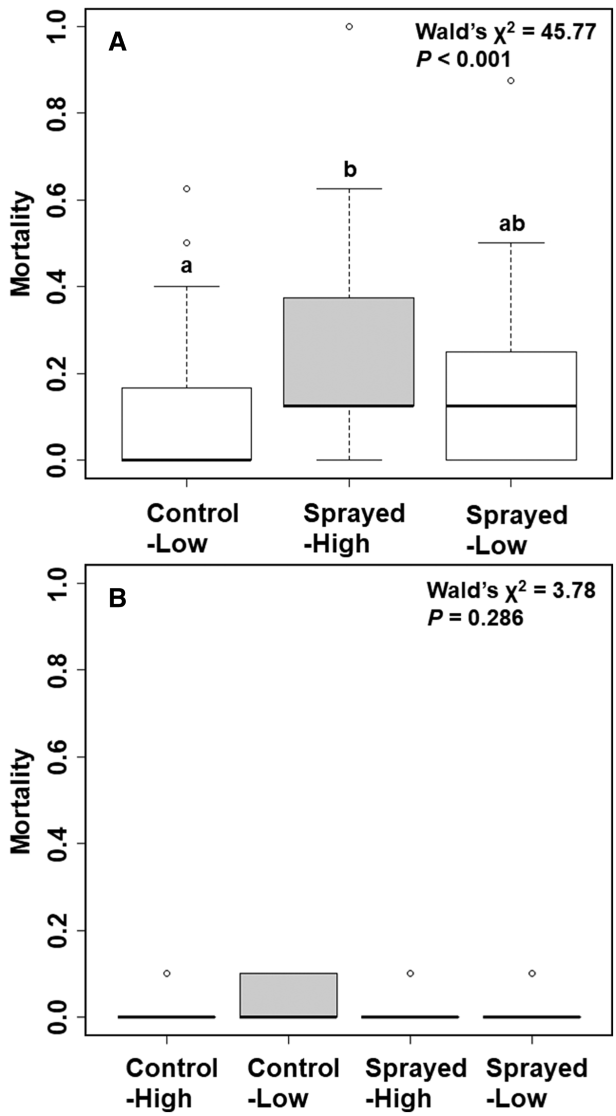

Figure 3 A comparison of the mortality of *Monochamus alternatus* (a) and honey bees (b) between thiacloprid-sprayed and unsprayed plots. In sprayed plots, small mesh cages were placed at low and high heights.

located on the tree (i.e., height). These variations were similarly observed in aerial spraying of thacloprid by unmanned aerial vehicles (Lee *et al.* 2019). Thiacloprid residues on leaves and branches of pine trees can also affect the survival of PSBs through ingestion toxicity. In addition, PSBs showed abnormal behaviors such as convulsions or inactivity due to thiacloprid both in field and laboratory level experiments. Thus, the aerial spraying of thiacloprid appears to be an effective control agent to reduce the population density of PSBs, either by contact or ingestion toxicities.

We expected that aerial spraying can hinder the expansion of the infected area of PWN, because the survival of PSBs was influenced by thiacloprid in our field and laboratory experiments. However, the number of dead pine trees caused by PWN at the end of field study showed no differences between the thiacloprid-sprayed and unsprayed plots. There are some possible explanations to understand the occurrence of dead trees caused by PWN in the thiacloprid sprayed area. First, different thiacloprid residues were found according to the spraying time and sampled heights. The difference in the spraying time is thought to be caused by various factors of spraying, such as variation of climate conditions. In particular, rainfall, temperature, and wind when aerial spraying was conducted may be, in part, a reason for the occurrence of dead pine trees in aerial sprayed plots. Second, frequency of spraying may be another factor in the efficacy of aerial spraying. In Korea, thiacloprid is repeatedly sprayed once every two weeks during spring and summer seasons. However, aerial spraying in this study was conducted once a month from June to August, and there was heavy rainfall after

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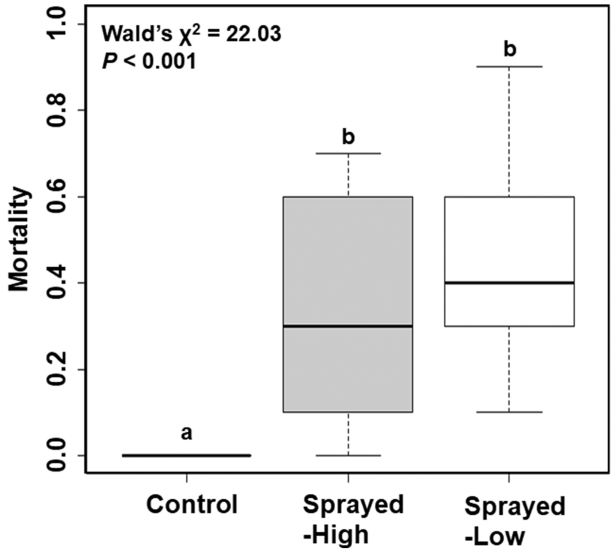

Figure 4 A comparison of the mortality of *Monochamus alternatus* that fed branches sampled from thiacloprid-sprayed (high and low) or unsprayed plots (control).

the second aerial spraying. Finally, the half-life of thiacloprid in soil is varied, ranging from 3.2 (Yu *et al.* 2007) to 27 days (Krohn 2001), which depends on soil types and environmental conditions. Therefore, efficacy of aerial spraying for reducing PWD in pine forests was limited in our study, and thus more case studies would be needed.

Effect of aerial spraying of thiacloprid on honey bees

Results of the study showed that no direct negative effect of thiacloprid on honey bees were observed, i.e., the mortality was observed ranging from 0.8 to 4.4%. In fact, thiacloprid exhibited relatively lower toxicity with LD_{50} values of 14.6 ug/bee compared to other insecticides (Iwasa *et al.* 2004). In addition, thiacloprid is reported as not dangerous to the honey bees unless they were fed (Iwasa *et al.* 2004; Maccagnani 2008). However, thiacloprid appeared to have sublethal effects on honey bees, such as reducing their foraging activity (Elbert *et al.* 2000) and resistance ability against pests and diseases (Vidau *et al.* 2011). These results indicated that honey bees may not be affected by aerial spraying directly, but there may be still a risk of exposure to toxicity when honey bees visit flowers in thiacloprid sprayed areas.

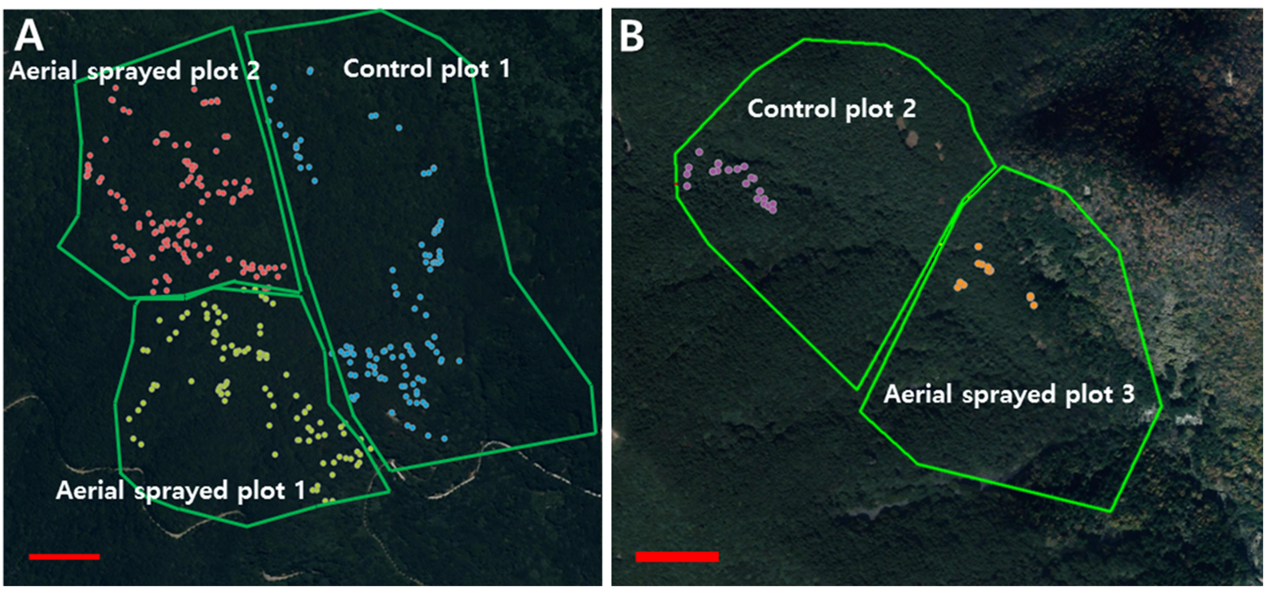

Figure 5 The location of the dead woods by PWD in October, 2016 (A, Mt. Geumjeong; B, Mt. Obong). Each point indicated the location of dead wood in the test area (scale bar = 100 m).

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Implication of aerial spraying method for protection of pine forests

This study suggested that aerial spraying of thiacloprid can be used as a chemical control method to reduce PSB population without the lethal effect of honey bees, although efficacy for the prevention of PWD in healthy pine forests was still uncertain. To enhance the efficacy of aerial spraying using thiacloprid on economically serious pests of pine forests, however, improving the quality control of aerial spraying methods, such as timing and frequency of spraying, would thus be of importance, given climate conditions during the summer season in Korea. This is due to the fact that aerosol type thiacloprid can drift by wind, and thiacloprid on leaves and branches of pines can be washed out by rains. In addition, environmental risk assessment for honey bees as well as other beneficial insects, such as solitary bees and predatory arthropods, would be necessary to improve ecosystem services.

Acknowledgments

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