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Egg Deposition of *Micromelalopha sieversi* (Staudinger) on Clones of *Populus* from Section *Aigeiros* Induces Resistance in Neighboring Plants

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Abstract: *Research Highlights*: We demonstrated that the resistance mechanisms of plants could be used to combat damage caused by pests in forestry plantations. *Background and Objectives*: Poplar is the main tree species used in plantations in northern China, with *Micromelalopha sieversi* (Staudinger) representing a major pest species causing defoliation. Here, we investigated whether two poplar clones could resist this pest species and the physiological mechanisms involved. *Materials and Methods*: Two clones of *Populus* from section *Aigeiros* were used, with '108' (*P.* × *euramericana* 'Guariento') being more attractive to *M. sieversi* than '111' (*P.* × *euramericana* 'Bellotto'). Three treatments were set up (oviposited plants, neighboring plants, and control plants) to determine whether resistance was induced in plants neighboring oviposited plants. *Results*: Significantly fewer eggs were oviposited on neighboring plants compared to control plants for both clones, with more eggs being laid on oviposited and control plants of '108' compared to '111'. β-Pinene was detected in oviposited and neighboring plants, but not control plants for either clone. Significantly higher concentrations of 3-carene was present in oviposited and neighboring plants of '108' and '111' compared to control plants at 24, 48, and 72 h after oviposition. Males, females, and mated females primarily responded to electroantennogram (EAG), methyl palmitate and 2-ethylhexyl acrylate at 50 ng/μL, and to 3-carene and β-pinene at 5 ng/μL, and to styrene at 10 ng/μL in EAG assays. When using these concentrations on plant leaves, 3-carene, β-pinene, and styrene significantly reduced the number of eggs laid on '108', while 3-carene and β-pinene were effective for '111'. *Conclusions*: Plants neighboring oviposited plants exhibited defense responses; 3-carene and β-pinene were used to transmit chemical signals (volatile cues) from oviposited plants to neighboring plants; which induced neighboring plants released volatiles as a defense mechanism to prevent egg laying.

Keywords: *Populus* × *euramericana* clones; *Micromelalopha sieversi* (Staudinger); oviposition; induced resistance; plant volatiles

1. Introduction

Plants are able to detect and respond to oviposition by insects by activating direct and indirect defense responses [1]. Many studies have demonstrated a variety of responses of plants to the early stages of insect attack [2]. Induced direct and indirect plant defenses in response to egg deposition have been determined. Egg deposition may induce the formation of a neoplasm that elevates eggs from the plant surface, so the exposed eggs easily drop off the leaves [3]. Leaves with eggs may form necrotic tissue to remove eggs and reduce the survival of hatching larvae [4,5]. Rice plants even produce an

ovicidal substance that kills the eggs of *Sogatella furcifera* (Horváth) [6–8]. Furthermore, cabbage leaves with the eggs of *Pieris brassicae* L. produce oviposition deterrents [9].

In response to herbivory, plants emit volatiles that may be quantitatively and qualitatively different from those emitted by intact plants [10]. Information-mediated indirect resistance might increase by attracting predatory and parasitoid organisms [11–15]. For instance, egg deposition by *Diprion pini* induces a significant increase in the amount of (E)-β-farnesene from *Pinus sylvestris*, attracting the egg parasitoid *Chrysonotomyia ruforum* [16]. This induced response is not strictly limited to the site of egg deposition, but egg-free leaves adjacent to the site of oviposition also release volatiles that are attractive to egg parasitoids, showing systematic induced resistance [15,17,18].

Phytophagous insects are able to identify chemical signals emitted by host plants and locate them. As long as there is a smell of potato leaves, potato beetles exhibit host directed behavior [19]. The odor from withering black poplar (*Populus nigra* L.) branches affect how sites are selected for hiding and oviposition, mating rates, and the fecundity of female moths of the cotton bollworm (*Helicoverpa armigera* Hübner) [20]. The monoterpene profiles of defoliated and undefoliated lodgepole pines (*Pinus contorta* Douglas) are associated with the oviposition preferences of *Panolis flammea* (D&S) [21]. The females of *Pyrrhalta maculicollis* Mots. preferentially lay eggs on uninfected leaves compared to heavily infected leaves due to changes in plant volatiles [22]. Following mating, female *Trichoplusia ni* (Hubner) moths primarily oviposit on undamaged cotton plants (*Gossypium hirsutum* L.) and cabbage plants (*Brassica oleracea* L.) [23].

The air transfer experiment is used to confirm the role of volatile cues in communication between plants [24]. Volatiles are released within minutes in response to tissue damage that induces the undamaged parts of damaged plant and neighboring plants [25]. Undamaged leaves of hybrid poplar (*Populus deltoides* × *nigra*) exposed to volatiles from herbivore-wounded leaves on the same stem exhibit elevated defensive responses to feeding by *Lymantria dispar* L. [26]. The systemic defenses of blueberry (*Vaccinium corymbosum* L.) are activated on undamaged branches when damaged branches release volatiles in response to damage by herbivores [27]. Some plants rely on volatile cues that are active over relatively short distances and might be subject to eavesdropping by other plants [28]. Conveyance of chemical information between damaged and undamaged plants has been detected [29]. For example, wild tobacco (*Nicotiana attenuata*) becomes more resistant to herbivores when grown in close proximity to clipped sagebrush (*Artemisia tridentata*) neighbors, with communication being airborne rather than soilborne [30]. Pairs of sagebrush (*A. tridentata*) plants that were grown up to 60 cm apart were influenced by the experimental clipping of a neighbor [31].

Thus, there is evidence that plants with eggs, including egg-laden leaves and egg-free leaves, activate direct and indirect defenses. However, there is limited information on the defenses of neighboring plants induced by oviposited plants. Here, we investigated the response of two clones of *Populus* from section *Aigeiros* to the eggs deposition of *M. sieversi* (Staudinger), including oviposited plants and nearby plants, and we examined whether the plants neighboring to oviposited plants can produce induced resistance and the mechanism of induced resistance. '108' (*Populus* × *euramericana* 'Guariento') is more susceptible than '111' (*P.* × *euramericana* 'Bellotto') to *M. sieversi* (Staudinger) [32].

2. Materials and Methods

2.1. Plants and Insects

In 2017, branches of '108' (*Populus* × euramericana 'Guariento') and '111' (*P.* × *euramericana* 'Bellotto') were cut from the forest farm in Daxing, Beijing. Each branch was cut into small segments (about 12–13 cm) with 3–4 buds, and was cultivated outside for 3–10 month in separate plastic pots filled with soil containing nutrients (Figure 1A). Plants of 4–5-month-old, with more than 20 leaves (Figure 1B), were transferred to the laboratory (26 ± 2 °C, 50% ± 5% relative humidity (RH), 16:8 h light:dark (L:D)) and were used for the experiments. Pupae of *M. sieversi* (Staudinger) were collected

in the field. Each pupae was placed separately in an incubator (28 ± 1 °C, 50 ± 1% RH, 16:8 h L:D) to pupate.

(**A**) (**B**)

Figure 1. (**A**) Seedlings from cuttings of '108' and '111'; (**B**) the seedling with leaves.

2.2. Oviposition Bioassay I

The experiments were carried out in two laboratories with the same natural conditions. There were three treatments: the oviposited plants, the plants neighboring oviposited plants, and the control plants. Two insect cages (150 cm × 75 cm × 75 cm) for four oviposited plants and four neighboring plants were set up in a laboratory, with a distance of about 50 cm between the plants (inside and between cages), Another insect cage was set up with four control plants in another laboratory. Twenty pairs of newly hatched male and female moths were allowed to mate freely and lay eggs in the cage for oviposited plants. After egg masses appeared on oviposited plants, 20 pairs of male and female moths were placed in the cages of neighboring and control plants. The number of eggs on the plants of the three treatments was counted at 24 h, 48 h and, 72 h after oviposition. '108' and '111' were tested separately, and the experiment was repeated four times. After analyzing how the positioning (1, 2, 3, 4) of oviposited plants, neighboring plants, and control plants affected the number of eggs in the same cage using the Kruskal-Wallis test of non-parametric test in SPSS 19.0 (IBM, New York, NY, USA), respectively, the changes of the number of eggs at three time periods were statistically analyzed. Finally, the sum number of eggs on the four plants in each cage at 72 h after oviposition was used for the statistical analysis.

2.3. Volatile Collection

Volatile compounds were collected from whole poplar plants, with and without the eggs of *M. sieversi*, using dynamic headspace sampling [33]. Before collecting volatiles, plants for oviposition were kept overnight inside cages with 20 pairs of female and male moths. Volatiles were collected for 8 h from oviposited plants, neighboring plants, and control plants at 24 h, 48 h, and 72 h after eggs were laid on oviposited plants. The upper eight to 10 leaves of plants with and without eggs were enclosed in sample bags (Ziploc, 25.0 cm × 32.5 cm) and the volatiles were collected using the atmospheric sampling instrument (QC-1S, Institute of Labor Protection Science, Beijing, China). The inlet port was connected to the sample tube (Chrompack, 0.6 mm × 16 mm, Porapak Q, 200 mg) by a silicone pipe. The outlet port was directly connected to the silicone tube. The sample tube and the silicone tube were connected to the outlet port and were sealed in the sample bag. Velocity of air flow was 500 mL·min^{-1}. After entrainment, volatiles were eluted with 4 mL hexane and were concentrated to 0.5 mL using N_2.

2.4. Coupled Gas Chromatography-Mass Spectrometry (GC-MS)

The concentrated extracts of the volatile substances were analyzed on GC-MS (Trace DSQ, Thermo, Boston, MA, USA) with a capillary column (DB-5, 30 m length, 0.25 mm i.d. (inner diameter), 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, USA). Ionization was performed by electron impact (70 eV, 230 °C). The oven temperature was maintained at 40 °C for 1 min, and then programmed at 4 °C·min^{-1} to 200 °C, where it was held for 5 min. The software (Xcalibur Data System, Ver.1.4 (Thermo, Boston, MA, USA)) began to collect data after 3 min into sample processing. The total scanning mass range was 41–650 amu (atomic mass unit), scanning five times per second. Compounds were identified by comparing the retention indices and mass spectra with those of authentic standards. We analyzed the relative contents of the main component over three time periods, and the relative contents of five terpenes and esters were compared in detail. The samples of five volatile compounds were tested by the following electroantennogram analyses.

2.5. Electroantennogram (EAG) Recording

EAG analyses were conducted as described by Kong et al. [33]. In brief, the metal electrodes were connected to the antennae of a male/female/mated female of *M. sieversi* with conductive adhesive, and the metal electrodes with the antennae were suspended at 0.5 cm in the mouth of the "L" glass tube, in a continuous and humid air flow (900 mL min^{-1}). The Pasteur tube that contained the filter paper with the sample was aimed at the small hole of the "L" glass tube, and the volatiles were blown into the glass tube with N_2. A piece of filter paper (6 cm × 0.5 cm) with 10 μL samples of different concentrations was placed in the Pasteur tube. The concentration of samples was 1 ng/ μL, 5 ng/ μL, 10 ng/μL, 50 ng/μL, and 100 ng/μL. The five concentrations of each sample were grouped together. Each group was controlled by 10 μL hexane. The five concentrations of samples were analyzed in the order of low to high. The stimulus time was 0.2 s, and the interval of two stimuli was 40 s. After three samples tests, one control was tested, and each concentration analyzed three times. The difference between the electroantennogram response value and the control mean value was the absolute value of the EAG test. The average of three absolute values of each concentration was analyzed three times before being compared. Males, females, and mated females were tested in the same way.

2.6. Volatile Treatment and Oviposition Bioassay II

The second egg-laying test was carried out using volatile compounds on the plants. Five samples were dissolved in 75% ethanol, and the concentrations were the maximum concentration of the active EAG for each chemical. The solution of each compound (10 μL) was dropped on the leaves of each plant, and 10 μL of 75% ethanol solution was applied to the control plants. The six insect cages containing the plants treated with five volatile compounds and control plants were placed in separate laboratories. Four plants were placed in each cage, 20 pairs of newly hatched male and female moths were allowed to mate freely and lay eggs in each cage. The sum number of eggs on the four plants in each cage at 72 h after oviposition was used for the statistical analysis. '108' and '111' were processed separately, and the experiment was repeated four times.

3. Results

3.1. Differences in Oviposition among Oviposited Plants, Neighboring Plants, and Control Plants

The results showed that there was no significant difference in the number of eggs on oviposited plants ($p = 0.936$), neighboring plants ($p = 0.755$), and control plants ($p = 0.637$) at different positions in each cage on '108'. There was also no significant difference in the number of eggs on oviposited plants ($p = 0.985$), neighboring plants ($p = 0.798$), and control plants ($p = 0.839$) at different positions in each cage on '111'. Therefore, for further statistical analysis, the four plants groups in each cage were considered as a replicate. The results showed that there was no significant difference in the number of eggs on oviposited plants ($F_{(2,9)} = 0.000$, $p = 1.000$), neighboring plants ($F_{(2,9)} = 0.010$, $p = 0.990$), and

control plants ($F_{(2,9)}$ = 0.000, p = 1.000) of '108' at 24 h, 48 h, and 72 h after oviposition (Figure 2A). There was also no significant difference in the number of eggs on oviposited plants ($F_{(2,9)}$ = 0.002, p = 0.998), neighboring plants ($F_{(2,9)}$ = 0.010, p = 0.990), and control plants ($F_{(2,9)}$ = 0.007, p = 0.993) of '111' at 24 h, 48 h, and 72 h after oviposition (Figure 2B). Therefore, the number of eggs on the three treated plants was counted at 72 h after oviposition. The number of eggs on the neighboring plants of both '108' ($F_{(2,9)}$ = 6.303, p = 0.019) and '111' ($F_{(2,9)}$ = 14.425, p = 0.002) was significantly lower than that on oviposited plants and control plants in the first bioassay (Figure 3A). The number of eggs on the oviposited (p = 0.048, n = 4) and control (p = 0.033, n = 4) plants of '108' was significantly higher than that on '111' (Figure 3B).

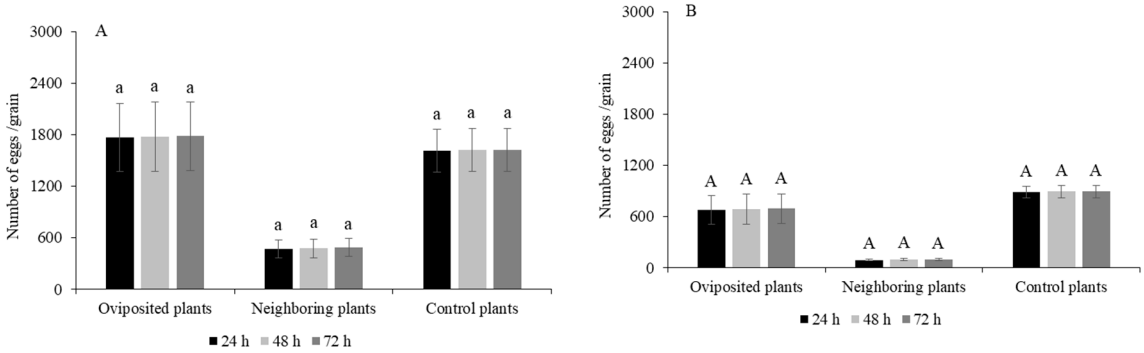

Figure 2. (**A**) The number of eggs on oviposited plants, neighboring plants, and control plants of '108' at 24 h, 48 h, and 72 h after oviposition, respectively. ANOVA was used to compare the numbers. Multiple comparisons and significant means were separated using the Tukey HSD (Honestly Significant Difference) test. Note: different lowercase letters indicate a significant difference between the three time periods of each treatment (p < 0.05). (**B**) Number of eggs on oviposited plants, neighboring plants, and control plants of '111' at 24 h, 48 h, and 72 h after oviposition, respectively. ANOVA was used to compare the numbers. Multiple comparisons and significant means were separated using the Tukey HSD test. Note: different uppercase letters indicate a significant difference between the three time periods of each treatment (p < 0.05).

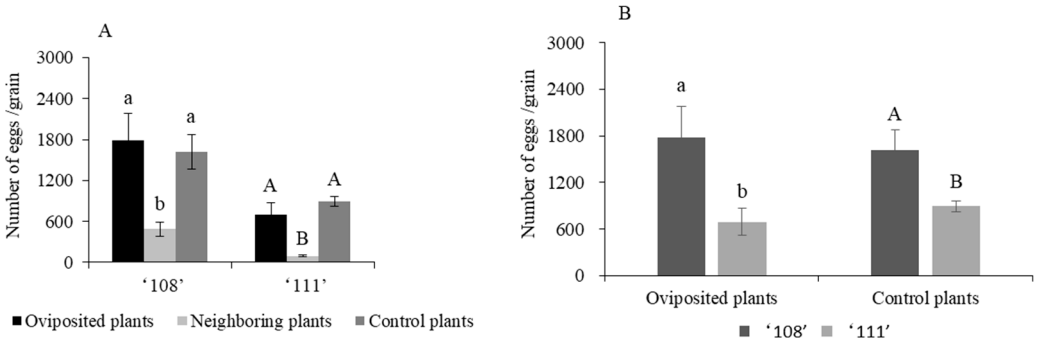

Figure 3. (**A**) The number of eggs on oviposited, neighboring, and control plants of '108' and '111'. ANOVA was used to compare the numbers. Multiple comparisons and significant means were separated using the Tukey HSD test. Note: different lowercase letters indicate a significant difference between three treatments of '108' (p < 0.05), and different uppercase letters indicate a significant difference between three treatments of '111' (p < 0.05). (**B**) Number of eggs on the oviposited or control plants of '108' and '111' was compared using independent-samples T test. Note: different lowercase letters indicate a significant difference between oviposited plants of '108' and '111' (p < 0.05), and different uppercase letters indicate a significant difference between control plants of '108' and '111' (p < 0.05).

3.2. Comparison of Volatiles Emitted from Oviposited Plants, Neighboring Plants, and Control Plants

Coupled gas chromatography-mass spectrometry revealed that there were differences in the relative content of the main volatile components in three treatments of '108' and '111'. Ten volatile components were detected in the processing of most '108' samples (Table 1). p-Xylene, styrene, 3-carene, 2-ethylhexyl acrylate, 2,6,10-trimethyl tetradecane, $C_{26}H_{42}O_4$ (unknown), and methyl palmitate were detected in every processed sample. β-Pinene was detected in oviposited and neighboring plants but was not detected in control plants. 3-carene was significantly higher in oviposited and neighboring plants than in control plants at 24 h, 48 h, and 72 h. The relative content of styrene and 2-ethylhexyl acrylate in oviposited plants was significantly higher than that in control plants at 24 h and 48 h, while the content of these compounds was significantly lower than that in control plants at 72 h. Methyl palmitate was significantly lower in oviposited plants than in control plants at 48 h. Styrene was significantly lower in neighboring plants than in control plants at 48 h and 72 h. 2-Ethylhexyl acrylate was significantly lower in neighboring plants than in control plants at 24 h and 48 h. Methyl palmitate was significantly lower in neighboring plants than in control plants at 24 h, 48 h, and 72 h. In addition, 1,3-diethylbenzene and 1,4-diethylbenzene both only had a high relative content in neighboring plants at three time periods.

Eight volatile components were detected in most of the processed '111' samples (Table 2). p-Xylene, styrene, 3-carene, 2-ethylhexyl acrylate, 2,6,10-trimethyl tetradecane, $C_{26}H_{42}O_4$ (unknown), and methyl palmitate were detected in every process. β-Pinene was detected in oviposited and neighboring plants, but was not detected in control plants, similar to '108'. The relative content of 3-carene in oviposited and neighboring plants was significantly higher than that in control plants at 24 h, 48 h, and 72 h, similar to '108'. The relative content of styrene in oviposited plants was significantly lower than that in control plants at 48 h and 72 h. 2-Ethylhexyl acrylate was significantly higher in oviposited than in control plants at 24 h. Methyl palmitate was significantly higher in oviposited plants than in control plants at 48 h and 72 h, while it was significantly lower at 24 h. The relative content of styrene in neighboring plants was significantly lower than that of the control at 24 h and 72 h. 2-Ethylhexyl acrylate was significantly lower in neighboring plants than in the control at 24 h and 48 h. Methyl palmitate was significantly higher in neighboring plants than in the control at 48 h.

Table 1. Main volatile components of oviposited plants, neighboring plants, and control plants after 24 h, 48 h, and 72 h of '108'. The relative content of the same chemical components of three treatments in each time period was compared by ANOVA. Multiple comparisons and significant means were separated using the Tukey HSD test. Note: Different lowercase letters in the same line show significant differences between the three treatments in the same time period ($p < 0.05$).

Compound Composition of '108'	Oviposited-24 h	Neighboring-24 h	Control-24 h	Oviposited-48 h	Neighboring-48 h	Control-48 h	Oviposited-72 h	Neighboring-72 h	Control-72 h
	Relative Content (%)								
p-Xylene	3.79 ± 0.08 a	1.32 ± 0.06 b	1.05 ± 0.09 b	4.45 ± 0.19 a	1.26 ± 0.08 b	0.85 ± 0.04 b	2.21 ± 0.06 b	3.41 ± 0.19 a	1.81 ± 0.12 b
Styrene	8.08 ± 0.28 a	2.70 ± 0.46 b	3.19 ± 0.10 b	8.46 ± 0.06 a	2.70 ± 0.12 c	5.79 ± 0.13 b	2.77 ± 0.04 b	3.98 ± 0.22 b	6.09 ± 0.62 a
3-Carene	9.65 ± 0.58 a	4.63 ± 0.52 b	1.26 ± 0.17 c	14.22 ± 0.77 a	4.29 ± 0.10 b	1.85 ± 0.09 c	6.82 ± 0.12 b	9.26 ± 0.72 a	1.51 ± 0.06 c
β-Pinene	2.17 ± 0.12 a	0.42 ± 0.03 b	0.00 ± 0.00 c	1.63 ± 0.13 a	0.28 ± 0.01 b	0.00 ± 0.00 c	2.22 ± 0.07 a	2.49 ± 0.12 a	0.00 ± 0.00 b
1,3-Diethylbenzene	0.00 ± 0.00 b	17.44 ± 1.20 a	0.00 ± 0.00 b	0.00 ± 0.00 b	21.53 ± 0.75 a	0.00 ± 0.00 b	0.00 ± 0.00 b	18.12 ± 1.05 a	0.00 ± 0.00 b
1,4-Diethylbenzene	0.00 ± 0.00 b	22.35 ± 1.11 a	0.00 ± 0.00 b	0.00 ± 0.00 b	28.15 ± 1.54 a	0.00 ± 0.00 b	0.00 ± 0.00 b	12.03 ± 1.27 a	0.00 ± 0.00 b
2-Ethylhexyl acrylate	2.21 ± 0.06 a	0.55 ± 0.01 c	1.45 ± 0.03 b	5.73 ± 0.08 a	0.82 ± 0.03 c	1.78 ± 0.35 b	0.83 ± 0.08 b	1.74 ± 0.05 a	1.86 ± 0.10 a
2,6,10-Trimethyl tetradecane	5.26 ± 0.04 b	1.99 ± 0.16 c	12.80 ± 0.81 a	2.55 ± 0.12 b	1.33 ± 0.10 b	12.36 ± 0.79 a	1.36 ± 0.09 b	1.83 ± 0.08 b	13.33 ± 0.71 a
$C_{26}H_{42}O_4$ (Unknown)	6.75 ± 0.23 a	3.73 ± 0.19 b	6.63 ± 0.17 a	4.46 ± 0.14 b	2.80 ± 0.23 c	6.09 ± 0.12 a	3.85 ± 0.21 b	2.29 ± 0.13 c	6.81 ± 0.44 a
Methyl palmitate	61.71 ± 0.88 a	38.61 ± 1.97 b	67.61 ± 1.67 a	54.47 ± 0.42 b	28.38 ± 0.13 c	67.84 ± 1.42 a	69.90 ± 2.83 a	26.67 ± 1.54 b	63.32 ± 1.80 a

Table 2. Main volatile components of oviposited plants, neighboring plants, and control plants at 24 h, 48 h, and 72 h for '111'. ANOVA was used to compare the relative content of the same chemical components of the three treatments in each time period. Multiple comparisons and significant means were separated using the Tukey HSD test. Note: Different lowercase letters on the same line show significant differences between the three treatments in the same time period ($p < 0.05$).

Composition Compound of '111'	Oviposited-24 h	Neighboring-24 h	Control-24 h	Oviposited-48 h	Neighboring-48 h	Control-48 h	Oviposited-72 h	Neighboring-72 h	Control-72 h
	Relative Content (%)								
p-Xylene	8.90 ± 0.81 a	4.05 ± 0.10 b	2.05 ± 0.10 c	2.85 ± 0.09 b	3.61 ± 0.11 a	3.46 ± 0.07 a	3.32 ± 0.06 b	3.98 ± 0.18 a	2.07 ± 0.15 c
Styrene	15.52 ± 1.34 a	9.75 ± 0.64 b	17.82 ± 1.04 a	4.62 ± 0.18 b	7.82 ± 0.18 a	8.23 ± 0.25 a	3.15 ± 0.03 c	6.21 ± 0.22 b	8.31 ± 0.41 a
3-Carene	20.46 ± 1.13 a	12.53 ± 0.71 b	5.53 ± 0.17 c	7.97 ± 0.21 b	10.79 ± 0.12 a	5.14 ± 0.02 c	7.33 ± 0.06 b	9.82 ± 0.65 a	4.40 ± 0.06 c
β-Pinene	1.65 ± 0.09 a	0.18 ± 0.01 b	0.00 ± 0.00 c	0.36 ± 0.01 b	1.09 ± 0.04 a	0.00 ± 0.00 c	1.34 ± 0.07 a	0.94 ± 0.08 b	0.00 ± 0.00 c
2-Ethylhexyl acrylate	5.72 ± 0.18 a	3.55 ± 0.09 c	4.78 ± 0.09 b	7.77 ± 0.27 a	4.06 ± 0.03 b	8.85 ± 0.78 a	4.99 ± 0.14 a	6.54 ± 0.38 a	7.41 ± 0.88 a
2,6,10-Trimethyl tetradecane	1.20 ± 0.09 b	2.13 ± 0.04 a	2.00 ± 0.19 a	2.06 ± 0.02 b	2.23 ± 0.12 b	3.59 ± 0.21 a	1.14 ± 0.03 b	1.67 ± 0.10 b	3.52 ± 0.07 a
$C_{26}H_{42}O_4$ (Unknown)	2.63 ± 0.06 b	5.55 ± 0.08 a	5.77 ± 0.13 a	4.90 ± 0.17 b	5.28 ± 0.06 b	7.96 ± 0.18 a	3.18 ± 0.10 b	3.17 ± 0.10 b	6.97 ± 0.27 a
Methyl palmitate	41.52 ± 1.42 b	59.84 ± 0.23 a	59.66 ± 1.41 a	64.17 ± 1.25 a	63.48 ± 0.85 a	55.87 ± 0.50 b	72.95 ± 0.98 a	60.26 ± 1.18 b	59.49 ± 1.66 b

3.3. Electroantennogram (EAG) Response of the Males, Females, and Mated Females to the Five Volatile Compounds

There was a difference in the active EAG of males, females, and mated females with respect to the five compounds at different concentrations, including methyl palmitate, 3-carene, β-pinene, styrene, and 2-ethylhexyl acrylate. The EAG reaction of males to methyl palmitate ($F_{(4,10)}$ = 22.644, p = 0.000) and 2-ethylhexyl acrylate ($F_{(4,10)}$ = 6.990, p = 0.006) was greatest at 50 ng/μL. The EAG reaction of males to 3-carene ($F_{(4,10)}$ = 57.584, p = 0.000) and β-pinene ($F_{(4,10)}$ = 13.699, p = 0.000) at 5 ng/μL was significantly greater than that at other concentrations. The EAG reaction of males to styrene at 10 ng/μL was significantly greater than that at other concentrations ($F_{(4,10)}$ = 20.726, p = 0.000) (Figure 4).

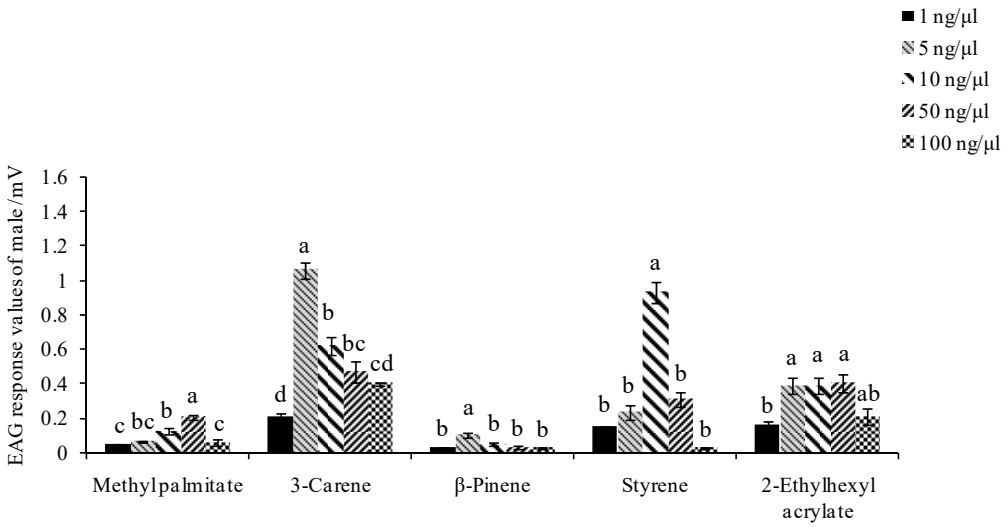

Figure 4. Electroantennogram responses of *Micromelalopha sieversi* males to different concentrations of the five volatile compounds. The concentration of the five compounds was 1 ng/μL, 5 ng/μL, 10 ng/μL, 50 ng/μL, and 100 ng/μL. ANOVA was used to compare the averages of three absolute values of each concentration for each chemical composition. Multiple comparisons and significant means were separated using the Tukey HSD test.

The EAG reaction of females to methyl palmitate (F(4,10) = 36.004, p = 0.000) and 2-ethylhexyl acrylate(F(4,10) = 10.763, p = 0.001) was greatest at 50 ng/μL. Females exhibited the largest response to 3-carene (F(4,10) = 53.307, p = 0.000) and β-pinene (F(4,10) = 3.769, p = 0.040) at 5 ng/μL respectively. Females exhibited the greatest response to styrene (F(4,10) = 25.381, p = 0.000) at 10 ng/μL (Figure 5).

There was no significant difference in the response of mated females to the EAG with different concentrations of styrene ($F_{(4,10)}$ = 1.911, p = 0.185), and the response was greatest at 10 ng/μL. The EAG reaction of mated females to methyl palmitate ($F_{(4,10)}$ = 13.968, p = 0.000) and 2-ethylhexyl acrylate ($F_{(4,10)}$ = 38.743, p = 0.000) was greatest at 50 ng/μL. The EAG reaction of mated females to 3-carene ($F_{(4,10)}$ = 55.409, p = 0.000) and β-pinene ($F_{(4,10)}$ = 110.77, p = 0.000) was greatest at 5 ng/μL, and was significantly greater than that of the other concentrations (Figure 6).

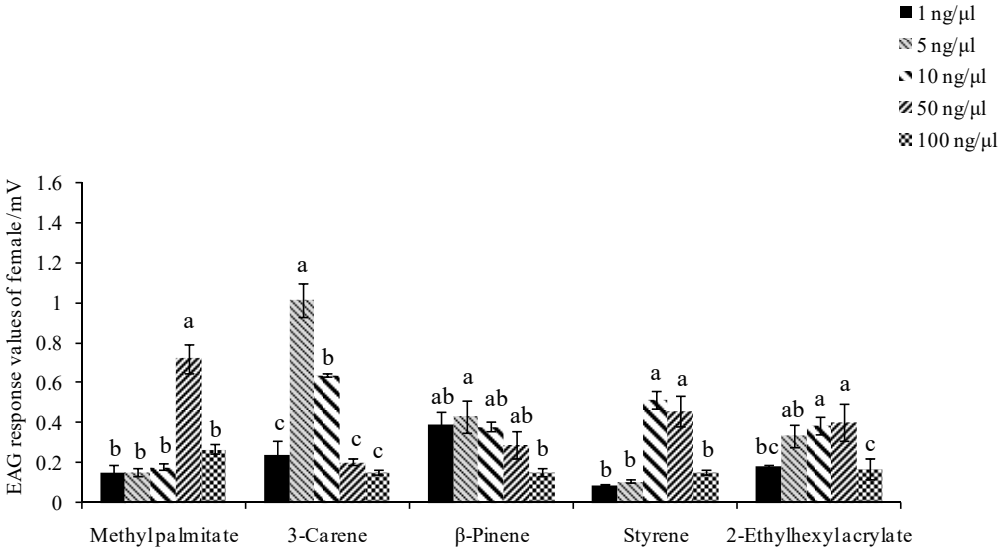

Figure 5. Electroantennogram responses of *Micromelalopha sieversi* females to different concentrations of the five volatile compounds. The concentration of the five compounds was 1 ng/µL, 5 ng/µL, 10 ng/µL, 50 ng/µL, and 100 ng/µL, ANOVA was used to compare the average of three absolute values of each concentration for each chemical composition. Multiple comparisons and significant means were separated using the Tukey HSD test.

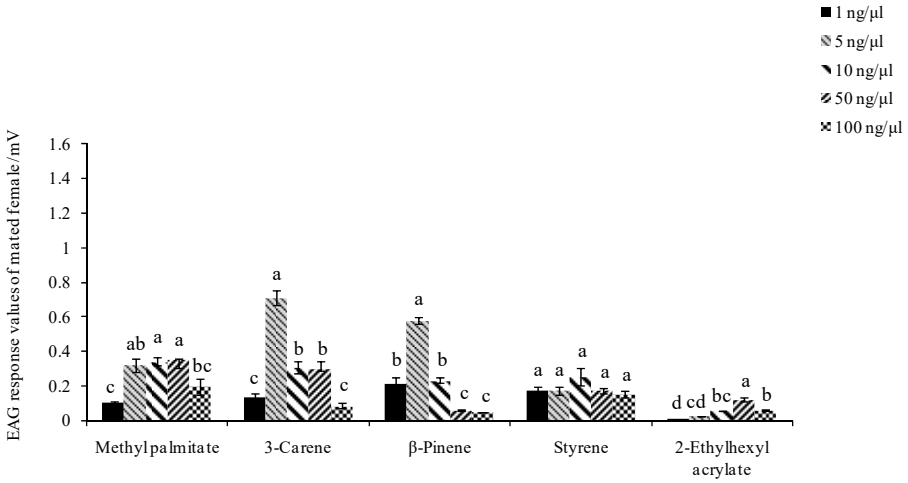

Figure 6. Electroantennogram responses of mated *Micromelalopha sieversi* females to different concentrations of the five volatile compounds. The concentration of the five compounds was 1 ng/µL, 5 ng/µL, 10 ng/µL, 50 ng/µL, and 100 ng/µL. ANOVA was used to compare the average of three absolute values of each concentration for each chemical composition. Multiple comparisons and significant means were separated using the Tukey HSD test.

The males, females, and mated females exhibited the greatest response to the EAG at 50 ng/µL of methyl palmitate and 2-ethylhexyl acrylate, 5 ng/µL of 3-carene and β-pinene, and 10 ng/µL of styrene, respectively.

3.4. Differences in Oviposition on the Plants Treated with Volatile Compounds

There was a difference between the number of eggs from the treated plants and control plants. The number of eggs on '108' plants treated with 3-carene, β-pinene, and styrene was significantly lower than that of the controls ($F_{(5,18)} = 5.496$, $p = 0.003$). There was no significant difference between the controls and plants treated with methyl palmitate, 2-ethylhexyl acrylate. '108' plants treated

with 3-carene had significantly fewer eggs than plants treated with methyl palmitate. There was no significant difference in the number of eggs laid on plants treated with β-pinene, styrene, and 2-ethylhexyl acrylate. The number of eggs on '111' plants treated with 3-carene and β-pinene was significantly lower than that of the controls ($F_{(5,18)} = 4.823$, $p = 0.006$). There was no significant difference in the number of eggs on the controls versus plants treated with methyl palmitate, 2-ethylhexyl acrylate and styrene. Plants treated with 3-carene and β-pinene had significantly fewer eggs than that treated with methyl palmitate (Figure 7).

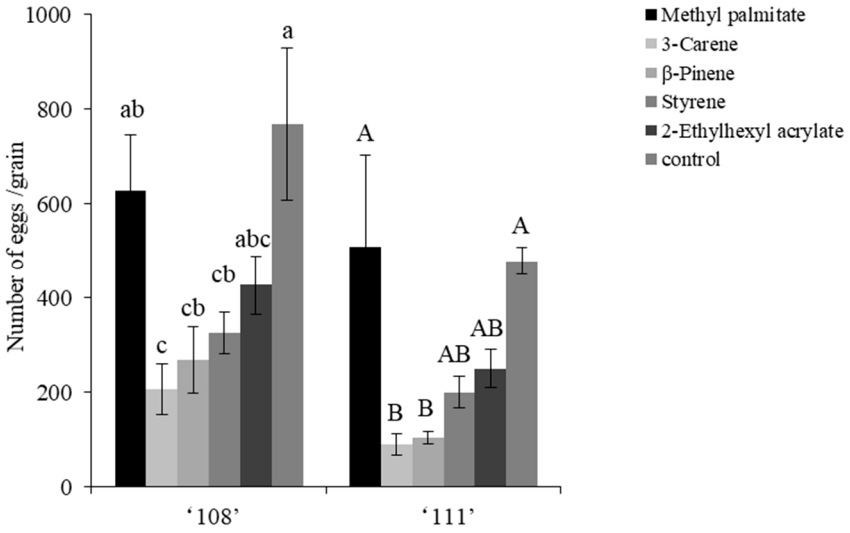

Figure 7. Number of eggs on '108' and '111' plants treated with the five compounds. The compounds were methyl palmitate, 3-carene, β-pinene, styrene, and 2-ethylhexyl acrylate. ANOVA was used to compare the number of eggs. Multiple comparisons and significant means were separated using the Tukey HSD test. Note: Different lowercase letters indicate a significant difference between six treatments on '108' ($p < 0.05$); different uppercase letters indicate a significant difference between six treatments on '111' ($p < 0.05$).

4. Discussion

The present study demonstrated that neighboring plants had significantly fewer eggs than control plants. These results indicate that neighboring plants of two clones of *Populus* from section *Aigeiros* were probably induced to change the relative content and composition of volatile compounds that resist oviposition by *M. sieversi*, when that of oviposited plants changed. Some plants rely on volatile cues that are active over relatively short distances and might be subject to eavesdropping by other plants [28]. For example, wild tobacco (*N. attenuata*) became more resistant to herbivores when grown in close proximity to clipped sagebrush (*A. tridentata*) neighbors within 15 cm [30]. Furthermore, in another study, pairs of sagebrush (*A. tridentata*) plants that were up to 60 cm apart were influenced by the experimental clipping of their conspecific neighbor [31]. However, few studies have evaluated egg deposition induced resistance of conspecific neighboring plants. In this experiment, the two areas of oviposited plants and neighboring plants were about 50 cm apart. The neighboring plants clearly produced induced resistance. Whether greater distances between the two areas would produce induced resistance, along with the interaction between the plants in same area requires further verification. In addition, once egg masses appeared on '108' and '111' clones, the number of eggs basically remained unchanged until the end of the egg incubation period. Previous research demonstrated that egg-free leaves neighboring those laden with eggs on the same plant show systemically induced resistance [15,17,18]. The results showed that the oviposited plants of two *P. nigra* clones produced systemic induced resistance.

We recorded a difference in the composition and relative content of the volatiles collected from oviposited plants, neighboring plants, and control plants of '108' and '111' at 24 h, 48 h, and 72 h after egg-laying. The quantity and quality of terpenoid volatiles on bean plants [14], elm trees [13], and pine needles [16] changes with the presence of egg masses. In this study, β-pinene and 3-carene caused a significant reduction in the amount of eggs from *M. sieversi* (Staudinger). Thus, this compound had a clear repellent effect on adults. In a previous study, β-pinene and 3-carene, which are volatile compounds from the flowers and leaves of *Rosa multiflora* var. *cathayensis*, showed some antibacterial activity [34]. 3-carene is often used as an active ingredient in repellents [35]. β-pinene and 3-carene deter the presence of *Monochamus alternatus* (Hope) adults on plants, which could noticeably reduce the number of grooves that the insect carves on the trunk [36]. 3- carene has a repellent effect on the females of *Dendrolimus superans*, while β-pinene induces both males and females at low levels but causes them to evade plants at high levels [37]. When the needles of *Pinus massoniana* Lamb release high concentrations of β-pinene, the females of *Dendrolimus punctatus* Walker lay significantly fewer eggs on the needles [38]. Our results confirmed these studies. In addition, the number of eggs laid on the '108' treated with styrene decreased significantly, indicating that it had some repellent effect on adults. Styrene is the main volatile component of *Salix ohsidare*, *Melia azedarach*, and *Acer negundo*, and might be an attractant to *Anoplophora glabripennis* [39]. Thus, styrene affects the behavior of different insects differently. Compared to the control, there was no significant difference in the number of eggs laid on '108' and '111' clones treated by methyl palmitate and 2-ethylhexyl acrylate. Thus, methyl palmitate and 2-ethylhexyl acrylate had no obvious attraction or repellent effect to *M. sieversi*. Methyl palmitate is a fatty acid methyl ester that is found in many plants and has bactericidal activity [40]. Methyl palmitate extracted from *Juglans regia* L. has strong acaricidal activity, with 10 mg/mL methyl palmitate having a 97.9% mortality rate on *Tetranychus cinnabarinus* (Boisduval) and only 57.2% on its eggs [41]. Insect eggs may be considered microbial pathogens at least at the molecular level [42]. Methyl palmitate increased or decreased in the oviposited and neighboring plants of '108' and '111'. Thus, the change of the relative content of methyl palmitate in two clones of *Populus* from section *Aigeiros* might play a role in plant response to pathogen but did not affect the oviposition behavior of *M. sieversi*. The EAG response and olfactory behavior of *Apriona germari* (Hope) were tested using 2-ethylhexyl acrylate from mulberry (*Morus alba* L.) [43] and *Juglans mandshurica* [44], both of which attract *A. germari* (Hope). In this experiment, it did not affect the oviposition behavior of *M. sieversi*. Thus, 2-ethylhexyl acrylate has different effects on the behavior of different insects.

Some studies suggest that there is no difference between the sexes in the EAG response to plant odors [45,46]. The EAG responses of females and males to 2-ethylhexyl acrylate in this experiment was consistent with this suggestion. In addition, males and females might react differently to the EAG response of the same compound. The EAGs of female moths to some volatiles are often about 2-fold greater than those observed for male moths [47,48]. In this study, the response of female and mated females to different concentrations of methyl palmitate and β-pinene confirmed this observation. Also, each sex exhibits stronger EAG responses to specific volatile components in insects. For example, the antennae of *Ceratitis capitata* Wied females respond more to dihydrocarvone, 4-ethyl acetophenone, and carvone than males. In comparison, the antennae of males respond more to limonene oxide than females [49]. Hexyl butyrate and (E)-2-hexenyl butyrate elicited greater EAGs in the males of *Lygus lineolaris* than females; females were more sensitive to the monoterpene geraniol than males [50]. In the current study, the EAG response of female to 50 ng/μL methyl palmitate was significantly greater than that of males, whereas the EAG response of males to 100 ng/μL 3-carene of was significantly greater than that of females. Whether a female was mated also affected the EAG response. The EAG responses of *Dioryctria abietivorella* females to monoterpenes generally increased with age and mating, and were greater than those of males of the same age [51]. In this study, mated females exhibited weaker responses to 3-carene, β-pinene, styrene, and 2-ethylhexyl acrylate than did males and unmated females; however, certain concentrations of methyl palmitate generated more responses in mated

females compared to males and unmated females. The results showed that both females and males might be affected by the volatiles of plants during their search for hosts.

At present, studies on the response of various organisms in the tritrophic system to induced-oviposition are mainly focused on synomones from oviposition-induced plant, which are semiochemical cues used by egg parasitoids during host selection. For example, the induction time for *Brassica oleracea*, *Murgantia histrionica*, and *Trissolcus brochymenae* was less than 24 h [52]. The egg parasitoid *Telenomus podisi* responds quickly to induced volatiles from soybean when damaged following oviposition by *Euschistus heros* [53]. *Oomyzus gallerucae* is a major egg parasitoid of the elm leaf beetle, and it responds to volatiles produced by elm leaves with eggs at 3 h after egg deposition by herbivores [12,17]. This experiment confirmed that plants are able to detect changes to volatile composition from conspecific plant in a short time. The eggs of *M. sieversi* usually require just three days to hatch after being laid. Our study detected a significant induction response between neighboring plants and oviposited plants within 24 h. The results showed that the neighboring plants of both two clones of *Populus* from section *Aigeiros* could detect changes to volatile composition from oviposited plants and quickly respond to these changes.

5. Conclusions

In this study, significantly fewer eggs were laid on plants neighboring oviposited plants. Thus, neighboring plants were induced to change the relative content and composition of volatile compounds to deter oviposition by *M. sieversi*. Resistance could be induced by changing the composition and relative content of volatiles in neighboring plants by these plants detecting changes to the volatiles of oviposited plants. β-pinene and 3-carene exhibited a clear repellent effect on the adults of *M. sieversi*, so the development of a relevant repellent is feasible. The study could provide new ideas for future pest resistance afforestation.

Author Contributions: L.G. and Z.Z. conceived and designed the experiments; L.G. and F.L. collected the pupae in the wild; L.G. performed the experiments and wrote the paper; S.Z. and X.K. conducted the data analysis.

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