forests

MDPI

Article

Nematicidal Properties and Chemical Composition of *Pinus rigida* Mill. Resin against Pinewood Nematodes

Hwan-Su Hwang, Yi-Re Kim, Jung-Yeon Han and Yong-Eui Choi *

Department of Forest Resources, College of Forest and Environmental Sciences, Kangwon National University, Chuncheon 200-701, Korea; whansu3368@hanmail.net (H.-S.H.); yiraekim@kangwon.ac.kr (Y.-R.K.); jyhan@kangwon.ac.kr (J.-Y.H.)

* Correspondence: yechoi@kangwon.ac.kr

Abstract: Pitch pine (*Pinus rigida* Mill.) is native to North America and has a strong resistance to pine wood nematodes (PWNs). The PWN resistance mechanism of this tree species has yet to be discovered. In this work, we found that the spreading of inoculated PWNs in the branch of *P. rigida* was significantly suppressed compared to those in the branches of *Pinus densiflora* (Sieb. et Zucc.) and *Pinus koraiensis* (Sieb. et Zucc.). Dipping of PWNs in the resins isolated from *P. rigida* significantly suppressed the PWN mobility and conferred significantly higher PWN mortality compared to those in the resins from *P. densiflora* and *P. koraiensis*. All PWNs dipped in *P. rigida* resin were killed after six days, but more than 50% of the PWNs dipped in the resin from *P. densiflora*, and *P. koraiensis* were still alive after six days. The phytochemical analysis of resins revealed that *P. rigida* resin contained little or no amount of sesquiterpenes compared to those from *P. densiflora* and *P. koraiensis*. However, *P. rigida* resin contained rich amounts of diterpenes, among which dehydroabietic aldehyde, methyl dehydroabietate, and methyl abietate were uniquely detected. Particularly, two pinosylvin stilbenes (*trans* and *cis*-3,5-dimethoxystilbene) were accumulated in *P. rigida* resin, which were not detected in the resins from *P. densiflora* and *P. koraiensis*. *cis*-3,5-Dimethoxystilbene showed high nematicidal activity but not in *trans*-3,5-dimethoxystilbene. Conclusively, PWN resistance of *P. rigida* may be due to the toxic chemicals in the resin, in which *cis*-3,5-dimethoxystilbene may contribute to PWN toxicity. This work is the first demonstration that resin from PWN-resistant *P. rigida* directly affected PWN mobility and mortality, probably due to toxic phytochemicals in the resin.

Keywords: *Pinus rigida*; pinewood nematode; resin; pinosylvin stilbene; abietane diterpene

Citation: Hwang, H.-S.; Kim, Y.-R.; Han, J.-Y.; Choi, Y.-E. Nematicidal Properties and Chemical Composition of *Pinus rigida* Mill. Resin against Pinewood Nematodes. *Forests* **2022**, *13*, 1131. https://doi.org/10.3390/f13071131

Academic Editor: Julio Javier Diez

Received: 14 June 2022
Accepted: 15 July 2022
Published: 18 July 2022

1. Introduction

Pinewood nematode (PWN; *Bursaphelenchus xylophilus*) severely damages various pine species worldwide [1]. The nematodes mainly move through cortical and xylem resin canals in the stem [2]. They proliferate by eating plant tissues such as epithelial cells and parenchyma cells in pine trees, which eventually leads to water deficiency (embolization) by blocking tracheids [3]. It is considered the main factor of pine wilt disease [4]. Several methods have been used to prevent pine wilt disease. The representative control methods for the disease are preventing an insect vector (*Monochamus* species) by spraying insecticides such as thiacloprid during the hatching season of the vector [5]. Another method is the injection of nematicides such as abamectin into pine trees [6,7]. However, the application of these control methods is not successful in preventing the spread of pine wilt disease (PWD) [8]. A breeding strategy that selects resistant pine trees that survive after PWN infection is one method to induce PWD-resistant lines in several pine species [9,10].

The pinewood nematode is native to North America but was first reported in Japan [11]. Many *Pinus* species in East Asian countries, including Korea, China, and Japan, have seriously suffered from PWN infection, whereas North American pine species, such as *Pinus elliottii* Engelm., *Pinus rigida* Mill., and *Pinus taeda* L., are highly resistant to PWNs [12].

The detailed mechanism of PWN resistance in American pine species is not yet known. For this reason, it is imperative to investigate the mechanisms of differences in resistance that exist among pine species. In previous studies, researchers suggested that plant defense compounds, pre-existing and/or induced by PWN infection, may contribute to PWN resistance [13,14]. A water-soluble repellent for PWNs was found in the bark of *P. taeda* [15]. *Pinus massoniana* Lamb., *Pinus strobus* L., and *Pinus palustris* Mill. contain nematicidal substances (mainly water-insoluble pinosylvin stilbenes) in their heartwood [16]. Generally, pinosylvin stilbenes do not accumulate in fresh tissue of pine branches and needles but accumulate mainly in heartwood, which prevents wood tissues from decaying due to fungi [17]. There are various types of stilbenoid compounds in pine species, among which some show strong nematicidal activity [13,14,16].

It is now well accepted that PWNs in susceptible pine species inoculated by the insect vector quickly migrate downwards to the main stem and colonize the whole tree through the resin canal system [2,18], particularly through the thicker resin canals of the phloem and cortex [19]. In contrast, PWN migration has been found to be slower or even completely blocked not only in PWN-resistant conifer species [20,21] but also in PWN-resistant genetic variants [22]. Oku et al. [20] reported that PWN migration was highly inhibited in PWN-inoculated stems of a *P. rigida* × *P. taeda* hybrid, which is also categorized as a highly resistant pine species against PWNs. Son et al. [23] compared PWN migrations with PWN-susceptible (*Pinus thunbergii* Parl.) and PWN-resistant (*P. strobus* and *P. rigida*) pine trees. PWNs inoculated on the stem top actively migrated downwards through both cortical resin canals and xylem resin canals in PWN-susceptible pine (*P. thunbergii*). In contrast, PWN migration was highly inhibited, and the migration of PWNs was particularly restricted in xylem resin canals in resistant pine species (*P. strobus* and *P. rigida*). However, further investigation to understand the mechanism of resin in PWN resistance has not been clearly elucidated.

Pitch pine (*P. rigida*) is native to eastern North America and was introduced for forest restoration in South Korea in 1907. Since then, there have been no reports of PWD infection of pitch pine in South Korea. PWNs inoculated on branches failed to migrate in *P. rigida* [23]. However, it is unknown what kind of resistance mechanism would affect resistance against PWNs in *P. rigida* plants. In general, resin is a representative defense substance in conifers [24], and PWN infection highly affects the resin secretion of pine trees [25].

The role of resin in defending against PWNs in PWN-resistant pine species has not been investigated in detail. We postulated that PWN resistance in *P. rigida* may be due to the nematicidal properties of resin. To address this question, we investigated the role of *P. rigida* resin in PWN mobility and mortality. Moreover, we investigated the difference in chemical compositions between the resins from PWN-susceptible pine (*P. densiflora* and *P. koraiensis*) and PWN-resistant pine (*P. rigida*), and investigated the nematicidal activity of phytochemicals (*trans* and *cis*-3,5-dimethoxystilbene) accumulated only in the resin of *P. rigida*.

2. Materials and Methods

2.1. PWN Propagation

PWNs were cultured on *Botrytis cinerea* grown on potato dextrose agar (PDA) medium for 7 days [26]. PWNs were subcultured at 25 °C in darkness for 10 days periodically. Proliferating PWNs were isolated by the Baermann funnel method [27].

2.2. PWN Migration on PWN-Inoculated Stems

Freshly taken stem segments (10 cm long and 9 mm diameter) of *P. densiflora*, *P. koriensis*, and *P. rigida* were inoculated with PWNs (~200 nematodes) to estimate PWN distribution after PWN infection. The PWNs were inoculated in small drilled holes (4 mm) at the base of branches. In order to prevent the drying of samples, PWN-inoculated stem segments were placed in 15 mL Falcon tubes. The inoculation portions, including the proximal

excised portion of stem segments, were sealed with Parafilm. After one day, the stems were divided into 2 cm pieces. The nematodes were separated from each stem segment by the Baermann funnel method, and the number of nematodes was counted by observation with a microscope (40×).

2.3. Resin Toxicity to PWNs

In order to examine the resin toxicity to PWNs, resins were collected from surfaces of excised branches of three-year-old pine saplings (*P. densiflora*, *P. koraiensis*, and *P. rigida*) obtained by germination of seeds. Fifty microliters of freshly collected resins were placed into a PCR tube to prevent water evaporation. After centrifugation of the PCR tube, approximately 50 nematodes were immersed into resin. After 3 days, the escape of PWNs from the resin was observed by microscopy.

2.4. Mortality of PWNs by Resin Treatment

Because PWNs escaped from the resin in the PCR tube, PWNs were cultured in resin that was inoculated onto a cell culture slide with a cover (Nunc® Lab-Tek® II—CC^{2}™ Chamber Slide™ system 8 wells, glass slide, 0.7 cm^2/well, and sterile) (Sigma–Aldrich Korea, Ltd., Seoul, Korea) to prevent escape from the resin. Approximately 50 nematodes were dipped into resin. The survival rate and migration distance of nematodes were observed by microscopy during 6 days of culture.

2.5. GC–MS Analysis of Resin Compositions in Pinus Species

The resin drops collected from needle tips from three *Pinus* species (*P. densiflora*, *P. koraiensis*, and *P. rigida*) were extracted by sonication in 100% methanol for 30 min. After centrifugation, the supernatant was filtered through a 0.45 μm membrane. An aliquot (5 μL) of each extraction was analyzed by GC (Agilent 7890A, Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 mm) and MSD system (Agilent 5975C) linked to a Triple-Axis detector; carrier gas: He (1.2 mL min^{-1}) and column temperature: 70–220 °C (5 °C min^{-1}), 220–320 °C (4 °C min^{-1}), and 320 °C (5 min hold). The peaks were identified by matching retention times and fragmentation of mass spectra with authentic standards and the mass spectral library of the GC–MS. The analyses were repeated three times, and GC chromatogram data were selected from one representative dataset. The percentage peak area method was used to analyze the area of the peak as a proportion of the total area of all detected peaks to analyze quantity.

2.6. PWN Toxicity of Two Pinosylvin Stilbenes

The *trans*-3,5-dimethoxystilbene standard for GC–MS analysis was purchased from Tokyo Chemical Industry Co., Ltd. (Toshima, Tokyo, Japan). Methyl dehydroabietate was purchased from Toronto Research Chemicals (TRC, Toronto, ON, Canada). *cis*-3,5-Dimethoxystilbene was isolated by the *n*-hexane-ethyl acetate (9:1) fraction of resin and identified by GC/MS.

Trans- and *cis*-3,5-dimethoxystilbene (100 μg/mL) and methyl dehydroabietate were dissolved in ethanol and diluted to 100 μg/mL by dilution using a 10 mg mL^{-1} concentration of 2-hydroxypropyl-b-cyclodextrin (HP-β-CD) solution, which was used as an emulsifier by He et al. [28]. Approximately 50 PWNs mixed with adults and J2 stage (2:3) were inoculated in each test solution in ibidi μ-Slide angiogenesis dishes (ibidi, Munich, Germany) and incubated for 2 days at 25 °C. HP-β-CD solution without the tested chemicals was used as a control. The immobilization of PWNs was determined by light microscopy. The experiment was performed in triplicate and repeated three times.

2.7. Statistics

All experiments were repeated in triplicate. Values in all data are presented as the average relative quantities ± standard error (SE). Statistical significance was measured

according to one-way ANOVA followed by *Duncan's post hoc analysis* at the 5% significance level.

3. Results

3.1. Migration of PWNs in Stems of P. densiflora, P. koraiensis and P. rigida

We investigated the distribution of PWNs in stems of PWN-susceptible (*P. densiflora* and *P. koraiensis*) and PWN-resistant (*P. rigida*) pine plants after inoculation with PWNs. Migration of inoculated PWNs in the branch of *P. rigida* was significantly suppressed compared to those in the branches of *P. densiflora* and *P. koraiensis*. PWNs rapidly spread upwards from the inoculated portion in susceptible *P. densiflora* and *P. koraiensis* after one day of inoculation (Figure 1). However, in *P. rigida* plants, most of the PWNs (92 ± 11.4%) remained near the inoculation point (Figure 1).

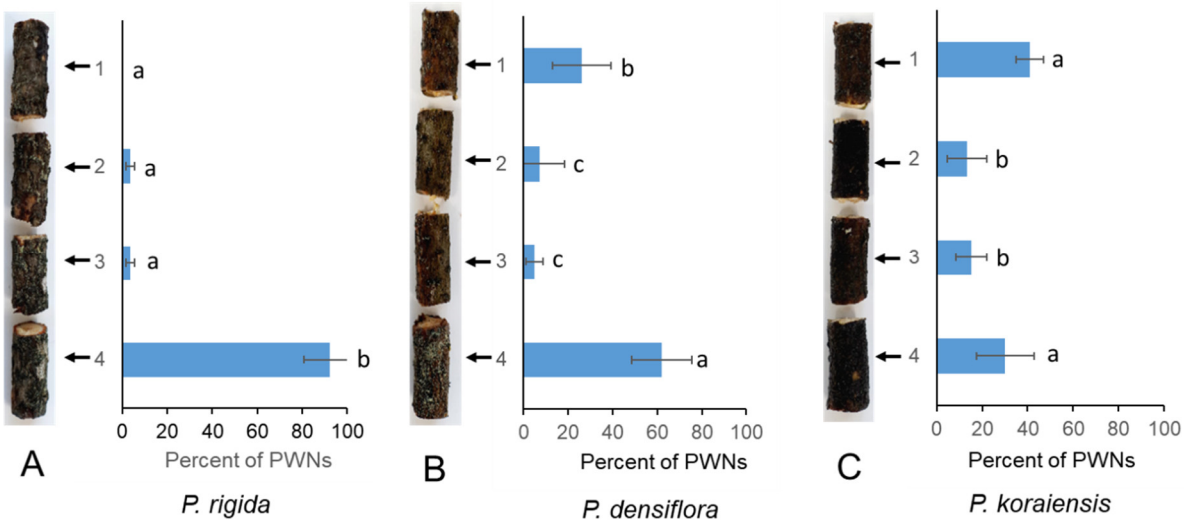

Figure 1. PWN migration in the stems of three *Pinus* species ((**A**) *P. rigida* Mill.), (**B**) *P. densiflora* (Sieb. et Zucc.), and (**C**) *P. koraiensis* (Sieb. et Zucc.) after one day of PWN inoculation. PWNs were inoculated into the small, drilled hole located at the base of the branch (number 4 portion). The experiment was repeated in triplicate. Error bars indicate the standard error of the mean (±SE) of three replicate measurements. Different letters above the bars indicate significantly different values ($p < 0.05$), calculated using one-way ANOVA followed by Duncan's post hoc analysis.

3.2. PWN Mobility in Resins from P. densiflora, P. koraiensis, and P. rigida

To examine the role of resin on PWN mobility, PWNs were immersed in the isolated resins from *P. densiflora*, *P. koraiensis*, and *P. rigida*. PWN mobility showed a significant reduction in resin from *P. rigida* compared to those in resins from *P. densiflora* and *P. koraiensis* (Figure 2). PWNs at 61 ± 8.9% and 48 ± 6.4% were located on the surfaces of the tube after escape from the resin after three days of culture, respectively (Figure 2A,B). However, more than 90.5 ± 11.2% of the nematodes inoculated in the resin from *P. rigida* failed to escape from the resin (Figure 2A,B). These results indicate that *P. rigida* resin contains toxic compounds immobilizing PWNs.

3.3. Mortality of PWNs in Resins from P. densiflora, P. koraiensis, and P. rigida

PWNs were directly inoculated into resins from *P. densiflora*, *P. koraiensis*, and *P. rigida* plants in cell culture chamber slides with covers to prevent free movement of PWNs outside of resin. Analysis of PWN mortality revealed that *P. rigida* resin showed significantly higher nematicidal activity than *P. densiflora* and *P. koraiensis* resins (Figure 3). There is no significant difference in resin toxicity between *P. densiflora* and *P. koraiensis* (Figure 3). The PWN mortalities in the resin from *P. densiflora* and *P. koraiensis* were 42.9% and 45.6%, respectively, after six days of treatment (Figure 3). On the other hand, 68% of the nematodes treated

with resin from *P. rigida* showed no movement after one hour (Figure 3). Subsequently, all nematodes in *P. rigida* resin were killed after six days (Figure 3).

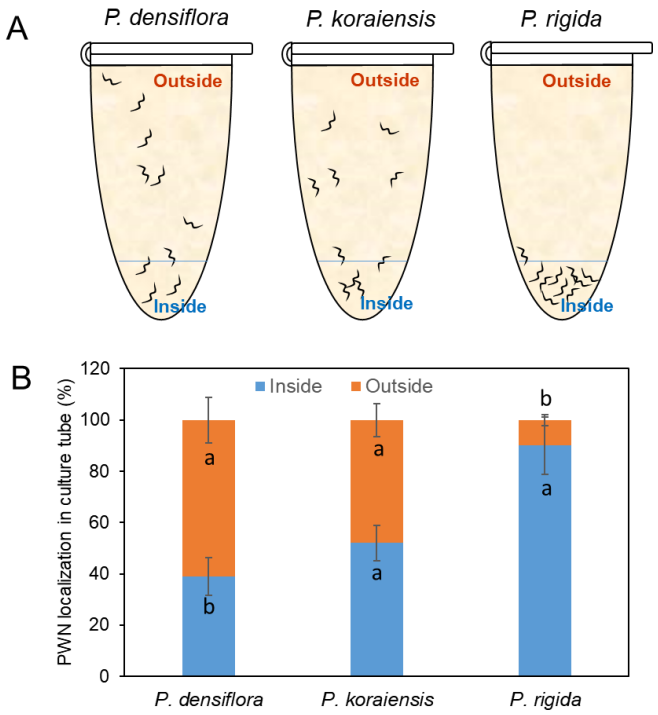

Figure 2. Movement of PWNs inside and outside of resin from three *Pinus* species (*P. densiflora*, *P. koraiensis*, and *P. rigida*) during 3 days of culture. (**A**) Location of PWNs in resin or outside of resin in a PCR tube. (**B**) Number of PWNs located in resin (inside) or outside of resin after 3 days. Experiment was repeated in triplicate. Error bars indicate the standard error of the mean (±SE) of three replicate measurements. Different letters above the bars indicate significantly different values ($p < 0.05$), calculated using one-way ANOVA followed by Duncan's post hoc analysis.

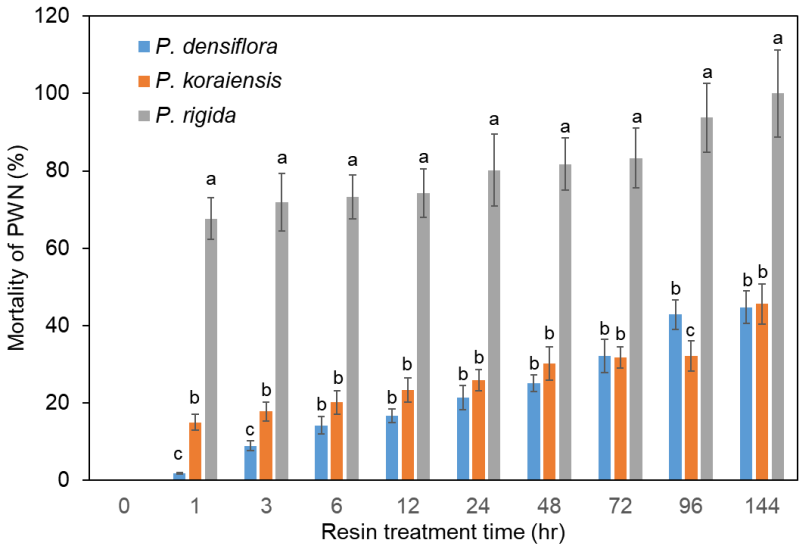

Figure 3. Mortality of PWNs in resin from three *Pinus* species. Mortality of PWNs in *P. densiflora* resin. Mortality of PWNs in *P. koraiensis* resin. Mortality of PWNs in *P. rigida* resin. Experiment was repeated in triplicate. Error bars indicate the standard error of the mean (±SE) of three replicate measurements. Different letters above the bars indicate significantly different values ($p < 0.05$), calculated using one-way ANOVA followed by Duncan's post hoc analysis.

3.4. Chemical Composition of Resins from P. densiflora, P. koraiensis, and P. rigida

We analyzed the resin components extracted from PWN-susceptible (*P. densiflora* and *P. koraiensis*) and PWN-resistant (*P. rigida*) pine plants by GC–MS. A total of 41 compounds were identified by comparison with the GC/MS library (Figure 4), of which 10 monoterpenoids, 12 sesquiterpenoids, 2 stilbenoids, and several abietane diterpenoids were detected (Table 1). *Pinus rigida* resin contained little to no sesquiterpenes compared to those of *P. densiflora* and *P. koraiensis*. Only β-cubebene was detected as a small peak in the GC chromatogram (Figure 4A). However, *P. rigida* resin contained a rich amount of diterpenes compared to *P. densiflora* and *P. koraiensis* resins, among which dehydroabietic aldehyde, methyl dehydroabietate, and methyl abietate were uniquely detected in *P. rigida* resin. In particular, methyl dehydroabietate showed the highest peak area among the diterpenoids (Table 1 and Figure 4). Two pinosylvin stilbenes (peak numbers 23 and 28 in Figure 4) were uniquely found in *P. rigida* resin. The stilbene peaks were identified as *trans*- and *cis*-3,5-dimethoxystilbene by comparison of retention time and mass spectra of standard compounds (Figure 5A–D). The two pinosylvin stilbenes had the same mass spectra (Figure 5C,D), but the retention times of the two compounds were different (Figure 5A). *Trans*-3,5-dimethoxystilbene (pinosylvin dimethyl ether) was determined to be one of the major substances in the resin from *P. rigida*.

Table 1. Identification of compounds was achieved using computer matching of the mass spectra with the NIST library or with mass spectra obtained from standard compounds.Compounds presented as dashed lines with amounts less than 0.05% are indicated. The dashed line indicates not detected and/or trace amounts. The bold characters/numbers indicate *P. rigida* Mill. resin-specific compounds. The values of peak area are mean of three replicates.

Peak Number	Retention Time (min)	Compound Name	Peak Area			
			Classfication	*P. densiflora*	*P. koraiensis*	*P. rigida*
1	4.66	α-Pinene	Mono	11.56	10.37	10.74
2	5.01	Camphene	Mono	0.50	3.64	0.50
3	5.76	β-Pinene	Mono	24.36	2.07	29.83
4	6.04	β-Myrcene	Mono	3.20	29.62	0.75
5	6.43	α-Phellandrene	Mono	0.37	–	0.32
6	6.80	3-Carene	Mono	–	0.36	–
7	7.18	β-Phellandrene	Mono	34.71	–	17.59
8	7.23	Limonene	Mono	–	1.92	–
9	8.86	Cyclohexene,4-methyl-3-(1-methylethylidene)-	Sesqui	0.49	1.08	–
10	13.18	Thymyl methyl ether	Sesqui	0.90	–	–
11	14.62	2-Camphanol acetate	Sesqui	0.41	6.60	–
12	16.37	α-Longipinene	Sesqui	0.35	0.36	–
13	17.04	Copaene	Sesqui		0.60	–
14	17.83	Longifolene	Sesqui	0.58	1.04	–
15	18.23	β-Caryophyllene	Sesqui	0.36	10.37	–
16	19.07	α-Caryophyllene	Sesqui	–	2.43	–
17	19.74	β-Cubebene	Sesqui	1.22	1.64	0.46
18	20.13	τ-Elemene	Sesqui	–	1.07	–
19	20.40	β-Bisabolene	Sesqui	–	0.26	–
20	20.65	(-)-δ-Cadinal lke	Sesqui	2.64	1.54	–
21	20.78	δ-Cadinene	Sesqui	0.34	2.18	–
22	29.95	Geranyl linalool	Diter	–	1.82	–
23	**30.58**	**Cis-3,5-dimethoxystilbene**	**Stilbene**	**–**	**–**	**0.58**
24	31.94	Thunbergol	Diter	2.17	2.93	–
25	33.47	18-Oxokauran-17-yl acetate	Diter	0.62	–	0.47
26	35.12	Pimara-7,15-dien-3-one	Diter	1.86	0.26	0.61
27	35.24	Pimara-7,15-dien-3-one-related	Diter	–	1.02	–
28	**35.36**	**Trans-3,5-dimethoxystilbene**	**Stilbene**	**–**	**–**	**9.36**
29	35.91	Androstane-3,17-dione	Diter	1.92	–	–
30	**36.05**	**Dehydroabietic aldehyde**	**Diter**	**–**	**–**	**0.32**
31	36.85	Methyl palustrate	Diter	0.84	–	–
32	**36.96**	**Unknown diterpene**	**Diter**	**–**	**–**	**3.13**
33	37.09	Agathadiol	Diter	1.92	–	–
34	**37.87**	**Methyl dehydroabietate**	**Diter**	**–**	**–**	**7.45**
35	38.64	Unknown diterpene	Diter	0.32	–	2.59
36	**38.92**	**Methyl abietate**	**Diter**	**–**	**–**	**0.70**
37	39.76	Cinnamyl cinnamate	Phenylpropan	0.62	–	1.74
38	40.49	Methyl noeabietate-like	Diter	0.58	–	0.91
39	42.13	Methyl 7,13,15-abietatrienoate	Diter	0.96	–	3.35
40	48.07	Methyl steviol	Diter	–	2.32	–
41	52.34	Ergosteryl acetate	Triter	4.75	0.47	0.96

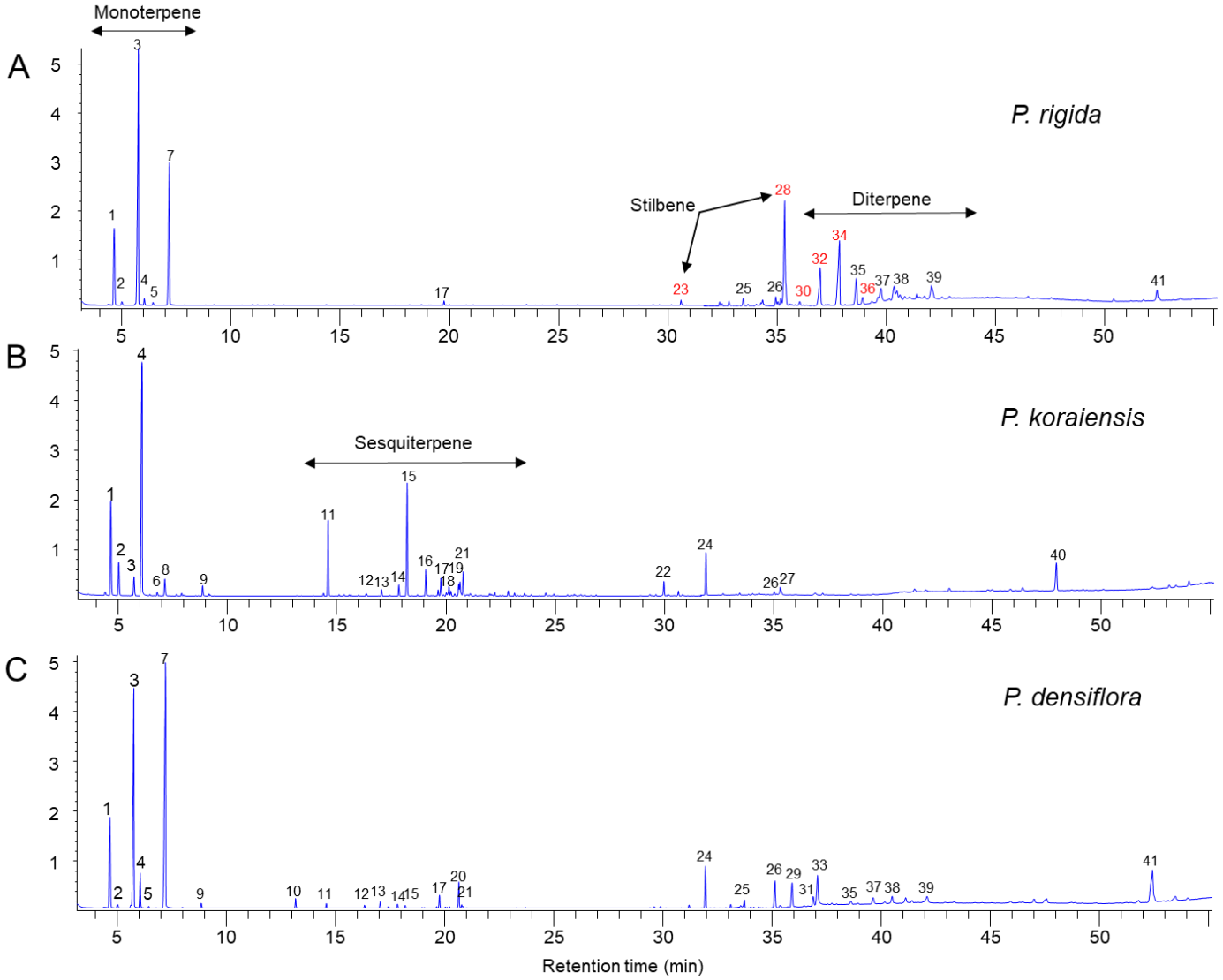

Figure 4. Chemical composition in resin extracts of three *Pinus* species (*P. rigida*, *P. koraiensis*, and *P. densiflora*) by GC–MS analysis. (**A**) GC chromatogram of *P. rigida* resin. (**B**) GC chromatogram of *P. koraiensis* resin. (**C**) GC chromatogram of *P. densiflora* resin.

The concentrations of *trans*- and *cis*-3,5-dimethoxystilbene were evaluated in different samples (leaf, stem bark, resin, and xylem). Both stem bark and resin showed higher amounts of *trans*- and *cis*-3,5-dimethoxystilbene than leaf and xylem tissue (Figure 5E).

3.5. Nematicidal Activity of 3,5-Dimethoxystilbene against PWNs

In order to investigate the PWN toxicity of the two pinosylvin stilbenes, PWNs were inoculated in 100 μg/mL *trans*- and *cis*-3,5-dimethoxystilbene. In PWNs treated with *trans*-3,5-dimethoxystilbene, there was no significant difference in the nematicidal activity compared to the control group (Figure 6), indicating no nematicidal activity in *trans*-3,5-dimethoxystilbene. In contrast, *cis*-3,5-dimethoxystilbene showed significantly high nematicidal activity against PWNs in comparison to the control and *trans*-3,5-dimethoxystilbene treatment. PWN mortality in *cis*-3,5-dimethoxystilbene was 78% after 24 h (Figure 6).

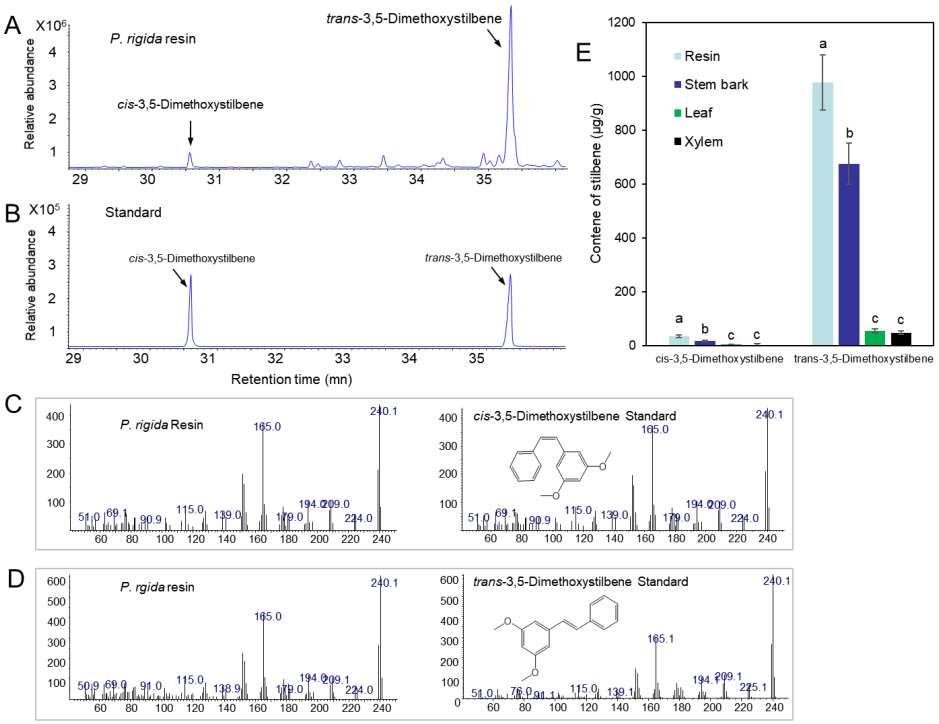

Figure 5. Identification of *trans*-3,5-dimethoxystilbene and *cis*-3,5-dimethoxystilbene and analysis of *trans*-3,5-dimethoxystilbene and *cis*-3,5-dimethoxystilbene content in different tissues of *P. rigida* plants. (**A**) GC chromatogram of *trans*-3,5-dimethoxystilbene and *cis*-3,5-dimethoxystilbene in resin from *P. rigida*. (**B**) GC chromatogram of standard *trans*-3,5-dimethoxystilbene and *cis*-3,5-dimethoxystilbene. (**C**) Mass spectra of a peak (30.58 min) detected in resin and *cis*-3,5-dimethoxystilbene standard. (**D**) Mass spectra of a peak (35.36 min) detected in resin and *trans*-3,5-dimethoxystilbene standard. (**E**) Content of *trans*-3,5-dimethoxystilbene and *cis*-3,5-dimethoxystilbene in leaf, resin, bark, and xylem. Error bars indicate the standard error of the mean (±SE) of three replicate measurements. Different letters above the bars indicate significantly different values ($p < 0.05$), calculated using one-way ANOVA followed by Duncan's post hoc analysis.

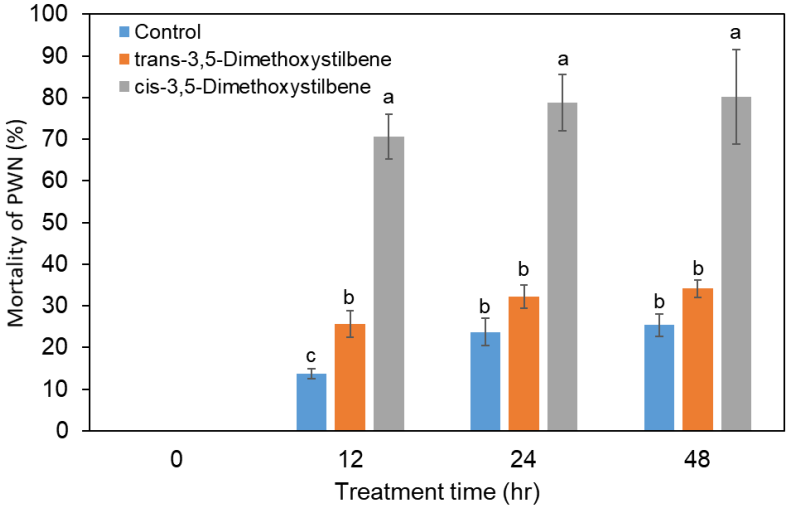

Figure 6. Nematicidal activity of *trans*-3,5-dimethoxystilbene and *cis*-3,5-dimethoxystilbene against *Bursaphelenchus xylophilus* during 48 h of treatment. Experiment was repeated in triplicate. Error bars indicate the standard error of the mean (±SE) of three replicate measurements. Different letters above the bars indicate significantly different values ($p < 0.05$), calculated using one-way ANOVA followed by Duncan's post hoc analysis.

4. Discussion

4.1. Inhibition of PWN Migration in Pine Stems

There were significant differences in the migration of PWNs in the branches of *P. densiflora*, *P. koraiensis* and *P. rigida* after inoculation with PWNs. PWNs were actively dispersed in the branches after PWN inoculation in PWN-susceptible *P. densiflora* and *P. koraiensis*. However, in the branches of *P. rigida*, most inoculated PWNs failed to migrate and remained at the inoculation point of PWNs. Similarly, Oku et al. [20] reported that PWN migration was highly inhibited in PWN-inoculated stems of a *P. rigida* × *P. taeda* hybrid, which is also categorized as a highly resistant pine species against PWNs. Son et al. [23] compared PWN migrations with PWN-susceptible (*P. thunbergii*) and PWN-resistant (*P. strobus* and *P. rigida*) pine trees. PWNs inoculated on the stem top actively migrated downwards through both cortical resin canals and xylem resin canals in PWN-susceptible pine (*P. thunbergii*). In contrast, PWN migration was highly inhibited, and the migration of PWNs was particularly restricted in xylem resin canals in resistant pine species (*P. strobus* and *P. rigida*).

The inhibition of PWN migration in resin canals in PWD-resistant pines can be explained by two possible factors. One is the presence of inhibitory compounds that affect the PWN movement [13,16,20,29]. Another possible interpretation has been proposed: anatomical differences in the lumen area of resin canals between susceptible and resistant pines. Kawaguchi [19] reported that the smaller lumen area in resistant *P. thunbergii* caused the restriction in PWN migration. However, Son et al. [23] and Mori et al. [30] did not see a relationship between anatomical differences in resin canals between PWD-resistant and PWD-susceptible pines. They suggested that the structures of cortical and xylem axial resin canals may not be a general and critical factor preventing PWN migration in resistant pines.

4.2. Resin Toxicity against PWNs

Resin plays an important role as a defense substance for preventing insect invasion or pathogens in conifers [3]. We suppose that suppression of PWN migration in PWN-resistant pine species might be caused by toxic phytochemicals in resins. However, the role of resin in defending against PWNs in PWN-resistant pine species has not been investigated in detail. To investigate the resin toxicity against PWNs, we collected the resins from PWN-susceptible (*P. densiflora* and *P. koraiensis*) and -resistant (*P. rigida*) pine trees and observed the mobility and mortality of PWNs by directly immersing PWNs in resins. We found that the mobility of PWNs was strongly suppressed in the resin from *P. rigida*. Moreover, *P. rigida* resin showed a significantly higher PWN mortality rate than *P. densiflora* and *P. koraiensis* resins. All the PWNs dipped in *P. rigida* resin were killed after six days. In contrast, more than 50% of PWNs dipped in the resins from *P. densiflora* and *P. koraiensis* were still alive until after six days. These results indicate that the resins from *P. rigida* directly affected the mobility and mortality of PWNs, probably due to the toxic nematicidal compounds in resin, by which *P. rigida* plants might be able to attain PWN resistance.

4.3. Chemical Composition of Resins Extracted from P. densiflora, P. koraiensis, and P. rigida

Resin is composed of various phytochemicals, including mainly terpenoids, which are basic substances that constitute or induce defense mechanisms [31]. In order to uncover the nematicidal compounds in *P. rigida* resin, phytochemical components extracted from resin from PWN-susceptible (*P. densiflora* and *P. koraiensis*) and PWN-resistant (*P. rigida*) pine plants were analyzed by *GC/MS*. Resins sampled from the three pine species mainly contained monoterpenes, sesquiterpenes, and diterpenes. *Pinus rigida* resin contained small amounts of sesquiterpenes compared to *P. densiflora* and *P. koraiensis*. *Pinus rigida* resin contained rich and various kinds of diterpenoids, among which dehydroabietic aldehyde, methyl dehydroabietate, methyl abietate, and one unknown compound were uniquely detected in *P. rigida* resin. Interestingly, *P. rigida* resin contained two stilbenoid compounds (*cis*- and *trans*-3,5-dimethoxystilbene), which are not found in the resin from *P. densiflora*

and *P. koraiensis*. The above results indicate that the toxicity of *P. rigida* resin against PWNs might be caused by the different chemical compositions in the resin.

4.4. Toxicity of 3,5-Dimethoxystilbene against PWNs

Generally, pinosylvin stilbenes are particularly rich in heartwood extracts preventing wood tissues from decaying by fungi [17]. In sapwood and needles, pinosylvin stilbenes seem to function as phytoalexins because these compounds are accumulated under abiotic and biotic stresses [32–35]. Suga [16] firstly reported that some pine stilbenoid compounds have strong nematicidal activity. Recently, the two pinosylvin stilbenes (pinosylvin monomethyl ether and dihydropinosylvin monomethyl ether) in *P. strobus* had strong nematicidal activity against PWNs and the accumulation of these stilbenes highly enhanced by PWN infection [14]. In this work, we found that *Pinus rigida* resin contained *trans*- and *cis*-3,5-dimethoxystilbene (pinosylvin dimethyl ether and cis-pinosylvin dimethyl ether) in resin even under normal growth conditions without PWN infection. The occurrence of the two stilbenes was also reported in the bark extract of *P. banksiana* [36], and *P. banksiana* is moderately resistant to PWNs [3].

We investigated the biological activity of 3,5-dimethoxystilbene against PWNs. PWNs were treated with the two isoforms of 3,5-dimethoxystilbene (*cis*- and *trans*-3,5-dimethoxystil bene). *cis*-3,5-Dimethoxystilbene showed significantly higher nematicidal activity compared to *trans*-3,5-dimethoxystilbene, which is similar to the result of Suga et al. [16]. They reported that *cis*-3,5-dimethoxystilbene at 100 μg mL^{-1} showed strong nematicidal activity against PWNs, but *trans*-3,5-dimethoxystilbene did not. Although *cis*-3,5-dimethoxystilbene showed PWN toxicity in an in vitro test, the presence of *cis*-3,5-dimethoxystilbene (67 μg/g) in *P. rigida* resin may partially contribute to achieving PWN resistance due to insufficient concentrations.

In addition to the occurrence of 3,5-dimethoxystilbene in *P. rigida* resin, *Pinus rigida* resin contained rich and various abietane diterpenes together with several unknown diterpenes. There is no report on the nematicidal activity of pine diterpenoids against PWNs. However, some diterpenoids isolated from coniferous plants were reported to have strong nematicidal activity in some nematode species. Abieta-7,13-diene, ferruginol (abieta-8,11,13-trien-12-ol), and sugiol (12-Hydroxyabieta-8,11,13-trien-7-one) isolated from *Juniperus* berries have strong nematicidal activity against malarial nematodes (*Plasmodium falciparum*), and totarol (14-isopropyl podocarpa-8,11,13-trien-13-ol) has nematicidal activity against *Caenorhabditis elegans* [37]. Although we did not analyze the PWN toxicity of diterpenoids that were uniquely detected in *P. rigida* resin, the occurrence of various abietane diterpenoids accumulated in *P. rigida* resin may be involved in additional PWN resistance together with *cis*-3,5-dimethoxystilbene.

5. Conclusions

In this study, we found that dipping in the resin isolated from *P. rigida* strongly suppressed PWN mobility and conferred high PWN mortality. In GC–MS analysis of resin extracts from three pine species (*P. densiflora, P. koraiensis*, and *P. rigida*), two types of pinosylvin stilbenes (*cis*- and *trans*-3,5-dimethoxystilbene) were detected only in *P. rigida* resin, and several diterpenoids were also particularly rich in *P. rigida* resin. In the test of PWN toxicity of the two isoforms of 3,5-dimethoxystilbene (*cis*- and *trans*-3,5-dimethoxystilbene), *cis*-3,5-dimethoxystilbene showed higher nematicidal activity than *trans*-3,5-dimethoxystilbene. From our results, we suggest that toxic phytochemicals accumulated in the resin from *P. rigida*, such as *cis*-3,5-dimethoxystilbene, may create PWN resistance in *P. rigida* pine.

Author Contributions: Y.-E.C. designed the research, and both H.-S.H. and Y.-E.C. wrote the paper. H.-S.H. and J.-Y.H. performed the analysis of secondary compounds by GC/MS. Y.-R.K. determined the PWN toxicity of the resin. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Korea Forest Service (Korea Forestry Promotion Institute), Republic of Korea (Grant No. 2021339A00-2123-CD02).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jones, J.T.; Haegeman, A.; Danchin, E.G.J.; Gaur, H.S.; Helder, J.; Jones, M.J.K.; Kikuchi, T.; Manzanilla-López, R.; Palomares-Rius, J.E.; Wesemael, W.M.L.; et al. Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol. Plant Pathol.* **2013**, *14*, 946–961. [CrossRef] [PubMed]
2. Ichihara, Y.; Fukuda, K.; Suzuki, K. Early symptom development and histological changes associated with migration of *Bursaphelenchus xylophilus* in seedling tissues of *Pinus thunbergii*. *Plant Dis.* **2000**, *84*, 675–680. [CrossRef] [PubMed]
3. Futai, K. Pine Wood Nematode, *Bursaphelenchus Xylophilus*. *Annu. Rev. Phytopathol.* **2013**, *51*, 61–83. [CrossRef] [PubMed]
4. Ikeda, T. Responses of water-stressed *Pinus thunbergii* to inoculation with avirulent pine wood nematode (*Bursaphelenchus xylophilus*): Water relations and xylem histology. *J. For. Res.* **1996**, *1*, 223–226. [CrossRef]
5. Linit, M.J. Nematode-vector relationships in the pine wilt disease system. *J. Nematol.* **1988**, *20*, 227–235.
6. James, R.; Tisserat, N.; Todd, T. Prevention of pine wilt of scots pine (*Pinus sylvestris*) with systemic abamectin injections. *Arboric. Urban For.* **2006**, *32*, 195–201. [CrossRef]
7. Sousa, E.; Naves, P.; Vieira, M. Prevention of pine wilt disease induced by *Bursaphelenchus xylophilus* and *Monochamus galloprovincialis* by trunk injection of emamectin benzoate. *Phytoparasitica* **2013**, *41*, 143–148. [CrossRef]
8. Kim, B.-N.; Kim, J.H.; Ahn, J.-Y.; Kim, S.; Cho, B.-K.; Kim, Y.-H.; Min, J. A short review of the pinewood nematode, *Bursaphelenchus xylophilus*. *Toxicol. Environ. Health Sci.* **2020**, *12*, 297–304. [CrossRef]
9. Kurinobu, S. Current status of resistance breeding of Japanese pine species to pine wilt disease. *For. Sci. Technol.* **2008**, *4*, 51–57. [CrossRef]
10. Ribeiro, B.; Espada, M.; Vu, T.; Nóbrega, F.; Mota, M.; Carrasquinho, I. Pine wilt disease: Detection of the pinewood nematode (*Bursaphelenchus xylophilus*) as a tool for a pine breeding programme. *For. Pathol.* **2012**, *42*, 521–525. [CrossRef]
11. Yano, S. Investigation on pine death in Nagasaki prefecture. *Sanrin-Kouhou* **1913**, *4*, 1–14.
12. Mamiya, Y. Pathology of the pine wilt disease caused by *Bursaphelenchus xylophilus*. *Annu. Rev. Phytopathol.* **1983**, *21*, 201–220. [CrossRef] [PubMed]
13. Hanawa, F.; Yamada, T.; Nakashima, T. Phytoalexins from *Pinus strobus* bark infected with pinewood nematode, *Bursaphelenchus xylophilus*. *Phytochemistry* **2001**, *57*, 223–228. [CrossRef]
14. Hwang, H.S.; Han, J.Y.; Choi, Y.E. Enhanced accumulation of pinosylvin stilbenes and related gene expression in *Pinus strobus* after infection of pine wood nematode. *Tree Physiol.* **2021**, *41*, 1972–1987. [CrossRef] [PubMed]
15. Futai, K. Responses of two species of *Bursaphelenchus* to the extracts from pine segments and to the segments immersed in different solvents. *Jpn. J. Nematol.* **1979**, *9*, 54–59.
16. Suga, T.; Ohta, S.; Munesada, K.; Ide, N.; Kurokawa, M.; Shimizu, M.; Ohta, E. Endogenous pine wood nematicidal substances in Pines, *Pinus Massoniana*, *P. Strobus* and *P. Palustris*. *Phytochemistry* **1993**, *33*, 1395–1401. [CrossRef]
17. Harju, A.M.; Venäläinen, M. Measuring the decay resistance of scots pine heartwood indirectly by the Folin-Ciocalteu assay. *Can. J. For. Res.* **2006**, *36*, 1797–1804. [CrossRef]
18. Son, J.A.; Komatsu, M.; Matsushita, N.; Hogetsu, T. Migration of pine wood nematodes in the tissues of *Pinus thunbergii*. *J. For. Res.* **2010**, *15*, 186–193. [CrossRef]
19. Kawaguchi, E. Relationship between the anatomical characteristics of cortical resin canals and migration of *Bursaphelenchus xylophilus* in stem cuttings of *Pinus thunbergii* seedlings. *J. Jpn. For. Soc.* **2006**, *88*, 240–244. [CrossRef]
20. Oku, H.; Shiraishi, T.; Chikamatsu, K. Active defense as a mechanism of resistance in pine against pine wilt disease. *Jpn. J. Phytopathol.* **1989**, *55*, 603–608. [CrossRef]
21. Nunes da Silva, M.; Lima, M.R.M.; Vasconcelos, M.W. Susceptibility evaluation of *Picea abies* and *Cupressus lusitanica* to the pine wood nematode (*Bursaphelenchus xylophilus*). *Plant Pathol.* **2013**, *62*, 1398–1406. [CrossRef]
22. Kuroda, K. Inhibiting factors of symptom development in several Japanese red pine (*Pinus densiflora*) families selected as resistant to pine wilt. *J. For. Res.* **2004**, *9*, 217–224. [CrossRef]
23. Son, J.A.; Matsushita, N.; Hogetsu, T. Migration of *Bursaphelenchus xylophilus* in cortical and xylem axial resin canals of resistant pines. *For. Pathol.* **2014**, *45*, 246–253. [CrossRef]
24. Martin, D.; Tholl, D.; Gershenzon, J.; Bohlmann, J. Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiol.* **2002**, *129*, 1003–1018. [CrossRef]
25. Bolla, R.I.; Nosser, C.; Tamura, H. Chemistry of response of pines to *Bursaphelenchus xvlophilus*: Resin acids. *Jpn. J. Nematol.* **1989**, *19*, 1–6.
26. Zhu, L.-H.; Ye, J.; Negi, S.; Xu, X.-L.; Wang, Z.-L.; Ji, J.-Y. Pathogenicity of aseptic *Bursaphelenchus xylophilus*. *PLoS ONE* **2012**, *7*, e38095. [CrossRef]

27. Hooper, D.J. Extraction of nematodes from plant material. In *Laboratory Methods for Work with Plant and Soil Nematodes*; Reference Book No. 402; Southey, J.F., Ed.; Ministry of Agriculture, Fisheries and Food, Her Majesty's Stationery Office: London, UK, 1986; pp. 51–58.
28. He, J.; Zheng, Z.P.; Zhu, Q.; Guo, F.; Chen, J. Encapsulation mechanism of oxyresveratrol by β-cyclodextrin and hydroxypropyl-β-cyclodextrin and computational analysis. *Molecules* **2017**, *22*, 1801. [CrossRef]
29. Yamada, T.; Ito, S.I. Chemical defense responses of wilt resistant pine species, *Pinus strobus* and *P. taeda*, against *Bursaphelenchus xylophilus* infection. *Nippon Shokubutsu Byori Gakkaiho* **1993**, *59*, 666–672. [CrossRef]
30. Mori, Y.; Miyahara, F.; Tsutsumi, Y.; Kondo, R. Relationship between resistance to pine wilt disease and the migration or proliferation of pine wood nematodes. *Eur. J. Plant Pathol.* **2008**, *122*, 529–538. [CrossRef]
31. Phillips, M.A.; Croteau, R.B. Resin-based defenses in conifers. *Trends Plant Sci.* **1999**, *4*, 184–190. [CrossRef]
32. Harju, A.M.; Venäläinen, M.; Laakso, T.; Saranpää, P. Wounding response in xylem of Scots pine seedlings shows wide genetic variation and connection with the constitutive defence of heartwood. *Tree Physiol.* **2009**, *29*, 19–25. [CrossRef] [PubMed]
33. Schoeppner, A.; Kindl, H. Stilbene synthase (pinosylvine synthase) and its induction by ultraviolet light. *FEBS Lett.* **1979**, *108*, 349–352. [CrossRef]
34. Gehlert, R.; Schöppner, A.; Kindl, H. Stilbene synthase from seedlings of *Pinus sylvestris*: Purification and induction in response to fungal infection. *Mol. Plant Microbe. Interact.* **1990**, *3*, 444–449. [CrossRef]
35. Rosemann, D.; Heller, W.; Sandermann, H. Biochemical plant responses to ozone: II. Induction of stilbene biosynthesis in Scots pine (*Pinus sylvestris* L.) seedlings. *Plant Physiol.* **1991**, *97*, 1280–1286. [CrossRef]
36. Rowe, J.W.; Bower, C.L.; Wagner, E.R. Extractives of jack pine bark: Occurrence of *cis*- and *trans*-pinosylvin dimethyl ether and ferulic acid esters. *Phytochemistry* **1969**, *8*, 235–241. [CrossRef]
37. Samoylenko, V.; Dunbar, D.C.; Gafur, M.A.; Khan, S.I.; Ross, S.A.; Mossa, J.S.; El-Feraly, F.S.; Tekwani, B.L.; Bosselaers, J.; Muhammad, I. Antiparasitic, nematicidal and antifouling constituents from *Juniperus* berries. *Phytother. Res.* **2008**, *22*, 1570–1576. [CrossRef]