Fumigant Antifungal Activity via Reactive Oxygen Species of *Thymus vulgaris* and *Satureja hortensis* Essential Oils and Constituents against *Raafaelea quercus-mongolicae* and *Rhizoctonia solani*

Jeong-Eun Kim 1,2, Ji-Eun Lee 1,3, Min-Jung Huh 1, Sung-Chan Lee 1,*, Seon-Mi Seo 1,4, Jun Hyeong Kwon 1 and Il-Kwon Park 1,4,*

1 Department of Forest Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea; ionlylove7@snu.ac.kr (J.-E.K.); jie0815@snu.ac.kr (J.-E.L.); shadma@snu.ac.kr (M.-J.H.); sungchan1225@empas.com (S.-C.L.); popcon24@naver.com (S.-M.S.); sibollr.o.k@gmail.com (J.H.K.)
2 LG Household & Health Care, Seoul 07795, Korea
3 Forest Insect Pests and Diseases Division, National Institute of Forest Science, Seoul 02455, Korea
4 Research Institute of Agriculture and Life Science, College of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea
* Correspondence: parkik1@snu.ac.kr

Received: 16 September 2019; Accepted: 1 October 2019; Published: 3 October 2019

Abstract: In this study, the fumigant antifungal activity of 10 Lamiaceae plant essential oils was evaluated against two phytopathogenic fungi, *Raffaelea quercus-mongolicae*, and *Rhizoctonia solani*. Among the tested essential oils, thyme white (*Thymus vulgaris*) and summer savory (*Satureja hortensis*) essential oils exhibited the strongest fumigant antifungal activity against the phytopathogenic fungi. We analyzed the chemical composition of two active essential oils and tested the fumigant antifungal activities of the identified compounds. Among the tested compounds, thymol and carvacrol had potent fumigant antifungal activity. We observed reactive oxygen species (ROS) generation in two fungi treated with thymol and carvacrol. Confocal laser scanning microscopy images of fungi stained with propidium iodide showed that thymol and carvacrol disrupted fungal cell membranes. Our results indicated that ROS generated by thymol and carvacrol damaged the cell membrane of *R. quercus-mongolicae* and *R. solani*, causing cell death.

Keywords: *Thymus vulgaris*; *Satureja hortensis*; antifungal activity; reactive oxygen species; thymol; carvacrol

1. Introduction

Oak wilt disease caused by a fungus that is symbiotic with ambrosia beetles is a serious problem in Korea [1]. This disease was first recorded in Sungnam city, Gyeonggi Province, the Republic of Korea in 2004 and has spread to several regions of the Korean peninsula [2]. *Raffaelea quercus-mongolicae* (K.H. Kim, Y.J. Choi, & H.D. Shin) was identified as the fungal pathogen of oak wilt disease in 2009 [3]. The ambrosia beetle, *Platypus koryoensis*, transfers *R. quercus-mongolicae* to new oak trees when they attack the host tree for breeding. Although *Quercus mongolicae* (Fischer) is the main tree species attacked by *P. koryoensis*, other *Quercus* species, such as *Q. acutissima* (Carruthers), *Q. aliena* (Blume), *Q. dentate* (Thunberg), *Q. serrata* (Thunberg), and *Q. variabilis* are also affected [4]. Damping-off is a tree disease that causes serious damage in nursery gardens. Fungal pathogens in the genera *Phytophthora*, *Fusarium*, *Rhizoctonia*, and *Pythium* cause this disease [5]. Among fungal pathogens of damping-off, *Rhizoctonia solani* Kuhn occurs around the world and causes serious damage to economically important vegetables, and fruit and forest trees [6,7].
Management of the two phytopathogenic fungi mainly depends on commercial fungicides in Korea [1,2,7]. Although effective, synthetic fungicides cause harm, such as environmental contamination, toxicity to non-target organisms, and development of resistance. These issues require research to find alternatives to conventional fungicides. Several studies have investigated plant essential oils as new, safe alternatives to synthetic fungicides because the biological activities of plant essential oils and their constituents have been reported [8–10]. Furthermore, many plant essential oils are considered safe for humans because of their wide use in the food and fragrance market.

In this study, we investigated the fumigant antifungal activities of 10 Lamiaceae plant essential oils and their constituents against two phytopathogenic fungi, *R. quercus-mongolicae* and *R. solani*. The aim was finding new, safe alternatives for conventional fungicides. To determine the antifungal mode of action, reactive oxygen species (ROS) generation and cell membrane integrity of two phytopathogenic fungi treated with constituents derived from thyme white and summer savory plant essential oils were studied.

2. Materials and Methods

2.1. Fungal Strain and Culture Conditions

The phytopathogenic fungi *Raffaelea quercus-mongolicae* and *Rhizoctonia solani* were supplied by the National Institute of Forest Science, Seoul, the Republic of Korea. Potato dextrose agar (PDA) (Difco, IL, USA) plates were prepared using Petri dishes (diameter 90 mm), and agar-myccelial plugs (diameter 5 mm) of *R. quercus-mongolicae* and *R. solani* were inoculated into the center of the dishes. Inoculated plates were incubated at 25 °C in the dark.

2.2. Plant Essential Oils and Chemicals

The 10 Lamiaceae plant essential oils studied are shown in Table 1. Basil, lavender, marjoram, pennyroyal, Spanish sage, spearmint, and thyme white plant essential oils were purchased from an online retailer Jinarome (www.jinarome.com). Hyssop, patchouli, and summer savory plant essential oils were from the online retailer Oshadhi Ltd. (www.ohsadhi.eu).

| Common Name   | Scientific Name               | Source       | Part              |
|---------------|-------------------------------|--------------|-------------------|
| Basil         | *Ocimum basilicum* L.         | Jinarome     | Leaves            |
| Hyssop        | *Hyssopus officinalis* L.     | Oshadhi      | plants            |
| Lavender      | *Lavandula angustifolia* Mill.| Jinarome     | Flowers           |
| Marjoram      | *Origanum majorana* L.        | Jinarome     | Fruits            |
| Patchouli     | *Pogostemon cablin* Benth.    | Oshadhi      | Leaves            |
| Pennyroyal    | *Mentha pulegium* L.          | Jinarome     | Plants            |
| Spanish Sage  | *Salvia lavandulaefolia* Vahl.| Jinarome     | Flowers           |
| Spearmint     | *Mentha spicata* L.           | Jinarome     | Flowering plants  |
| Summer Savory | *Satureja hortensis* L.       | Oshadhi      | Blossom/plants    |
| Thyme White   | *Thymus vulgaris* L.          | Jinarome     | Leaves            |

Reagents (−)-α-pinene (99%), (−)-camphen (80%), (+)-camphene (80%), (−)-β-pinene (99%), (+)-β-pinene (≥98.5%), myrcene (75%), p-cymene (99%), (+)-limonene (97%), and caryophyllene oxide (90%) were from Sigma-Aldrich (Milwaukee, WI, USA). Tokyo Kasei (TCI, Tokyo, Japan) supplied (−)-α-pinene (>95%), (−)-limonene (95%), terpinen-4-ol (>95%), carvacrol (>95%), and β-caryophyllene (>90%). We obtained α-terpinene (85%), γ-terpinene (≥97%), and thymol (≥99%) from Fluka (Buchs, Switzerland), and linalool (98%) from Wako Chemicals USA (Richmond, VA, USA).
2.3. Fumigant Antifungal Activity Bioassays

Fumigant antifungal activity bioassays followed the method of Kim and Park [11]. PDA (Difco, II, USA) plates were prepared using Petri dishes (diameter 90 mm). Agar-myccelial plugs (diameter 5 mm) of \( R. \ querqus-mongolicae \) and \( R. \ solani \) were inoculated into the center of the dishes. Lamiaceae plant essential oils or their constituents in 25% dimethyl sulfoxide (DMSO, Daejung, Gyeonggi Province, the Republic of Korea) and 1% Tween 80 (Daejung, Gyeonggi Province, the Republic of Korea) were applied to a paper disc (diameter 8 mm, Advantec, Tokyo, Japan) placed on agar-free dish lids (Figure 1). Treated Petri dishes were sealed with parafilm to minimize the leaking of the test oils and compounds. The control treatment was 25% DMSO and 1% Tween 80. After treatment, plates were incubated at 25 °C in the dark. The colony diameters were measured using Vernier calipers (CD-30C, Mitutoyo Corp., Kawasaki, Japan) after mycelial growth of controls completely covered dishes (average, 2–3 days for \( R. \ solani \), 5–7 days for \( R. \ querqus-mongolicae \)). All experiments were conducted five times. Inhibition by Lamiaceae plant essential oils or their constituents was calculated as % inhibition = \( C - \frac{T}{C} \times 100 \) where \( C \) is a hyphal extension (mm) on control plates and \( T \) is a hyphal extension (mm) of plates treated with plant oils or constituents.

2.4. Gas Chromatography

Gas chromatography (GC) (7890B, Agilent, CA, USA)—with flame ionization detector (FID) and DB-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent, CA, USA) was used to analyze the chemical composition of summer savory and thyme white essential oils. Oven temperatures were maintained at 40 °C for 5 min, raised to 250 °C at 6 °C/min, and kept at 250 °C for 5 min. Nitrogen was the carrier gas, and the flow rate was 1.0 mL/min. The retention indices (RIs) were calculated in relation to a homologous series of \( n \)-alkanes (\( C_8 - C_{22} \); DB-5MS) under the same GC operating conditions [12].

2.5. Gas Chromatography-Mass Spectrometry

Chemical compositions of summer savory and thyme white essential oils were further determined using a gas chromatography (Agilent 7890A)-mass spectrometer (GC-MS; Agilent 5975C MSD, Agilent Technologies, Santa Clara, CA, USA) and DB-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent, CA, USA). The oven temperature program was the same as for gas chromatography-flame ionization detector (GC-FID) analysis. Helium was the carrier gas, and the flow rate was 1.0 mL/min. The effluence from the DB-5 MS column was introduced directly into mass spectrometer (MS) through a transfer line (280 °C). The ionization was by electron impact (70 eV, source temperature 230 °C). The scan range was 41–400 amu. Most compounds of summer savory and thyme white plant essential oils were tentatively identified by comparing the mass spectra of the peaks with authentic samples in the National Institute of Standards and Technology mass spectral (NIST MS) library.
2.6. Assessment of Reactive Oxygen Species Generation

Intracellular ROS generation of *R. quercus-mongolicae* and *R. solani* was observed using the ROS-sensitive dye 2′,7′-dichlorofluorescin diacetate (≥97, Sigma-Aldrich, MO, USA). Nutrient agar (NA) (Difco, IL, USA) plates were prepared in Petri dishes (diameter 90 mm). Agar-mycelial plugs (diameter 5 mm) of *R. quercus-mongolicae* or *R. solani* were placed in the center of the dishes for incubation at 25 °C (one day for *R. solani* and four days for *R. quercus-mongolicae*). On agar-free lids, paper discs (diameter 8 mm, Advantec, Tokyo, Japan) with 10 mg carvacrol and thymol were placed. Controls received only 25% DMSO and 1% Tween 80. After treatment, treated and control NA plates were incubated for one day at 25 °C. After incubation, plugs from treated and control NA plate were stained with 3 µM 2′,7′-dichlorofluorescein diacetate for 1 h at 37 °C in the dark. Stained plugs were washed with phosphate-buffered saline (PBS, Sigma-Aldrich, MO, USA) diluted 10 times with distilled water, and observed with a confocal laser scanning microscope (CLSM) (excitation: 488 nm, emission: 490-560 nm, SP8X, Leica Microsystems, Germany).

2.7. Cell Membrane Integrity Assay

Cell membrane integrity of *R. quercus-mongolicae* and *R. solani* was measured by using propidium iodide (PI) (Thermofisher, MA, USA). PDA plates were prepared using Petri dishes (diameter 90 mm). Agar-mycelial plugs (diameter 5 mm) of *R. quercus-mongolicae* and *R. solani* were placed in the center of dishes for incubation at 25 °C (one day for *R. solani* and four days for *R. quercus-mongolicae*). On agar-free lids, paper discs with 10 mg carvacrol and thymol were placed. The controls only received 25% DMSO and 1% Tween 80. After treatment, treated and control PDA plates were incubated for one day at 25 °C. After incubation, plugs from PDA plates were stained with PBS 1 mL and PI (0.00625 µL for *R. quercus-mongolicae* and 0.05 µL for *R. solani*) for 15 min at 25 °C. Stained plugs were observed under CLSM (excitation: 561 nm, emission: 570–650 nm, SP8 X, Leica Microsystems).

2.8. Statistical Analysis

Antifungal activities of plant essential oils or their constituents were determined and transformed to arcsine square root values before analysis of variance. Treatment means were compared and separated by Tukey’s HSD test. Means ± standard error (SE) values for untransformed data were reported. We used R (ver. 3.5.1. R foundation for statistical computing, Austria) with R studio (ver. 1.1.456, R Studio Inc. MA, USA) for statistical analysis. Tukey’s HSD tests were performed with R package agricolae [13].

3. Results and Discussion

3.1. Fumigant Antifungal Activities of Lamiaceae Plant Essential Oils

Fumigant antifungal activities of 10 Lamiaceae plant essential oils against *R. quercus-mongolicae* are in Table 2. Among test oils, summer savory and thyme white essential oils showed the most potent antifungal activity. The activity was 91.50% for summer savory and 73.52% for thyme white oil at 1.25 mg/paper disc concentration and 54.40 and 50.18% at 0.625 mg/paper disc concentration, respectively. Spearmint plant essential oil had strong fumigant antifungal activity at 10 mg/paper disc concentration and moderate or weak fumigant antifungal activity at ≤5 mg/paper disc concentration. Antifungal activity of pennyroyal was 72.38% at 10 mg/paper disc concentration and 44.40% at 5 mg/paper disc concentration. Other plant essential oils had moderate or weak fumigant antifungal activity against *R. quercus-mongolicae* at 10 mg/paper disc concentration.
Fumigant antifungal activities of 10 Lamiaceae plant essential oils against *R. solani* are in Table 3. Among the tested oils, summer savory and thyme white essential oils showed 100% fumigant antifungal activity against *R. solani* at least 2.5 mg/paper disc concentration. The fumigant the antifungal activity of summer savory was higher than that of thyme white at 0.625 mg/paper disc concentration. However, antifungal activity of thyme white essential oil was stronger than that of summer savory at 0.3125 mg/paper disc concentration. Antifungal activities were 41.98% for summer savory and 54.38% for thyme white at 0.3125 mg/paper disc concentration. The Lamiaceae family is distributed widely around the world and has great economic value [14]. Fungicidal activity of thyme white essential oil against two phytopathogenic fungi, *Phytophthora cactorum*, and *Crypnonectria parasitica* has been reported [15]. Abdollahi et al. [16] insisted that thyme and summer savory plant essential oils exhibited strong antifungal activity against two postharvest fungi, *Penicillium digitatum*, and *Rhizopus stolonifer*. Although many antimicrobial activities of plant essential oils belonging to the Lamiaceae family have been reported [15–17], no studies report on the fumigant antifungal activity against *R. quercus-mongolicae* and *R. solani*.

Table 2. Fumigant antifungal activities of Lamiaceae plant essential oils against *R. quercus-mongolicae*.

| Essential Oils | Inhibition Rate (%), mean ± SE |
|---------------|--------------------------------|
|               | 10 | 5  | 2.5 | 1.25 | 0.625 | 0.3125 |
| Basil         | 58.42 ± 1.21b | 0d  | -3  |  -  |  -   |  -    |
| Hyssop        | 27.72 ± 2.38c | 0d  |  -  |  -  |  -   |  -    |
| Lavender      | 37.46 ± 8.53c | 0d  |  -  |  -  |  -   |  -    |
| Marjoram      | 37.30 ± 5.77c | 0d  |  -  |  -  |  -   |  -    |
| Patchouli     | 24.60 ± 4.27c | 0d  |  -  |  -  |  -   |  -    |
| Pennyroyal    | 72.38 ± 2.29b | 44.40 ± 0.35c | 34.62 ± 2.82b | 27.96 ± 2.70c | 0b   |
| Spanish sage  | 90.96 ± 1.77b | 0d  |  -  |  -  |  -   |  -    |
| Summer savory | 91.76 ± 0.75a | 57.50 ± 1.53b | 37.06 ± 1.94b | 15.98 ± 2.29d | 0b   |
| Thyme white   | 100a | 100a | 100a | 91.50 ± 1.60a | 54.40 ± 0.78a | 34.86 ± 4.01 |
|               | F9.40 = 60.81 | F9.40 = 71.10 | F3.16 = 4.69 | F3.16 = 60.81 | F3.16 = 60.81 | F3.16 = 60.81 |
|               | p < 0.0001 | p < 0.0001 | p < 0.0001 | p < 0.0001 | p < 0.0001 | p < 0.0001 |

1 mg/paper disc. 2 Means within a column followed by the same letters are not significantly different (Tukey’s HSD test). 3 Not tested.

Table 3. Fumigant antifungal activities of Lamiaceae plant essential oils against *R. solani*.

| Essential Oils | Inhibition Rate (%), mean ± SE |
|---------------|--------------------------------|
|               | 10 | 5  | 2.5 | 1.25 | 0.625 | 0.3125 |
| Basil         | 100a | 54.40 ± 1.04e | 20.20 ± 1.24d | 0d  |  -  |  -    |
| Hyssop        | 63.96 ± 1.33d | 36.64 ± 1.54e | 11.96 ± 1.14e | 0d  |  -  |  -    |
| Lavender      | 86.38 ± 0.64b | 62.86 ± 1.13c | 19.28 ± 1.98d | 0d  |  -  |  -    |
| Marjoram      | 0e  |  -   |  -   |  -  |  -  |  -    |
| Patchouli     | 0e  |  -   |  -   |  -  |  -  |  -    |
| Pennyroyal    | 100a | 87.96 ± 0.90b | 36.42 ± 2.29c | 21.96 ± 0.96c | 17.70 ± 0.70c | 0c   |
| Spanish sage  | 73.08 ± 0.95c | 57.50 ± 0.96d | 23.52 ± 0.88d | 0d  |  -  |  -    |
| Summer savory | 100a | 91.30 ± 0.89b | 44.18 ± 1.12b | 25.28 ± 1.07c | 0d  |  -    |
| Thyme white   | 100a | 100a | 100a | 91.76 ± 2.07a | 82.18 ± 1.00a | 41.98 ± 0.81b |
|               | F9.40 = 5264 | F7.32 = 631.3 | F3.16 = 730.9 | F3.16 = 1562 | F3.16 = 1926 | F2.12 = 346.4 |
|               | p < 0.0001 | p < 0.0001 | p < 0.0001 | p < 0.0001 | p < 0.0001 | p < 0.0001 |

1 mg/paper disc. 2 Means within a column followed by the same letters are not significantly different (Tukey’s HSD test). 3 Not tested.
3.2. Chemical Composition of Summer Savory and Thyme White Essential Oils

The chemical compositions of the active essential oils of summer savory and thyme white were analyzed using GC and GC-MS (Table 4). The most abundant compound in summer savory oil was carvacrol (39.84%) followed by γ-terpinene (34.63%), p-cymene (10.72%), α-terpinene (3.51%), and β-caryophyllene (2.40%). Other compounds were less than 2% of the oil. In thyme white essential oil, p-cymene (30.16%) was the most abundant compound followed by thymol (25.32%), γ-terpinene (11.44%), linalool (9.49%), α-pinene (5.25%), and carvacrol (3.46%). Other compounds, such as β-caryophyllene, camphene, and limonene were less than 2% each. The chemical compositions of summer savory and thyme white have been reported [18,19]. Moosavi-Nasab et al. [18] reported that the main components of S. hortensis oil were carvacrol at 54.7% and γ-terpinene at 26.9%. These results were consistent with our study. Zambonelli et al. [19] reported the chemical composition of Thymus vulgaris plant oils extracted from whole plants cultivated in different geographical areas of Italy and France. Thymol and p-cymene were two main components of thyme oil, although the most abundant component differed according to the area. The composition of carvacrol was low in all analyzed thyme oils. Although a few differences were observed in the chemical composition of the components in summer savory and thyme white essential oils between our study and prior studies [18,19], the major components were the same. The dates of harvest, storage period, extraction method, or climate could influence the chemical compositions of plant essential oils [19,20].

Table 4. Chemical composition of summer savory and thyme white plant essential oils.

| No. | Compound            | Retention Index | Composition Rate (%) |
|-----|---------------------|-----------------|----------------------|
|     |                     | DB-5 Summer Savory | Thyme White          |
| 1   | α-Pinene            | 930             | 1.34                 | 5.25                 |
| 2   | Camphene            | 946             | -                    | 1.41                 |
| 3   | β-Pinene            | 973             | 0.43                 | -                    |
| 4   | Myrcene             | 989             | 1.81                 | 3.35                 |
| 5   | α-Terpinene         | 1014            | 3.51                 | -                    |
| 6   | p-Cymene            | 1022            | 10.72                | 30.16                |
| 7   | Limonene            | 1027            | 0.63                 | 1.03                 |
| 8   | γ-Terpinene         | 1057            | 34.63                | 11.44                |
| 9   | Linalool            | 1100            | -                    | 9.49                 |
| 10  | Terpinen-4-ol       | 1179            | 0.50                 | -                    |
| 11  | Thymol              | 1290            | 0.21                 | 25.32                |
| 12  | Carvacrol           | 1298            | 39.84                | 3.46                 |
| 13  | β-Caryophyllene     | 1415            | 2.40                 | 1.75                 |
| 14  | Caryophyllene oxide | 1577            | -                    | 1.15                 |
| Sum |                     | 96.04           | 93.81                |

1 Not detected.

3.3. Fumigant Antifungal Activities of Constituents from Summer Savory and Thyme White Essential Oils

Fumigant antifungal activities of constituents from summer savory and thyme white plant essential oils against R. quercus-mongolicae and R. solani are in Table 5; Table 6. Among test compounds, thymol and carvacrol exhibited the strongest fumigant antifungal activities against the phytopathogenic fungi. In a test with R. quercus-mongolicae, fumigant antifungal activities were 100% for thymol and 81.28% for carvacrol at 0.625 mg/paper disc and 53.30% and 30.68% at 0.3125 mg/paper disc, respectively. Terpinen-4-ol had moderate antifungal activity at a concentration of more than 0.625 mg/paper disc, but weak antifungal activity at 0.3125 and no activity at 0.15625 mg/paper disc. Other constituents of summer savory and thyme white plant essential oils had weak or no antifungal activities at 2.5 and 1.25 mg/paper disc concentration. In a test with R. solani, thymol and carvacrol showed a 100% inhibition rate at concentration more than 0.625 mg/paper disc concentration. Inhibition
was 78.64% for thymol and 81.96% for carvacrol at 0.3125 mg/paper disc concentration and 51.28% and 55.50% at 0.15625 mg/paper disc concentration, respectively. Fumigant antifungal activities of terpinen-4-ol, (+)-α-pinene, (−)-α-pinene, and linalool ranged from 71.30–42.62% at 2.5 mg/paper disc concentration, and less than 40% at 1.25 mg/paper disc concentration. Other constituents of summer savory and thyme white essential oils showed weak or no fumigant antifungal activities against R. solani at 2.5 mg/paper disc concentration. In our study, thymol and carvacrol showed very strong fumigant antifungal activities against the two phytopathogenic fungi. Thymol and its isomer carvacrol are phenolic monoterpenoids found in the Lamiaceae family. Reported biological effects for thymol and carvacrol include antispasmodic, antioxidant, anti-inflammatory, and anticarcinogenesis activity [21,22]. Among identified constituents of thyme white and summer savory plant essential oils, the antifungal activity of phenolic monoterpenes (thymol and carvacrol) was much higher than the activity of monoterpane hydrocarbons (α-pinene, camphene, β-pinene, myrcene, α-terpinene, p-cymene, limonene, γ-terpin), alcohols (terpinen-4-ol and linalool) and sesquiterpenes (β-caryophyllene, caryophyllene oxide). These results were very similar to the results of Kim et al. [15], that phenol (thymol, carvacrol, phenylpropanoids) compounds generally exhibited strong fumigant antifungal activity against the two phytopathogenic fungi, Phytophthora cactorum, and Cryphonectria parasitica, compared to hydrocarbon monoterpenes.

| Compounds | Inhibition Rate (% mean ± SE) |
|-----------|--------------------------------|
|           | 2.5 ± 1                        |
|           | 1.25                           |
|           | 0.625                          |
|           | 0.3125                         |
|           | 0.15625                        |
| (+)-α-Pinene | 0e                              |
| (−)-α-Pinene | 0e                             |
| (+)-Camphene  | 0e                             |
| (−)-Camphene  | 0e                             |
| (+)-β-Pinene  | 0e                             |
| (−)-β-Pinene  | 0e                             |
| α-Terpine   | 0e                             |
| β-Pinene    | 0e                             |
| (+)-Terpinene | 0e                            |
| (−)-Terpinene | 0e                            |
| Terpinen-4-ol | 15.48 ± 2.25d                 |
| Linalool    | 74.84 ± 0.89b                  |
| Thymol      | 59.28 ± 3.37b                  |
| Carvacrol   | 47.06 ± 1.47c                  |
| Caryophyllene oxide | 21.74 ± 1.04c             |
| F17,72 = 1601 | 28.40 ± 2.45a              |

1 mg/paper disc. 2 Means within a column followed by the same letters are not significantly different (Tukey’s HSD test). 3 Not tested.
Table 6. Fumigant antifungal activities of constituents from summer savory and thyme white essential oils against R. solani.

| Compounds             | Inhibition Rate (%), mean ± SE |
|-----------------------|--------------------------------|
|                       | 2.5 1 | 1.25 | 0.625 | 0.3125 | 0.15625 |
| (+)-α-Pinene          | 47.52 ± 1.84c 2 | 33.52 ± 1.37b | 21.52 ± 1.35b | 0b | - |
| (-)-α-Pinene          | 47.30 ± 1.88c | 18.38 ± 1.10c | 0d | - | - |
| (+)-Camphene          | 0f    | -    | -    | -     | - |
| (-)-Camphene          | 0f    | -    | -    | -     | - |
| (+)-β-Pinene          | 0f    | -    | -    | -     | - |
| (-)-β-Pinene          | 0f    | -    | -    | -     | - |
| Myrcene               | 0f    | -    | -    | -     | - |
| α-Terpineene          | 0f    | -    | -    | -     | - |
| p-Cymene              | 0f    | -    | -    | -     | - |
| (+)-Limonene          | 22.64 ± 0.44d | 0d | - | - | - |
| (-)-Limonene          | 0f    | -    | -    | -     | - |
| γ-Terpineene          | 0f    | -    | -    | -     | - |
| Linalool              | 42.62 ± 1.74c | 0d | - | - | - |
| Terpinen-4-ol         | 71.30 ± 1.84b | 32.40 ± 1.29b | 15.28 ± 1.32c | 0b | - |
| Thymol                | 100a  | 100a | 100a | 78.64 ± 1.34a | 51.28 ± 6.98 |
| Carvacrol             | 100a  | 100a | 100a | 81.96 ± 3.23a | 55.50 ± 0.92 |
| β-Caryophyllene       | 15.74 ± 1.77e | 0d | - | - | - |
| Caryophyllene oxide   | 0f    | -    | -    | -     | - |

F17,72 = 1338, F7,32 = 2997, F4,30 = 3331, F3,16 = 703.9, F1,8 = 0.359
p < 0.0001, p < 0.0001, p < 0.0001, p < 0.0001, p = 0.566

1 mg/paper disc. 2 Means within a column followed by the same letters are not significantly different (Tukey’s HSD test). 3 Not tested.

Carvacrol and thymol have delocalized electron and hydroxyl groups. Utlee et al. [23] suggested that the hydroxyl group and delocalized electron of carvacrol and thymol are essential for antifungal activity. Delocalized electrons in double bonds facilitate carvacrol and thymol releasing cations from hydroxyl groups when undissociated carvacrol and thymol diffuse through the cytoplasmic membrane to the cytoplasm. Carvacrol and thymol could then return to the outside of cells undissociated by carrying potassium or other cation from the cytoplasm. This hypothesis is supported by the observation of the efflux of K⁺ and the influx of H⁺ in Bacillus cereus treated with carvacrol [24]. The decrease in the ΔpH might eliminate the proton motive force for adenosine triphosphate (ATP) synthesis in the cell. Utlee et al. [23] reported that the antifungal activities of carvacrol methyl ester and p-cymene, a precursor of carvacrol, were lower than carvacrol because of a lack of hydroxyl groups. Antifungal activity was also lower for menthol than carvacrol because of a lack of delocalized electrons in menthol. Our study had the same result. Phenolic monoterpenes with hydroxyl groups and delocalized electrons exhibited very strong fumigant antifungal activity against R. querqus-mongolicae and R. solani compared to compounds without hydroxyl groups and delocalized electrons. The only difference in the chemical structure of thymol and carvacrol is the position of hydroxyl groups. In our study, thymol had higher antifungal activity than carvacrol against R. querqus-mongolicae. However, no significant difference was observed in the antifungal activity against R. solani. Lambert et al. [25] reported the hydroxyl group position of thymol and carvacrol did not cause differences in antimicrobial activity. However, positional differences in the hydroxyl group of thymol and carvacrol caused slight changes in the antifungal activity against Candida isolates in another study [26]. Our study and previous studies indicate that the effect on antifungal activity on the hydroxyl position of thymol and carvacrol varies according to fungal species.

3.4. Effect of Carvacrol and Thymol on Reactive Oxygen Species Generation

Intracellular ROS generation of R. querqus-mongolicae and R. solani treated with carvacrol and thymol is in Figure 2. A strong fluorescence signal was detected from R. querqus-mongolicae and R. solani treated with carvacrol and thymol (Figure 2d,f,j,l) compared to controls (Figure 2b,h). Representative molecules of ROS include hydroxyl radical (•OH), superoxide anion (O₂⁻), and hydrogen peroxide...
(H₂O₂) [27]. ROS are important in cell signaling and homeostasis. However, the antibacterial or antifungal action of many antibiotics and antifungal agents is related to the accumulation of ROS in bacteria and fungi, especially in Candida species [27–30]. Studies suggest that antifungal agents induce ROS formation in filamentous fungi such as Rhizoctonia, Fusarium, and Aspergillus species [31,32]. ROS-related antifungal activities of plant essential oils or their constituents are documented [33,34]. Shen et al. [33] reported that thymol treatment induces ROS and nitric oxide in Aspergillus flavus spores, causing spore death. Tian et al. [34] demonstrated that ROS is an important mediator of the antifungal action of dill oil against A. flavus. Our study and prior studies indicated that thymol and carvacrol induce ROS in R. querqus-mongolicae and R. solani, and accumulation of ROS might cause fungal cell damage. However, further research is necessary to elucidate the pathway of ROS generation by thymol and carvacrol in R. querqus-mongolicae and R. solani.

![Confocal laser scanning microscopy images of ROS generation](image-url)

**Figure 2.** Confocal laser scanning microscopy images of ROS generation. Bright mode (a) and confocal image (b) of untreated R. querqus-mongolicae. Bright mode (c) and confocal image (d) of R. querqus-mongolicae treated with thymol. Bright mode (e) and confocal image (f) of R. querqus-mongolicae treated with carvacrol. Bright mode (g) and confocal image (h) of untreated R. solani. Bright mode (i) and confocal image (j) of R. solani treated thymol. Bright mode (k) and confocal image (l) of R. solani treated with carvacrol. Scale bar = 50 µm.
3.5. Effect of Carvacrol and Thymol on Cell Membrane Integrity

CLSM images of *R. quercus-mongolicae* and *R. solani* stained with PI are shown in Figure 3. A strong fluorescence signal indicating dead cells stained with PI was seen for *R. quercus-mongolicae* and *R. solani* treated with carvacrol and thymol (Figure 3d,f,j,l). No similar fluorescence signal was observed in untreated *R. quercus-mongolicae* and *R. solani* (Figure 3b,h). PI molecules penetrate only corrupted cell membranes to stain cells [35]. This dye is used for testing plasma membrane integrity [36]. Xu et al. [37] observed PI-stained mycelia of *Alternaria aternata* treated with cinnamaldehyde, but no staining in untreated *A. aternata*. CLSM images of *R. quercus-mongolicae* and *R. solani* stained with PI confirmed that thymol and carvacrol damaged the cell membranes of *R. quercus-mongolicae* and *R. solani*. Based on our results, we concluded that ROS generated by thymol and carvacrol damaged cell membranes of *R. quercus-mongolicae* and *R. solani*, and this might cause cell death. However, we did not determine the ROS generation pathway in this study. Future studies on ROS generation pathways are necessary to determine the antifungal mode of action of thymol and carvacrol against *R. quercus-mongolicae* and *R. solani*.

![Confocal laser scanning microscopy images of cell membrane integrity.](image_url)

**Figure 3.** Confocal laser scanning microscopy images of cell membrane integrity. Bright mode (a) and PI stained (b) of untreated *R. quercus-mongolicae*. Bright mode (c) and PI-stained (d) of *R. quercus-mongolicae* treated with thymol. Bright mode (e) and PI stained (f) of *R. quercus-mongolicae* treated with carvacrol. Bright mode (g) and PI-stained (h) of untreated *R. solani*. Bright mode (i) and PI-stained (j) of *R. solani* treated with thymol. Bright mode (k) and PI-stained (l) of *R. solani* treated with carvacrol. Scale bar = 50 μm.
4. Conclusions

Thyme white and summer savory plant essential oils showed strong fumigant antifungal activity against two phytopathogenic fungi, *R. quercus-mongolicae* and *R. solani*. Among the identified constituents from the two plant essential oils, fumigant antifungal activities of two phenolic monoterpenes, thymol and carvacrol, were stronger than the activities of monoterpene hydrocarbons, alcohols, and sesquiterpenes. We concluded that intracellular ROS generated by thymol and carvacrol damaged cell membranes and this might have caused cell death of *R. quercus-mongolicae* and *R. solani*. The practical use of plant essential oils and their constituents as control agents against the phytopathogenic fungi *R. quercus-mongolicae* and *R. solani* will require the development of formulations and toxicity tests for non-target organisms.

Author Contributions: Conceptualization—J.-E.K. and I.-K.P.; Funding acquisition—I.-K.P.; Investigation—J.-E.K., J.-E.L., M.-J.H., S.-C.L., S.-M.S. and J.H.K.; Supervision—I.-K.P.; Writing—original draft—J.-E.K. and I.-K.P.; Writing—review & editing—J.-E.K. and I.-K.P.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by Korea government (MSIT) (NRF-2018R1D1A1B07047458).

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

References

1. Kim, J.; Lee, S.G.; Shin, S.C.; Kwon, Y.D.; Park, I.K. Male-produced aggregation pheromone blend in *Platypus koryoensis*. *J. Agric. Food Chem.* 2009, 57, 1406–1412. [CrossRef] [PubMed]
2. Park, I.K.; Nam, Y.; Seo, S.T.; Kim, S.W.; Jung, C.S.; Han, H.R. Development of a mass trapping device for the ambrosia beetle, *Platypus koryoensis*, an insect vector of oak wilt disease in Korea. *J. Asia-Pacific Entomol.* 2016, 19, 39–43. [CrossRef]
3. Kim, K.H.; Choi, Y.J.; Seo, S.T.; Shin, H.D. Raffaelea quercus-mongolicae sp. Nov. associated with *Platypus koryoensis* on oak in Korea. *Mycotaxon* 2009, 110, 189–197. [CrossRef]
4. Lee, B.Y.; Chung, Y.J. *Insect pests of trees and shrubs in Korea*; Seongandang: Seoul, Korea, 1997; p. 145.
5. Lee, J.E. Antimicrobial Activity and Mode of Action of Four Plant Essential Oils, Their Constituents and trans-Cinnamaldehyde Derivatives against Three Tree Pathogens. Master’s Thesis, Seoul National University, Seoul, Korea, 2018.
6. Stępniewska-Jarosz, S.; Mańka, M.; Asiegbu, F. Studies on anastomosis groups of *Rhizoctonia solani* isolates causing disease in two forest nurseries in Poland. *For. Pathol.* 2006, 36, 97–109. [CrossRef]
7. Kim, J.K.; Koh, S.H.; Koo, C.D.; Kim, K.W.; Kim, J.K.; Kim, J.J.; Park, G.S.; Park, Y.C.; Park, I.K.; Byun, B.K.; et al. *Forest Protection*; Haengmoonsa: Seoul, Korea, 2019.
8. Isman, M.B. Plant essential oils for pest and disease management. *Crop Prot.* 2000, 19, 603–608. [CrossRef]
9. Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M. Biological effects of essential oils—A review. *Food Chem. Toxicol.* 2008, 46, 446–475. [CrossRef]
10. Ribeiro-Santos, R.; Andrade, M.; Melo, N.R.D.; Sanches-Silva, A. Use of essential oils in active food packing: Recent advances and future trends. *Trends Food Sci. Tech.* 2017, 61, 132–140. [CrossRef]
11. Kim, E.; Park, I.K. Fumigant antifungal activity of Myrtaceae essential oils and constituents from *Leptospermum petersonii* against three *Aspergillus* species. *Molecules* 2012, 17, 10459–10469. [CrossRef]
12. Van den Dool, H.; Kratz, P.D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr. A* 1965, 11, 463–471. [CrossRef]
13. Mendiburu, F.D. Agricola: Statistical Procedures for Agricultural Research Version 1.2-8. Available online: http://tarwi.lamolina.edu.pe/~fjmendiburu (accessed on 3 October 2019).
14. Trivillini, A.; Lucchesini, M.; Maggini, R.; Mosadegh, H.; Villamarin, T.S.S.; Vernieri, P.; Mensuali-Sodi, A.; Pardossi, A. Lamiaceae phenols as multifaceted compounds: Bioactivity industrial prospects and role of “positive-stress” *Ind. Crops Prod.* 2016, 83, 241–254. [CrossRef]
15. Kim, J.; Lee, Y.S.; Lee, S.G.; Shin, S.C.; Park, I.K. Fumigant antifungal activity of plant essential oils and components from West Indian bay (*Pimenta racemose*) and thyme (*Thymus vulgaris*) oils against two phytopathogenic fungi. *Flavour Fragr. J.* 2008, 23, 272–277. [CrossRef]
16. Abdollahi, A.; Hassani, A.; Ghostaa, Y.; Meshkatalsadat, M.H.; Shabani, R. Screening of antifungal properties of essential oils extracted from sweet basil, fennel, summer savory and thyme against postharvest phytopathogenic fungi. J. Food Saf. 2011, 31, 350–356. [CrossRef]

17. Kordali, S.; Cakir, A.; Ozer, H.; Cakmakci, R.; Kesdek, M.; Mete, E. Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish Origanum acutifolia and its three components, carvacrol, thymol and p-cymene. Bioresour. Technol. 2008, 99, 8788–8795. [CrossRef] [PubMed]

18. Moosavi-Nasab, M.; Saharkhiz, M.J.; Ziae, E.; Moayedi, F.; Koshani, R.; Azizu, R. Chemical compositions and antibacterial activities of five selected aromatic plants essential oils against food-borne pathogens and spoilage bacteria. J. Essent. Oil Res. 2016, 28, 241–251. [CrossRef]

19. Zambonelli, A.; D’Aulrio, A.Z. Chemical composition and fungicidal activity of commercial essential oils of Thymus vulgaris L. J. Essent. Oil Res. 2004, 16, 69–74. [CrossRef]

20. Galabosi, B.; Peura, P. Agrobotanical features and oil content of wild and cultivated forms of caraway (Carum carvi L.). J. Essent. Oil. Res. 1996, 8, 389–397. [CrossRef]

21. Salehi, B.; Mishr, A.P.; Shukla, I.; Sharifi-Rad Contreas, M. d. M.; Sgura-Carretro, A.; Fathi, H.; Nasrabadi, N.N.; Kobafard, F.; Sharifi-Rad, J. Thymol, thyme, and other plant sources: Health and potential uses. Phytother. Res. 2018, 32, 1688–1706. [CrossRef]

22. Sharifi-Rad, M.; Varoni, E.M.; Iriti, M.; Martorell, M.; Setzer, W.N.; Contreras, M. d. M.; Salehi, B.; Soltani-Nejad, A.; Rajabi, S.; Tajbakhsh, M.; et al. Carvacrol and human health: A comprehensive revies. Phytther. Res. 2018, 32, 1675–1687. [CrossRef]

23. Ultee, A.; Bennik, M.H.J.; Moezelaar, R. The phenolic hydroxy group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Appl. Environ. Microbiol. 2002, 68, 1561–1568. [CrossRef]

24. Ultee, A.; Kets, E.P.W.; Smid, E.J. Mechanisms of action of carvacrol on the food-borne pathogen Bacillus cereus. Appl. Environ. Microbiol. 1999, 65, 4606–4610.

25. Lambert, R.J.W.; Skandamis, P.N.; Coote, P.J.; Nychas, G.J. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 2001, 91, 453–462. [CrossRef] [PubMed]

26. Ahmad, A.; Khan, A.; Akhtar, F.; Yousuf, S.; Xess, I.; Khan, L.A.; Manzoor, N. Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against Candida. Eur. J. Clin. Microbiol. Infect Dis. 2011, 30, 41–50. [CrossRef] [PubMed]

27. Dwyer, D.J.; Kohanski, M.A.; Collins, J.J. Role of reactive oxygen species in antibiotic action and resistance. Curr. Opin. Microbiol. 2009, 12, 482–489. [CrossRef] [PubMed]

28. Kohanski, M.A.; Dwyer, D.J.; Hayete, B.; Lawrence, C.A.; Collins, J.J. A common mechanism of cellular death induced by bacteriadicidicrobial. Cell 2007, 130, 797–810. [CrossRef]

29. Mello, E.O.; Ribeiro, S.F.E.; Carvalho, A.O.; Santos, I.S.; Da Cunha, M.; Santa-Catarina, C.; Gomes, V.M. Antifungal activity of PvD1 defensin involves plasma membrane permeabilization, inhibition of medium acidification, and induction of ROS in fungi cells. Curr. Biol. 2011, 21, 1209–1217. [CrossRef]

30. Yan, L.; Li, M.; Cao, Y.; Gao, P.; Cao, Y.; Wang, Y.; Jiang, Y. The alternative oxidase of Candida albicans causes reduced fluconazole susceptibility. J. Antimicrob. Chemother. 2004, 59, 764–773. [CrossRef]

31. Cheng, J.J.; Park, T.S.; Choi, L.G.; Fischl, A.S.; Ye, X.S. Induction of apoptosis by sphingoid long-chain bases in Aspergillus nidulans. Mol. Cell Biol. 2003, 23, 163–177. [CrossRef]

32. Qi, G.; Zhu, F.; Du, P.; Yang, X.; Qiu, D.; Yu, Z.; Chen, J.; Zhao, X. Lipopeptide induces apoptosis in fungal cells by a mitochondria-dependent pathway. Peptides 2010, 31, 1978–1986. [CrossRef]

33. Shen, Q.; Zhou, W.; Li, H.; Hu, L.; Mo, H. ROS involves the fungicidal actions of thymol against spores of Aspergillus flavus via the induction of nitric oxide. PLoS ONE 2016, 11, e0155647. [CrossRef]

34. Tian, J.; Ban, X.; Zeng, H.; He, J.; Chen, Y.; Wang, Y. The mechanism of antifungal action of essential oil from dill (Anethum graveolens L.) on Aspergillus flavus. PLoS ONE 2012, 7, e30147. [CrossRef]

35. Jin, Y.; Zhang, T.; Samaranayake, Y.H.; Fang, H.H.P.; Yip, H.K.; Samaranayake, L.P. The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in Candida albicans biofilms. Mycopathologia 2005, 159, 353–360. [CrossRef] [PubMed]
36. Wang, T.M.; Shi, G.X.; Shao, J.; Wu, D.Q.; Yan, Y.Y.; Zhang, M.X.; Cui, Y.Y.; Wang, C.Z. In vitro antifungal activity of baicalin gains *Candida albicans* biofilms via apoptotic induction. *Microb. Pathog.* 2015, 87, 21–29. [CrossRef] [PubMed]

37. Xu, L.; Tao, N.; Yang, W.; Jing, G. Cinnamaldehyde damaged the cell membrane of *Alternaria alternata* and induced the degradation of mycotoxins in vivo. *Ind. Crop. Prod.* 2018, 112, 427–433. [CrossRef]