Antibacterial Activity of Cinnamaldehyde and Estragole Extracted from Plant Essential Oils against \textit{Pseudomonas syringae pv. actinidiae} Causing Bacterial Canker Disease in Kiwifruit

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\textit{Pseudomonas syringae pv. actinidiae} (Psa) causes bacterial canker disease in kiwifruit. Antibacterial activity of plant essential oils (PEOs) originating from 49 plant species were tested against Psa by a vapor diffusion and a liquid culture assays. The five PEOs from \textit{Pimenta racemosa}, \textit{P. dioica}, \textit{Melaleuca linariifolia}, \textit{M. cajuputii}, and \textit{Cinnamomum cassia} efficiently inhibited Psa growth by either assays. Among their major components, estragole, eugenol, and methyl eugenol showed significant antibacterial activity by only the liquid culture assay, while cinnamaldehyde exhibited antibacterial activity by both assays. The minimum inhibitory concentrations (MICs) of estragole and cinnamaldehyde by the liquid culture assay were 1,250 and 2,500 ppm, respectively. The MIC of cinnamaldehyde by the vapor diffusion assay was 5,000 ppm. Based on the formation of clear zones or the decrease of optical density caused by these compounds, they might kill the bacterial cells and this feature might be useful for managing the bacterial canker disease in kiwifruit.

\textbf{Keywords:} cinnamaldehyde, estragole, liquid culture assay, \textit{Pseudomonas syringae pv. actinidiae}, vapor diffusion assay

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environmentally friendly way. For instance, Wilson et al. (1997) showed that essential oils from species of *Allium* and *Capsicum* have antifungal activities against *Botrytis cinerea*, which causes gray mold on grapes, strawberries, and tomatoes. In the case of plant-pathogenic bacteria, essential oils extracted from the fruits of *Cuminum cyminum* L. and *Carum carvi* L. were observed to have antibacterial activities against the genera *Clavibacter*, *Erwinia*, *Xanthomonas*, *Ralstonia*, and *Agrobacterium*, which cause various plant diseases (Iacobellis et al., 2005). Kotan et al. (2010) demonstrated that the essential oils of *Satureja spicigera* and *Thymus fallax* inhibit bacterial growth of the plant-pathogenic bacteria of *Clavibacter michiganensis* subsp. *michiganensis*, *Enterobacter*, *Pseudomonas sp.*, and *Xanthomonas sp.*, which cause diseases in tomatoes, potatoes, lettuce, apricots, cabbage, and so on. Recent studies have shown that some PEOs inhibited the growth of antibiotic-resistant pathogens (Mulyaningsih et al., 2010). In this study, the antibacterial activities of PEOs from forty-nine species against Psa were examined in order to determine how effective these PEOs might be for managing bacterial canker disease in kiwifruit.

The strains of Psa used in this study were obtained from Dr. Young Jin Koh at Sunchon National University. KBE9 and YCS3 strains of Psa, classified as biovar 2, and the strain SYS1, classified as biovar 3, were used in this study (Koh et al., 2010, 2012). These were cultured at 26°C on tryptic soy agar (TSA) or broth (TSB). And the forty-nine species of PEOs used in this experiment are listed in Table 1. Commercially available essential oils were purchased from G.R. Davis (Riverstone, NSW, Australia), Jin Aromatics (New York, NY, USA) and Osahdhi (Weinstrasse, Bühl/Baden, Germany). Other PEOs were extracted by using

| Plant species           | Plant part | Yield* or Sources† (%) |
|-------------------------|------------|------------------------|
| Acorus gramineus        | Root       | 0.07                   |
| Ammi visnaga            | Flowering plant | Oshadhi           |
| Amomum kravanh          | Seed       | 5.5                    |
| Anethum graveolens      | Seed       | Jin Amore              |
| Artemisia capillaris    | Whole plant| 0.21                   |
| Asarum sieboldii        | Root       | 0.45                   |
| Calacalia ainsliaeflora | Fruit      | 0.89                   |
| Carum carvi             | Seed       | 0.45                   |
| Cinnamomum cassia       | Bark       | 0.17                   |
| Coriandrum sativum      | Herb       | Oshadhi                |
| Coriandrum sativum      | Fruit      | Oshadhi                |
| Cuminum cyminum         | Seed       | Jin Aromatics          |
| Eucalyptus citriodora   | Leaf       | G.R. Davis             |
| Eucalyptus dives        | Leaf       | G.R. Davis             |
| Eucalyptus globulus     | Leaf       | G.R. Davis             |
| Eucalyptus polybractea  | Leaf       | G.R. Davis             |
| Eucalyptus radiate      | Leaf       | G.R. Davis             |
| Eucalyptus smithii      | Leaf       | G.R. Davis             |
| Juniperus chinensis     | Leaf       | 0.35                   |
| Juniperus chinensis var. globosa | Leaf | 0.31               |
| Juniperus chinensis var. kaizuka | Leaf | 0.23          |
| Juniperus chinensis var. sargentii | Leaf | 0.35               |
| Juniperus chinensis var. sargentii | Leaf | 0.32               |
| Juniperus rigida        | Leaf       | 0.14                   |
| Kaempferia galangal     | Root       | 0.36                   |
| Kunzea ericoides        | Leaf       | Oshadhi                |
| Leptospermum petersonii | Leaf       | G.R. Davis             |
| Leptospermum scoparium   | Leaf       | Oshadhi                |
| Melaleuca cajuputii     | Leaf       | Jin Amore              |
| Melaleuca distisiflora  | Leaf       | G.R. Davis             |
| Melaleuca linarifolia   | Leaf       | G.R. Davis             |
| Melaleuca quinquenervia | Leaf       | G.R. Davis             |
| Melaleuca uncinata      | Leaf       | G.R. Davis             |
| Myristica fragrans      | Fruit      | 2.9                    |
| Myrtle communis         | Flowering plant | Oshadhi           |
| Paeonia suffruticosa    | Root       | 0.54                   |
| Pimenta dioica          | Berries    | Jin Amore              |
| Pimenta racemose        | Leaf       | Jin Aromatics          |
| Pinus densiflora        | Leaf       | 0.12                   |
| Pinus koraiensis        | Leaf       | 0.33                   |

*All numbers indicate yield (w/w).
†Company names to purchase oils; Osahdhi (Weinstrasse, Bühl/Baden, Germany), G.R. Davis (Riverstone, NSW, Australia), Jin Amore (New York, NY, USA) and Jin Aromatics (Anyang, Korea).
steam-distillation. Coniferous plant species were collected at Hongneung arboretum in Seoul, and medicinal plant species were purchased at Kyungdong medicinal market in Seoul. In brief, plant parts (about 900–3,220 g for each sample), which have been used as aroma sources or have been known to contain oils, were powdered in a blender and then diluted with distilled water (800 ml) in a 2 l flask and steam distilled (100°C). Yields of essential oils extracted via steam distillation in the laboratory are given in Table 1.

The statistical analysis was performed using the SAS software, version 5.1 (SAS Institute, Cary, NC, USA), and showed the validity of the results obtained in this study. All of the screening assays were repeated three times and analyzed by Duncan’s multiple range test ($P < 0.05$, $n = 3$).

To determine the PEOs that show antibacterial activity against Psa, PEOs were purchased or extracted from various plant species as listed in Table 1, and their antibacterial activity was determined by two different assays, the vapor diffusion assay and the liquid culture assay. For the vapor diffusion assay, Psa strains were streaked on a TSA medium plate (petri dish of 90 mm diameter) from outside to inside, as shown in Fig. 1A (Song et al., 2013). A filter paper disc ($8 \times 0.7$ mm; Advantec MFS Inc., Tokyo, Japan) was placed at the center of the plate cover, and 10 µl of undiluted 100% PEOs (or extracted compounds) were dropped on the filter paper disc. The plate cover with the oil disc was covered with the streaked bottom medium plate, and the combined plate was kept upside-down. After sealing with parafilm, the plate was incubated for 48 hours at 26°C. The length of the clean zones from the center of plate to the bacterial colonies was measured as an indication of antibacterial activity. Among the forty-nine PEOs tested, three PEOs from Melaleuca linariifolia, M. cajuputii, and Cinnamomum cassia showed significant antibacterial activities against three Psa strains, as measured by the vapor diffusion assay (Fig. 1). The PEO from C. cassia showed the strongest antibacterial activity and its average inhibition zone length was between 1.2 and 2.2 cm. The strength of the antibacterial activities were not statistically different between the Psa strains because the inhibition lengths were statistically similar. For the PEOs coming from M. linariifolia and M. cajuputii, the average length of the inhibition zones were not larger than 1 cm. Other PEOs, like C. sativum, did not show significant antibacterial activity against Psa.

For the liquid culture assay, as previously described (Song et al., 2013), the bacterial pre-cultures were prepared in liquid TSB at 26°C overnight. These were diluted to 1/100 in TSB, and cultured at 26°C until a value of 0.5 for the optical density measured at a wavelength of 600 nm (OD$_{600}$; equivalent to $5 \times 10^6$ colony-forming unit/ml) was obtained, as measured with NanoDrop (Thermo Scientific, Wilmington, DE, USA). The PEOs (or extracted compounds) were diluted to 10% dimethyl sulfoxide (DMSO) for 5,000 ppm as a final concentration. The 180 µl of bacterial suspension were mixed with 20 µl of each PEO or each compound at the 96 well plate. The negative control for this assay was the bacterial culture that was amended with the same amount of 10% DMSO only. Blank wells were also measured through liquid TSB. The plates were kept shaking at 120 rpm and 26°C. Bacterial growth was determined at OD$_{600}$ using TECAN Infinite M200 (Tecan Group Inc., Männedorf, Switzerland), and was measured at 0, 1, 2, 4, 8, 16, and 24 hours after treatment with the PEOs (or extracted components). Among

![Image](image-url)
Table 2. List and composition ratio of separated components of three plant essential oils

| Components            | Composition ratio (%) |
|-----------------------|-----------------------|
|                       | Cinnamomum cassia     | Pimenta racemosa   | Pimenta dioica |
| Cinnamadehyde         | 90.00                 | 46.25              | 86.44          |
| Eugenol               | 25.88                 | 77.70              |                |
| Myrcene               | 10.49                 | 0.38               | 0.18           |
| 4-Allylphenol         | 1.05                  | 0.26               | 0.75           |
| β-Caryophyllene       | 0.30                  | 0.33               | 0.13           |
| Limonene              | 0.13                  | 0.93               | 0.99           |
| Linalool              | 0.05                  | 0.14               |                |
| Methyl eugenol        | 0.05                  | 0.05               |                |
| α-Pinene              | 0.05                  | 0.05               |                |
| 6-Methyl-5-hepten-2-one| 0.05                  | 0.05               |                |
| 1.8-Cineole           | 0.05                  | 0.05               |                |
| α-Phellandrene        | 0.05                  | 0.05               |                |
| β-Cymene              | 0.05                  | 0.05               |                |
| α-Humulene            | 0.05                  | 0.05               |                |
| Terpinen-4-ol         | 0.05                  | 0.05               |                |
| α-Terpineol           | 0.05                  | 0.05               |                |
| Estragole             | 0.05                  | 0.05               |                |
| Geraniol              | 0.05                  | 0.05               |                |
| Copaene               | 0.05                  | 0.05               |                |
| Sum                   | 90.00                 | 93.55              | 99.28          |
the PEOs tested, the \textit{C. cassia} PEO showed the strongest antibacterial activity measured by the liquid culture assay because it reduced bacterial concentration by up to about $OD_{600}$ 0.1 (Fig. 2) and made bacterial cultures appear clear 24 hours after treatment. PEOs from \textit{Pimenta racemosa} and \textit{P. dioica} also showed significant antibacterial activity as measured by the liquid culture assay. These results indicate that five PEOs can inhibit growth of Psa strains, regardless of biovar 2 or 3, \textit{in vitro}. Further, the results indicate that the assay method used might affect the efficiency in finding the effective PEOs against Psa.

PEOs are mixtures of many compounds, and their antibacterial activities generally come from particular components. To find the particular components responsible for antibacterial activities, the components of the three PEOs originating from \textit{C. cassia}, \textit{P. racemosa}, and \textit{P. dioica}, which showed the greatest antibacterial activity against Psa strains as measured by either the vapor diffusion assay or the liquid culture assay, were separated. The components found in the PEOs and their corresponding composition ratios are listed in Table 2. All of these components were tested by the vapor diffusion assay and the liquid culture assay in order to find the compound(s) responsible for the antibacterial activity against Psa. Cinnamaldehyde, which is the major component in \textit{C. cassia}, showed significant antibacterial activities in the vapor diffusion assay against Psa strains (Fig. 3). The length of the inhibitory zone averaged between 2.33 and 3.13 cm. Cinnamaldehyde also inhibited bacterial growth in the liquid culture assay (Fig. 4). Eugenol, which is the major component of both PEOs originated from \textit{P. racemosa} and \textit{P. dioica}, also showed significant antibacterial activity as measured by the liquid culture assay. These results indicate that five PEOs can inhibit growth of Psa strains, regardless of biovar 2 or 3, \textit{in vitro}. Further, the results indicate that the assay method used might affect the efficiency in finding the effective PEOs against Psa.

**Fig. 3.** Antibacterial activity of cinnamaldehyde and three phenolic compounds by the vapor diffusion assay against \textit{Pseudomonas syringae pv. actinidiae} (Psa). The assays were performed as described in Fig. 1. The letters to the right of the numbers show the results from Duncan’s multiple range test ($P < 0.05, n = 3$). Each error bar represents a standard error.

**Fig. 4.** Antibacterial activity of cinnamaldehyde and three phenolic compounds by the liquid culture assay against \textit{Pseudomonas syringae pv. actinidiae} (Psa). The assays were performed as described in Fig. 2. The four indicated species of separated components showed significant antibacterial activity against the Psa strains (A, KBE9; B, YCS3; C, SYS1). The letters to the right of the numbers show the results from Duncan’s multiple range test ($P < 0.05, n = 2$). Each error bar represents a standard error. $OD_{600}$, the optical density measured at a wavelength of 600 nm; Con, control.
dioica, showed significant antibacterial activity against Psa strains in the liquid culture assay. Methyl eugenol, which is present in both PEOs (Table 2), also showed significant antibacterial activity against Psa in the liquid culture assay. Interestingly, although estragole is a very minor component of the P. racemose PEO, it showed the strongest activity in the liquid culture assay regardless of Psa strains (Fig. 4). None of the other components showed antibacterial activity against Psa strains.

The minimum inhibitory concentration (MIC) is important for the application of PEOs and their components exhibiting antibacterial activity against Psa. Thus, the MICs of cinnamaldehyde and estragole were determined. The PEO components showing effective antibacterial activities were diluted twofold with 10% DMSO and were used for the vapor diffusion assay (10,000, 5,000, 2,500, 1,250, 625, and 312.5 ppm) or the liquid culture assay (2,500, 1,250, 625, 312.5, and 78.125 ppm). The lowest concentrations of the compounds showing antibacterial activity were determined as the MIC in both assays. The amount of cinnamaldehyde serially diluted was halved, starting from 10,000 down to 312.5 ppm, and the samples were tested by the vapor diffusion assay. Although 625, 1,250, or 2,500 ppm samples of cinnamaldehyde showed some activity, they were not significantly different from the control (Table 3). However, the 5,000 ppm solution was the lowest concentration to appear in the inhibition zone, which was significantly different from the control; this indicated that the 5,000 ppm concentration was the MIC of cinnamaldehyde, according to the vapor diffusion assay (Table 3). The MIC of cinnamaldehyde was also measured by the liquid culture assay, and its concentration was similarly serially diluted from 2,500 to 78.125 ppm. As shown in Table 4, the 1,250 ppm solution of cinnamaldehyde was the lowest concentration that consistently showed significant antibacterial activity against all Psa strains in the liquid culture assay. The same assay was performed with serially diluted estragole, which indicated

### Table 3. Minimum inhibitory concentration of the cinnamaldehyde in the vapor diffusion assay against Psa strains

| Concentration of cinnamaldehyde (ppm) | KBE9     | YCS3     | SYS1     |
|---------------------------------------|----------|----------|----------|
| Control                               | 0.00 ± 0.00<sup>c</sup> | 0.00 ± 0.00<sup>c</sup> | 0.00 ± 0.00<sup>c</sup> |
| 312.5                                 | 0.00 ± 0.00<sup>c</sup> | 0.00 ± 0.00<sup>d</sup> | 0.00 ± 0.00<sup>c</sup> |
| 625                                   | 0.17 ± 0.24<sup>bc</sup> | 0.17 ± 0.17<sup>cd</sup> | 0.00 ± 0.00<sup>c</sup> |
| 1,250                                 | 0.40 ± 0.22<sup>bc</sup> | 0.30 ± 0.08<sup>c</sup> | 0.00 ± 0.00<sup>c</sup> |
| 2,500                                 | 0.32 ± 0.32<sup>bc</sup> | 0.10 ± 0.08<sup>bc</sup> | 0.10 ± 0.14<sup>bc</sup> |
| 5,000                                 | 0.70 ± 0.08<sup>b</sup> | 0.60 ± 0.08<sup>c</sup> | 0.30 ± 0.24<sup>b</sup> |
| 10,000                                | 1.23 ± 0.09<sup>a</sup> | 1.17 ± 0.12<sup>a</sup> | 1.10 ± 0.16<sup>a</sup> |

Values are presented as mean ± standard deviation.
Different letters show results from Duncan’s multiple range test (<sup>P</sup> < 0.05, n = 3).

### Table 4. Minimum inhibitory concentrations of the estragole and cinnamaldehyde in the liquid culture assay against Psa strains

| Concentration of compounds (ppm) | Estragole | Cinnamaldehyde |
|---------------------------------|-----------|----------------|
|                                 | KBE9      | YCS3 | SYS1 | KBE9 | YCS3 | SYS1 |
| Control                         | 0.67 ± 0.01<sup>ab</sup> | 0.62 ± 0.00<sup>b</sup> | 0.45 ± 0.00<sup>a</sup> | 0.70 ± 0.02<sup>a</sup> | 0.66 ± 0.07<sup>a</sup> | 0.36 ± 0.05<sup>b</sup> |
| 78.1                            | 0.66 ± 0.01<sup>ab</sup> | 0.61 ± 0.00<sup>a</sup> | 0.43 ± 0.00<sup>a</sup> | 0.67 ± 0.00<sup>a</sup> | 0.63 ± 0.00<sup>ab</sup> | 0.43 ± 0.00<sup>ab</sup> |
| 156.2                           | 0.67 ± 0.02<sup>ab</sup> | 0.61 ± 0.01<sup>b</sup> | 0.44 ± 0.01<sup>a</sup> | 0.7 ± 0.01<sup>ab</sup> | 0.63 ± 0.01<sup>ab</sup> | 0.43 ± 0.06<sup>ab</sup> |
| 312.5                           | 0.68 ± 0.01<sup>ab</sup> | 0.62 ± 0.00<sup>a</sup> | 0.44 ± 0.00<sup>a</sup> | 0.7 ± 0.01<sup>ab</sup> | 0.62 ± 0.01<sup>ab</sup> | 0.43 ± 0.00<sup>ab</sup> |
| 625                             | 0.71 ± 0.02<sup>a</sup> | 0.62 ± 0.01<sup>a</sup> | 0.45 ± 0.00<sup>a</sup> | 0.68 ± 0.01<sup>b</sup> | 0.58 ± 0.01<sup>b</sup> | 0.39 ± 0.00<sup>ab</sup> |
| 1,250                           | 0.64 ± 0.00<sup>ab</sup> | 0.55 ± 0.06<sup>b</sup> | 0.37 ± 0.05<sup>b</sup> | 0.52 ± 0.01<sup>c</sup> | 0.1 ± 0.00<sup>c</sup> | 0.18 ± 0.01<sup>c</sup> |
| 2,500                           | 0.62 ± 0.01<sup>b</sup> | 0.46 ± 0.03<sup>c</sup> | 0.17 ± 0.01<sup>c</sup> | 0.17 ± 0.01<sup>d</sup> | 0.09 ± 0.00<sup>c</sup> | 0.11 ± 0.00<sup>d</sup> |

Values are presented as mean ± standard deviation.
OD<sub>600</sub>, the optical density measured at a wavelength of 600 nm.
Different letters show results from Duncan’s multiple range test (<sup>P</sup> < 0.05, n = 3).
that the lowest concentration of estragole to show activity was 2,500 ppm (Table 4).

Cinnamaldehyde, the yellow aroma liquid, has been widely studied and used by many scientists because of its antimicrobial activity against diverse microorganisms (Ooi et al., 2006). That study also showed great antibacterial activity for cinnamaldehyde against eight bacterial species. The antibacterial mechanism of cinnamaldehyde has also been studied in (Di Pasqua et al., 2006; Gill and Holley, 2004). The target of cinnamaldehyde is mainly the bacterial membrane; the contact of cinnamaldehyde with the bacterial membrane might cause the loss of membrane functionality or cause the loss of channel proteins in the membrane, resulting in death of bacterial cells. Thus, bacteria could not grow around filter paper containing certain PEOs in the vapor diffusion assay and moreover, their growth in the liquid culture was decreased. Estragole is a phenylpropene, a plant secondary metabolite. The study of antibacterial activity of estragole is comparatively less than that of cinnamaldehyde, but nonetheless it has been shown to carry antibacterial activity (Shahat et al., 2011). The antibacterial activity of estragole was stronger in the liquid culture assay than that of cinnamaldehyde, but showed weaker activity than that of cinnamaldehyde when using the vapor diffusion assay. Eugenol can be found in PEOs originating from clove, cinnamon, allspice and basil (Catherine et al., 2012). It has been shown to have both an antioxidant and antimicrobial activity (Devi et al., 2010), but very little is known about its mode of action against the target microorganisms. Moreover, although the structure of methyl eugenol is similar to eugenol, its antibacterial activity has not been well determined yet in comparison to eugenol. In this study, methyl eugenol has been shown to have antibacterial activity against Ps. strains, similar to eugenol, for the first time.

To determine why certain compounds only showed antibacterial activity in either the vapor diffusion assay or the liquid culture assay, their structures, vapor pressure, boiling points, and solubility in water were studied based on information presented in previous studies. However, these physical characteristics of those compounds could not explain the different activities exhibited when using different assays. Thus, the underlying mechanisms of this feature remains to be determined.

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