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Impact of *Piper betle* leaf extract on grape downy mildew: Effects of combining 4-allylpyrocatechol with eugenol, α-pinene or β-pinene

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Abstract: Methanol extract of *Piper betle* leaves exhibited an inhibitory effect on grape downy mildew. This extract might contain more than two compounds which have different polarities that suppress grape downy mildew. Gas chromatograph-tandem mass spectrometry analysis identified 4-allylpyrocatechol, eugenol, α-pinene, and β-pinene in the methanol extract. Neither of the compounds suppressed grape downy mildew by single treatment. On the other hand, treatment with a combination of 4-allylpyrocatechol with eugenol, α-pinene or β-pinene enhanced the inhibitory effects on grape downy mildew and perfectly suppressed it. The complex extracted from *P. betle* leaves may be used in organic agriculture as an alternative to chemical fungicides in viticulture.

Keywords: grapevine; pinene; *Plasmopara viticola*

The production of grapes in Vietnam continues to increase (FAO 2017). Viticulture for commercial purposes started here in the late 1970s. Muscat Blanc, Alden, Cardinal, and Alphonse Lavallee were cultivated in the 1980s. Since then, only Cardinal has become widespread into the 1990s. At present, the main grape-producing area in Vietnam is Tinh Ninh Thuân, and the grape yield in Vietnam was 16 965 t in 2014 (Agroviet 2017). Since the climate of Tinh Ninh Thuân is hot and humid year-round, the damage to grapevines by phytopathogenic fungal diseases, for example, downy mildew, powdery mildew, rust and bunch rot, has become a subject of discussion in viticulture. A simple strategy against fungal diseases is the application of chemical fungicides. Since squalls occurring from August to November in Tinh Ninh Thuân promote the pandemic of fungal diseases, a large quantity of chemical fungicides is frequently needed for the control of fungal diseases. Concern over environmental pollution due to the application of chemical fungicides has plagued vine growers in Vietnam and has led to the introduction of alternatives to chemical fungicides in viticulture.

The research on alternatives to chemical fungicides has seen an upsurge of interest in scientific communities. One of the candidate alternatives is natural bioactive compounds extracted from plants (Soković *et al.* 2013). Natural plant bioactive compounds provide antifungal activity and plant resistance-inducing activity. The extracts from spices (e.g. turmeric, nutmeg, ginger, clove, oregano, cinnamon, anise, fennel, basil, black cumin, and black pepper) have shown antifungal activity against phytopathogenic fungi (Radwan *et al.* 2014). Hordenine, a phenethylamine alkaloid found in barley (Mann *et al.* 1963), induces a plant defense response through the jasmonate-dependent defense pathway without any direct effect on the growth of phytopathogenic fungi (Ishiai *et al.* 2016). In the context of alternatives to chemical fungicides, natural plant bioactive compounds are attracting the interest of scientific communities.

Piper betle L. has been extensively used in traditional flavouring agents as well as ethnomedicine in Southeast Asia. In Vietnam, chewing *P. betle* leaves is thought to be one of the best methods of

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maintaining oral hygiene. In fact, crude extracts of *P. betle* leaves suppressed bacterial and fungal activity (Bissa *et al.* 2007; Nair & Chanda 2008). In addition, 4-allylpyrocatechol isolated in *P. betle* leaf extract exhibited an inhibitory effect on clinical fungal species (Ali *et al*. 2010). Thus, the antifungal compounds in *P. betle* leaves warrant their application as antifungal agents in clinical fungal infections. In contrast, regarding the introduction of *P. betle* extracts in agriculture, there have been few studies on the control of phytopathogenic fungal infection into a host plant using the extract of *P. betle* leaves. A crude chloroform extract of *P. betle* leaves reduced the symptoms and disease development of *Fusarium oxysporum* f.sp. *lycopersicii* on tomato (Singha *et al*. 2011). However, no studies have been carried out to investigate the inhibitory effect of *P. betle* extracts on diseases in grapevines, although it has recently been demonstrated that the ethyl acetate extract of the common rush, *Juncus effusus*, which is used in traditional Chinese medicine, reduced the disease severity of grape downy mildew (Thuerig *et al*. 2016).

The objective of this study was to clarify whether the extracts of *P. betle* leaves suppress grape downy mildew. *Plasmopara viticola*, which causes grape downy mildew, is a high-risk phytopathogenic fungus. Since *P. viticola* possesses high potential to acquire chemical fungicide resistance (Heaney *et al*. 2000; Gisi *et al*. 2007), vine growers face several risks posed by the emergence of chemical-fungicide-resistant *P. viticola* populations and vigorously demand alternatives to chemical fungicides. Herein we present a report on the inhibitory effects of the extracts from *P. betle* leaves on the infection by *P. viticola*. We also demonstrate that 4-allylpyrocatechol contained in the methanol extract of *P. betle* leaves suppresses the severity of grape powdery mildew in combination with eugenol, α-pinene or β-pinene.

MATERIAL AND METHODS

Chemicals. 4-Allylpyrocatechol, eugenol, α-pinene, and β-pinene were purchased from Tokyo Chemical Industry (Tokyo, Japan).

Plant material. To maintain *Plasmopara viticola* (Berk. & MA Curtis) Berl. & DeToni, potted seedlings of *Vitis vinifera* cv. Koshu were cultivated in an incubator (27°C, 11.8 W/m^2 for 16 h a day). Leaves of *P. betle* Linn. were collected in Hanoi (Vietnam) in November 2015.

Preparation of crude extracts of P. betle leaves. *P. betle* leaves were washed using tap water and air-dried at 25°C for 24 hours. The leaves were finely chopped and then dried at 50°C until completely dry. Dried leaf samples were pulverised in liquid nitrogen. For methanol, ethanol or chloroform extraction, 10 g of the pulverised leaf sample was added in 40 ml of methanol, ethanol or chloroform and then left to settle at room temperature for 3 days. For hot water extraction, 10 g of the pulverised leaf samples was added to 40 ml of boiling water for 2 h and then left to settle at room temperature for 3 days. The solutions were filtrated through filter paper (No. 1; Advantec, Tokyo, Japan) and the filtrates were collected as samples of each extract.

Fractionation of P. betle leaves methanol extract by two methods. As is mentioned below, the methanol extract of *P. betle* leaves exhibited an inhibitory effect on the severity of grape downy mildew. For further fractionation of the methanol extract, two methods were applied (Kumar *et al*. 2010; Dwivedi & Tripathi 2014).

With the first method, 2 ml of MilliQ water (Millipore, Bedford, USA) was added to 10 ml of the methanol extract. Then, 5 ml of chloroform and 2 ml MilliQ water were added to the methanol extract. After mixing gently with inversion, a stable bilayer was formed. The chloroform layer was collected as the chloroform fraction. MilliQ water was added in the methanol layer until the methanol concentration was 60%. An amount of 5 ml of diethyl ether was added to the methanol layer, and a stable bilayer was formed after mixing gently with inversion. The diethyl ether layer was collected as the diethyl ether fraction. An amount of 5 ml of ethyl acetate was added to the methanol layer and a stable bilayer was formed after mixing gently with inversion. The ethyl acetate layer was collected as the ethyl acetate fraction. Finally, the remaining methanol layer was collected as the methanol fraction.

Using the second method, 5 ml of the methanol fraction was air-dried at room temperature. Dried samples (0.185 g) were dissolved in 5 ml of boiling water, mixed gently with inversion, and then left to settle at room temperature. The solution was filtered through filter paper No. 1 (Advantec) and the filtrate was collected as the hot water extract. Dried samples (0.185 g) were also dissolved in 5 ml of diethyl ether, ethyl acetate or chloroform by mixing gently with inversion. The solutions were filtered through filter paper No. 1 (and the filtrates were collected

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as the diethyl ether fraction, ethyl acetate fraction or chloroform fraction, respectively.

Bioassay for evaluating the severity of grape downy mildew. An *in vivo* bioassay using grape leaf disks was performed to evaluate the severity of grape downy mildew, as described previously with minor modification (Furuya *et al*. 2010). Briefly, the fourth to seventh leaves, counted from the shoot tip, were collected from the potted seedlings of *V. vinifera* cv. Koshu. Five leaf disks having a diameter of 15 mm were cut out from the leaves and placed upside down on moistened filter paper in square Petri dishes (140 × 100 mm). *P. betle* leaf extracts and their fractions were diluted with MilliQ water 10 times (10%) or 100 times (1%). 10 µl of the sample were dropped onto four locations on the abaxial surface of leaf disk. MilliQ water, 10% methanol, 10% ethanol, 10% chloroform, 10% diethyl ether, and 10% ethyl acetate were used in control experiments. The leaf disks treated with each sample were dried at room temperature in a flow cabinet overnight. Spores of *P. viticola* were washed off with sterile water from the symptoms of grape downy mildew on leaves of the potted seedlings in the laboratory. The spore suspension was adjusted to a concentration of 50 000 spores/ml with sterile water. The spore suspension (10 µl) was dropped at the same locations as the pretreated samples on the leaf disks. The Petri dishes containing the inoculated leaf disks were placed in a plastic box containing moistened paper towel to achieve approximately 100% humidity. The box was incubated under darkness for 24 h and then in an incubator (22°C, 11.8 W/m^2 for 15 h a day). The downy mildew symptom on each disk was assessed when it occupied more than two-thirds of the leaf disk treated with water. The disease severity was scored in accordance with the symptom index of 0 to 5 as follows: 0 – no symptoms; 1 – white symptom occupies up to 1/6 of the disk; 2 – white symptom occupies up to 1/3 of the disk; 3 – white symptom occupies up to half of the disk; 4 – white symptom occupies up to two-thirds of the disk; 5 – white symptom occupies more than two-thirds of the disk. The *in vivo* bioassay was performed in five independent experiments.

Light-microscope observation. The leaf disks showing the inhibitory effect on grape downy mildew because of treatment with 10% methanol extract were treated with FAA solution (5% formalin, 5% acetic acid, 45% alcohol, 45% water) at room temperature for 24 hours. After treatment of the disks with the clearing agent Visikol for Plant Biology (Visikol, New Brunswick, USA) for 12 h at room temperature in the dark, infection processes, including germination, penetration into stomata and conidiophore formation, of *P. viticola* on the leaf disks were observed under a light microscope.

Identification and quantitation of 4-allylpyrocatechol, eugenol, α-pinene and β-pinene in methanol extract. *P. betle* leaves were pulverized in liquid nitrogen. 40 g of the pulverised leaf samples were added to 120 ml of methanol and then left to settle at room temperature for 3 days. The methanol extract was filtered with a 0.2 µm filter (Takara, Otsu, Japan). The identification and quantitative analysis of 4-allylpyrocatechol, eugenol, α-pinene, and β-pinene in the filtrates were performed with the GCMS-TQ8030 gas chromatograph-tandem mass spectrometer (Shimadzu, Kyoto, Japan) using a polar Restek Stabilwax column (Restek, Bellefonte, USA) for 4-allylpyrocatechol and eugenol and a nonpolar methyl silicone column Restek RTx-1 (Restek) for α-pinene and β-pinene. Quantitation was performed twice with two independent methanol extracts.

Inhibitory effect of 4-allylpyrocatechol, eugenol, α-pinene and β-pinene on grape downy mildew. Leaf disks were placed upside down on moistened filter paper in square Petri dishes (140 × 100 mm). 4-Allylpyrocatechol, eugenol, α-pinene, and β-pinene were prepared at 1, 0.1, or 0.01 mg/ml with 10% methanol. 10 µl of solution were dropped at four locations on a leaf disk. MilliQ water was used as control. The treated leaf disks were dried for 6 h at room temperature in a flow cabinet. 10 µl of 50 000 spores/ml *P. viticola* spore suspension were dropped at the same locations as the pretreatment compounds on the leaf disks. The Petri dishes containing the inoculated leaf disks were placed in a plastic box containing moistened paper towel to achieve approximately 100% humidity. The box was incubated under darkness for 24 h and then for 7 days in an incubator (22°C, 11.8 W/m^2 for 15 h a day). Downy mildew symptoms on each disk were assessed in accordance with the above-mentioned symptom index. The bioassay was performed in five independent experiments.

Combined treatment using 4-allylpyrocatechol with eugenol, α-pinene or β-pinene. Leaf disks were placed upside down on moistened filter paper in square Petri dishes (140 × 100 mm). For combined treatment, solutions of 0.1 mg/ml 4-allylpyrocatechol with eugenol, α-pinene, and β-pinene (0.1, 0.05, 0.033, 0.025, 0.02, 0.016, 0.014, 0.012, 0.011, 0.01 mg/ml) were prepared with 10% methanol. MilliQ water was used as control. 10 µl of solution were dropped at

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four locations on the leaf disk. The treated leaf disks were dried for 6 h at room temperature in a flow cabinet. 10 µl of 50 000 spores/ml *P. viticola* spore suspension were dropped at the same locations as the pretreatment compounds on the leaf disks. The Petri dishes containing the inoculated leaf disks were placed in a plastic box containing moistened paper towel to achieve approximately 100% humidity. The box was incubated under darkness for 24 h and then for 7 days in an incubator (22°C, 11.8 W/m^2 for 15 h a day). Downy mildew symptoms on each disk were assessed in accordance with the above-mentioned symptom index. The bioassay was performed in five independent experiments.

Statistical analysis. Data from the indicated independent experiments are presented as means ± standard deviations. Statistical analysis was performed by Tukey's multiple comparison test with MS Excel 2012 statistics software (Social Survey Research Information, Tokyo, Japan).

RESULTS

Inhibitory effect of P. betle leaves methanol extract on grape downy mildew. The inhibitory effect of methanol, ethanol, chloroform or hot water extracts of *P. betle* leaves on grape downy mildew was evaluated (Figure 1). Since chloroform induced browning on leaf disks, the chloroform extract could not be evaluated. Ethanol extract did not exhibit the inhibitory effect on grape downy mildew. Inhibitory effects on grape downy mildew were observed on leaf disks treated with 10% methanol extract or 10% hot water extract. The inhibitory effects were dose-dependent.

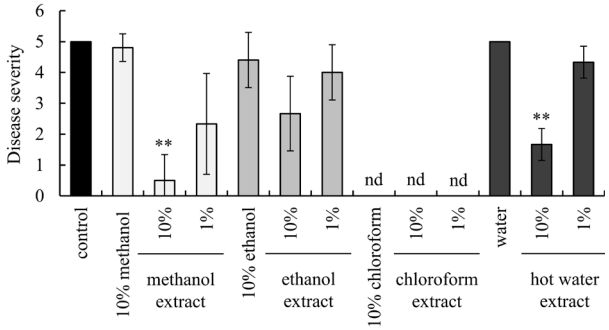

Figure 1. Suppression of grape downy mildew by crude extracts of *P. betle* leaves

Leaf disks were treated with the indicated crude extracts of *P. betle* leaves and inoculated with *P. viticola*; disease severity of grape downy mildew on leaf disks was scored in accordance with the symptom index of 0 to 5, as described in the Material and Methods part. Bars indicate means ± standard deviations of data from five leaf discs; $^{**}P < 0.05$ compared with control and 10% solvents; control – no treatment; nd – not determined

Methanol, a control for the methanol extract, did not affect the infection process of *P. viticola*. Spore germination (Figure 2A) and hyphal growth (Figure 2B) were excellent on the disks treated with methanol. Methanol extract inhibited the hyphal growth from germinated spores (Figure 2C), while spores without germination were sometimes observed after treatment with methanol extract (Figure 2D). Conidiophores from stomata were observed on leaf disks treated with methanol (Figure 2E), but not with methanol extract (Figure 2F).

These results suggested that *P. betle* leaves contain compounds that suppress the disease severity of grape downy mildew through the inhibition of *P. viticola* growth.

Inhibitory effect of each faction on grape downy mildew. Since the inhibitory effect of the methanol

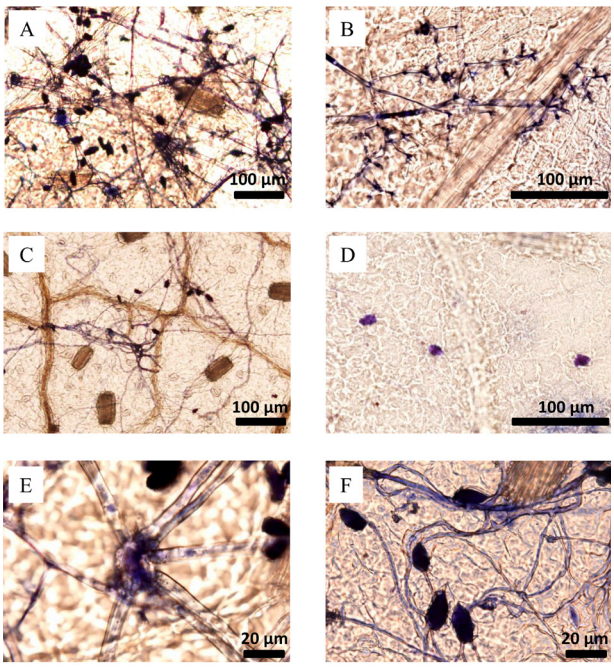

Figure 2. Antagonistic activity of the *P. betle* leaves methanol extract against *P. viticola* infection on grape leaves: (**A**) 10% methanol – colony formation was observed, (**B**) 10% methanol – conidiophores and new spores were observed, (**C**) 10% methanol extract – only small colonies were observed, (**D**) 10% methanol extract – ungerminated spores were sometimes observed, (**E**) 10% methanol – conidiophores emerged from stomata, and (**F**) 10% methanol extract – conidiophores were not observed

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extract of *P. betle* leaves on grape downy mildew was stronger than that of the hot water extract (Figure 1), the methanol extract was subjected to further fractionation. Two fractionation methods were used for the methanol extract of *P. betle* leaves. The sample prepared by the first method demonstrated that the diethyl ether fraction and the ethyl acetate fraction suppressed the disease severity of grape downy mildew (Figure 3A). The inhibitory effect on grape downy mildew was also maintained in the remaining methanol layer after organic solvent fractionation with diethyl ether, ethyl acetate, and chloroform. The sample prepared by the second method also demonstrated that the diethyl ether fraction and ethyl acetate fraction suppressed the disease severity of grape downy mildew (Figure 3B). An inhibitory effect was also detected upon treatment with a hot water fraction of the methanol extract. Regardless of the fractionation method, the ethyl acetate fraction exhibited the highest inhibitory effect on grape downy mildew among all fractions. Chloroform ultimately induced browning on leaf disks. Although we could not exclude the possibility that the same compound was present in different fractions, *P. betle* leaves might contain more than two compounds which have different polarities that suppress the disease severity of grape downy mildew.

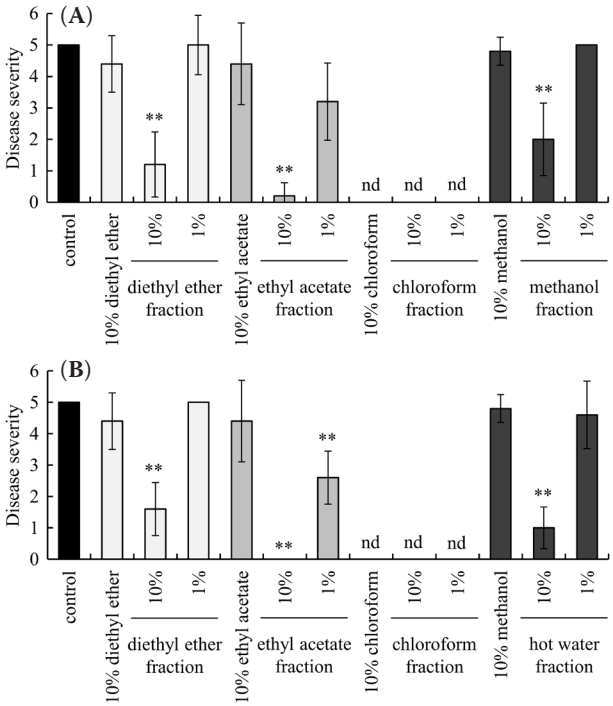

Figure 3. Suppression of grape downy mildew by fractions of the *P. betle* leaves methanol extract: (**A**) the first fractionation method, (**B**) the second fractionation method

Leaf disks were treated with the indicated crude fractions of the methanol extract of *P. betle* leaves and inoculated with *P. viticola*; disease severity of grape downy mildew on leaf disks was scored in accordance with the symptom index of 0 to 5, as described in the Material and Methods part; bars indicate means ± standard deviations of data from five leaf disks; $^{**}P < 0.05$ compared with control and 10% solvents; control – no treatment; nd – not determined

Inhibitory effect of 4-allylpyrocatechol, eugenol, α-pinene or β-pinene on grape downy mildew. We evaluated the inhibitory effects of 4-allylpyrocatechol, eugenol, α-pinene, and β-pinene (Figure 4), which were identified in the methanol extract of *P. betle* leaves by gas chromatograph-tandem mass spectrometer analysis, on the severity of grape downy mildew. The concentrations of 4-allylpyrocatechol, eugenol, α-pinene, and β-pinene in the methanol extract prepared in the present study were 2 800 ± 424, 17 ± 1.4, 20.5 ± 0.7, and 0.79 ± 0.03 μg/ml, respectively.

The *in vivo* bioassay using leaf disks demonstrated that eugenol and α-pinene did not decrease the severity of grape downy mildew at any concentration tested (Figure 5). Although β-pinene appeared to inhibit *P. viticola* infection at 0.1 mg/ml, 1 mg/ml of β-pinene induced browning on leaf disks. In contrast, 4-allylpyrocatechol decreased the severity of grape downy mildew at 1 mg/ml.

Combined effects of 4-allylpyrocatechol with eugenol, α-pinene or β-pinene. Since the methanol extract of *P. betle* leaves might contain more than two compounds (Figure 3), the combined effects of 4-allylpyrocatechol with eugenol, α-pinene or β-pinene were evaluated. The solutions for the combination treatment contained 0.1 mg/ml 4-allylpyrocatechol, which did not decrease the severity of grape downy mildew (Figure 4), and the indicated concentration of eugenol, α-pinene or β-pinene, respectively (Figure 6). The combination of 4-allylpyrocatechol with eugenol, α-pinene or β-pinene enhanced the inhibitory effects on grape downy mildew compared with each compound applied separately (Figure 6). The combined effects were dose-dependent.

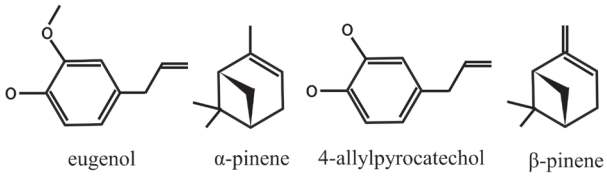

Figure 4. Chemical structures of eugenol, 4-allylpyrocatechol, α-pinene, and β-pinene

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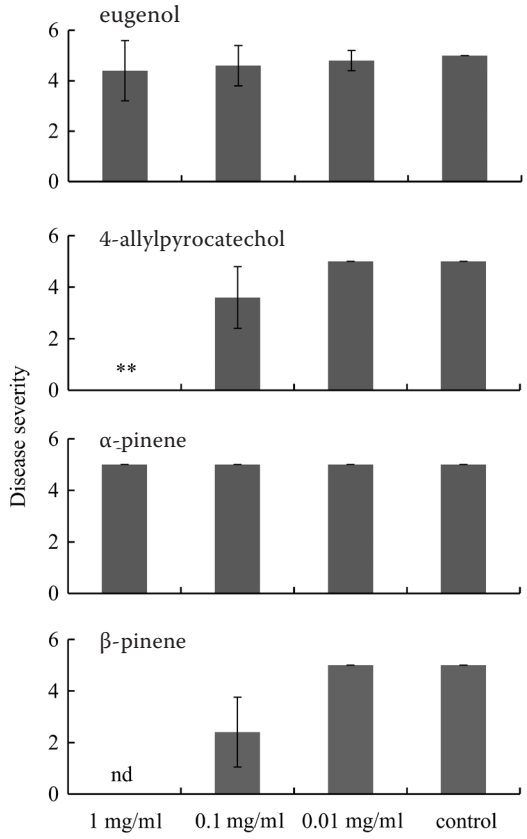

Figure 5. Single treatment with eugenol, 4-allylpyrocatechol, α-pinene or β-pinene

Leaf disks were treated with the indicated concentrations of eugenol, 4-allylpyrocatechol, α-pinene or β-pinene and inoculated with *P. viticola*; disease severity of grape downy mildew on leaf disks was scored in accordance with the symptom index of 0 to 5, as described in the Material and Methods part; bars indicate means ± standard deviations of data from five leaf disks; $^{**}P < 0.05$ compared with control and 10% solvents; control – no treatment; nd – not determined

DISCUSSION

The concern over environmental pollution as well as the emergence of fungicide-resistant phytopathogens led to the introduction of alternative pest management strategies to the use of synthetic chemical fungicides. New pesticide registration procedures, such as the Food Quality Protection Act from the United States Environmental Protection Agency, have reduced the number of accessible synthetic chemical fungicides in agriculture (Dayan *et al*. 2009). Natural bioactive compounds have received great attention as one of the alternative pest management strategies to the use of synthetic chemical fungicides. A great deal of research is being continued on natural bioactive compounds to reduce the application of synthetic chemical fungicides in agriculture (Radwan *et al*. 2014; Ishiai *et al*. 2016). In this context, the aim of this study was to contribute to the reduction in the dependence on synthetic chemical fungicides in agriculture through the use of *P. betle* leaves. *P. betle* leaf is an extraordinary reservoir of bioactive phenolic compounds with antibacterial and antioxidant activities (Rekha *et al*. 2014). In fact, an extract of *P. betle* leaves shows antifungal activities to phytopathogens. *In vitro* antifungal assays demonstrated that the methanol extract of *P. betle* inhibited hyphal growth of *Colletotrichum gloeosporioides*, which causes anthracnose on fruit crops including grapevine (Johnny *et al*. 2010).

In the present study, we focused on natural bioactive compounds of *P. betle* leaves and examined their abilities to suppress grape downy mildew. A great number of active compounds have been detected in organic solvent extracts of *P. betle* (Hossain *et al*. 2008; Kumar *et al*. 2010; Fazal *et al*. 2014; Rekha

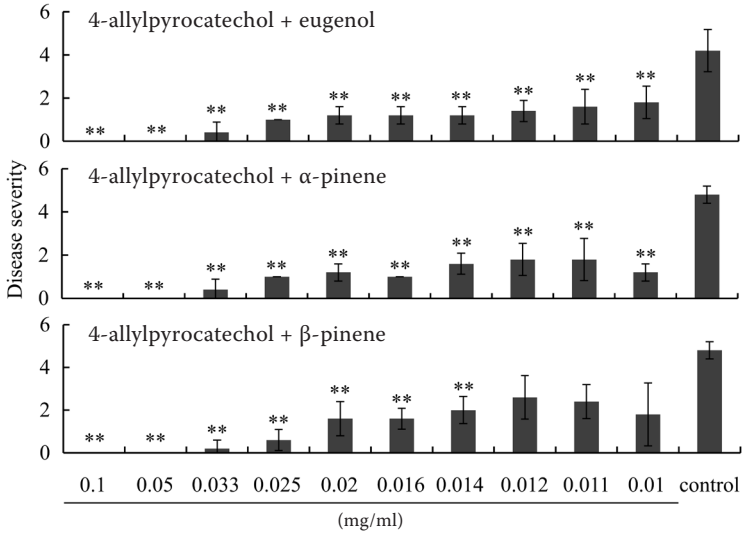

Figure 6. Combined treatment of 4-allylpyrocatechol with eugenol, α-pinene or β-pinene

Leaf disks were treated with 0.1 mg/ml 4-allylpyrocatechol with the indicated concentration of eugenol, α-pinene or β-pinene and inoculated with *P. viticola*; disease severity of grape downy mildew on leaf disks was scored in accordance with the symptom index of 0 to 5, as described in the Material and Methods part; $^{**}P < 0.05$ compared with control and 10% solvents; control – no treatment; nd – not determined

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et al. 2014). For example, 4-allylpyrocatechol is the most abundant active compound in *P. betle* leaf extract and has an antimicrobial property (Ali *et al*. 2010). To evaluate whether the compounds in *P. betle* leaf extract exhibit the inhibitory effect on grape downy mildew, purified materials were needed both in quality and in quantity. Since we could not purify the compounds in *P. betle* leaf extract satisfyingly for the evaluation in the present study, we selected four commercial compounds (eugenol, 4-allylpyrocatechol, α-pinene, and β-pinene) which have antimicrobial properties.

Eugenol possesses antimicrobial activities to the bacterial pathogens *Escherichia coli* and *Listeria monocytogenes* (Pérez-Conesa *et al*. 2011). When the biofilms formed by *E. coli* and *L. monocytogenes* were treated with eugenol, a large number of dead cells were observed in the biofilms. Eugenol also inhibited filamentous growth of *Candida albicans* in the biofilms (He *et al*. 2007). 4-Allylpyrocatechol killed *C. albicans* and *C. glabrata* and also inhibited the growth of biofilm generated by *C. albicans* (Ali *et al*. 2010). Inhibitory effects of α-pinene and β-pinene on *C. albicans* biofilm formation were also observed (Rivas da Silva *et al*. 2012). How do these compounds exert antimicrobial activities? The crude aqueous extract of *P. betle* induced plasma membrane damage and coagulation of the nucleoid in *Streptococcus mutans* (Nalina & Rahim 2007). 4-Allylpyrocathechol caused membrane disruption in *C. albicans* cells (Ali *et al*. 2010). The antifungal activity of eugenol to the phytopathogen *Botrytis cinerea* is due to the alteration of plasma membrane permeability, leading to hyphal shriveling (Wang *et al*. 2010). Pinene inhibited the pumping of protons and K^+ transport through an increase in the fluidity of the mitochondrial membrane of yeast (Uribe *et al*. 1985). Thus, the presumed active site of these compounds might be cellular membranes in the microorganisms. However, the membrane constitution of oomycetes, in which *P. viticola* is categorised, is different from that of other fungi (Latijnhouwers *et al*. 2003). Oomycetes have less sterol in the membranes and contain distinctive lipid molecules with unusual structures and long fatty acids instead of sterols in the membranes. No inhibitory effect of eugenol, 4-allylpyrocatechol, α-pinene or β-pinene on *P. viticola* infection into grapevine leaves was observed in this study, although a high concentration (1 mg/ml) of 4-allylpyrocatechol decreased the severity of grape downy mildew. The variation in the membrane constitution might create an inconsistency between the present study and previous reports that a single treatment of individual compounds affected the cell viability of target microorganisms.

Interestingly, a combined treatment of 4-allylpyrocatechol with eugenol, α-pinene or β-pinene enhanced the inhibitory effects on grape downy mildew. So far, we have been unable to determine any positive reason why the synergistic activities were exhibited. The combined treatment of ciprofloxacin with α-pinene or β-pinene yielded a synergistic inhibitory activity against methicillin-resistant *Staphylococcus aureus* growth (Rivas da Silva *et al*. 2012). Further investigation of the combined treatment in field tests may provide new information on the most suitable application of *P. betle* extracts for protection against grape downy mildew in viticulture.

In conclusion, our results suggested that the inhibitory effect of the *P. betle* leaves methanol extract on grape downy mildew resulted from a mixture of more than two compounds. Therefore, the complex extracts in methanol may be used in organic agriculture as alternatives to chemical fungicides in viticulture.

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