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Antifungal activity of essential oil and its constituents from *Calocedrus macrolepis* var. *formosana* Florin leaf against plant pathogenic fungi

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Abstract

Resistance to conventional fungicides causes the poor disease control of agriculture. Natural products from plants have great potential as novel fungicide sources for controlling pathogenic fungi. In this study antipathogenic activity of the leaf essential oil and its constituents from *Calocedrus macrolepis* var. *formosana* Florin were evaluated *in vitro* against six plant pathogenic fungi. Chemical analysis of leaf oil by GC/MS allowed identification of α-pinene (44.2%), limonene (21.6%), β-myrcene (8.9%), β-caryophyllene (8.2%), caryophyllene oxide (2.4%), α-cadinol (1.6%), β-pinene (1.2%), and T-muurolol (1.1%) as main components. Sesquiterpenoid components of the oil were more effective than monoterpenoid components of the oil. In particular, T-muurolol and α-cadinol strongly inhibited the growth of *Rhizoctonia solani* and *Fusarium oxysporum*, with the IC_{50} values < 50 μg ml^{-1}. These compounds also efficiently inhibited the mycelial growths of *Colletotrichum gloeosporioides*, *P. funerea*, *Ganoderma australe* and *F. solani*. These results showed that T-muurolol and α-cadinol possess antifungal activities against a broad spectrum of tested plant pathogenic fungi and could be used as potential antifungal agents for the control of fungal diseases in plants.

Keywords: *Calocedrus macrolepis* var. *formosana* Florin; Plant pathogenic fungi; Antifungal activity; T-Muurolol; α-Cadinol

1. Introduction

Plant pathogens include fungi, nematodes, bacteria, and viruses which can cause diseases or damages in plants (Montesinos, 2003). Among these pathogens, fungi are the main pathogen and cause many diseases of plants. Pathogenic fungi also cause yield losses in numerous economically important crops (Fletcher et al., 2006). Hussein et al. (2002) found that most of grains in maize fields were infected with *Fusarium* species, and soil can be considered as one of the important inoculum sources for these species. *Ganoderma* is a polyporoid fungus and has a worldwide distribution. Sankaran et al. (2005) reported that *Ganoderma* causes a decrease of production and death of various plants, such as cash crops and trees in India. Several fungi have been found to induce post-harvest spoilage of sweet potato, which is associated with decrease in starch, total sugar and organic acid (Ray and Ravi, 2005). Diseases caused by fungi are also the serious problem in forest management. Damping-off of seedlings caused by *Rhizoctonia solani* were frequently observed on many woody perennial plants (Chang, 1997). *Colletotrichum gloeosporioides* causes anthracnose disease of trees and results in leaf spots and defoliation (Chang et al., 1997). Trees infected with *F. solani* showed root crown rot, dieback and wilt (Fu and Chang, 1999; Demirci and Maden, 2006).

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Chemical treatments with soil amendments are the main solutions for pathogenic disease control of plants (Montesinos, 2003; Nunes et al., 2001). Synthetic fungicides are helpful to sustaining crop production by protecting plants from fungal diseases. Resistance to fungicides is one of critical causes of poor disease control of agriculture (Steffens et al., 1996; Aguin et al., 2006; Ishii, 2006). There are, therefore, needs to develop alternative agents for the control of pathogenic fungal diseases in plants. Biocontrol activities of enzymes were also investigated against pathogenic fungi (Prabavathy et al., 2006; Chang et al., 2007). In addition, several studies on the fungitoxic activities of plant secondary metabolites have been reported (Muller-Riebau et al., 1995; Ojala et al., 2000; Kordali et al., 2003; Nunez et al., 2006; Field et al., 2006; Lee, 2007).

Pitarokili et al. (2003) reported that volatile essential oil of *Salvia fruticosa* was effective against phytopathogenic fungi, including *R. solani*, *Sclerotinia sclerotiorum*, and *F. solani*. Leaf essential oils of *Thymbra spicata*, *Satureja thymbra*, and *Origanum minitiflorum*, growing wild in Turkey, were strongly inhibitory to the growth of the soil-borne plant disease-causing fungi *F. moniliforme*, *R. solani*, *S. sclerotiorum*, and *Phytophthora capsici* (Muller-Riebau et al., 1995). Antifungal diterpenoids were isolated from the bark extract of *Cryptomeria japonica*, and the antifungal activities of these components were evaluated against phytopathogenic fungi (Kofujita et al., 2006).

Calocedrus macrolepis var. *formosana* Florin (Taiwan incense cedar), coniferous evergreen tree, is one of the precious endemic woods in Taiwan, it belongs to the family of Cupressaceae. The chemical composition of *C. macrolepis* var. *formosana* leaf essential oil and its antifungal activity against wood-rot fungi has been previously reported by our research group (Cheng et al., 2004). In continuation of our investigation on bioactivities of the leaf oil and its constituents from *C. macrolepis* var. *formosana* against several important plant pathogenic fungi were evaluated in this study.

2. Methods

2.1. Plant materials

Leaves were collected from *Calocedrus macrolepis* var. *formosana* Florin tree (41 years of age) in the Experimental Forest of National Taiwan University. The species was identified, and a voucher specimen was deposited in the laboratory of wood chemistry (School of Forestry and Resource Conservation, National Taiwan University).

2.2. Plant pathogenic fungi

Six common plant pathogenic fungi were selected for the present study and purchased from Bioresource Collection and Research Center (BCRC) of Taiwan. *Fusarium oxysporum* f. sp. *melonis* Snyder & Hansen (BCRC32121) and *Rhizoctonia solani* Kuhn (BCRC31626) are usual damping-off pathogens of plants. *Pestalotiopsis funerea* (Desmazieres) Steyaert (BCRC35266) and *Colletotrichum gloeosporioides* Penzig (BCRC35003) cause many leaf diseases, such as anthracnose. *Ganoderma australe* (Fries) Paterson (BCRC36246) and *Fusarium solani* (Martius) Saccardo (BCRC32458) are the important root rot pathogens of plants. Each fungal strain was cultured in potato dextrose agar (PDA, Difco Company).

2.3. Distillation of sample plant

Fresh leaves of *C. macrolepis* var. *formosana* were hydrodistilled in a Clevenger-type apparatus for 8 h to obtain essential oil. Leaves were in boiling water during distillation. Yield of leaf essential oil was 0.3%.

2.4. GC/MS analysis of essential oil

The composition of the oil was analyzed using Hewlett–Packard 6890 gas chromatograph and Agilent Technologies HP 5973N mass spectrometry equipped with a HP-1MS capillary column (100% Dimethylpolysiloxane; 30 m × 0.25 mm; film thickness 0.25 μm). Oven temperature was initially held at 50 °C for 5 min, then increased to 120 °C at a rate of 2 °C min^{-1} and increased to 220 °C at a rate of 5 °C min^{-1} and held for 5 min. Carrier gas was helium at a flow rate of 1 ml min^{-1}, and the split ratio was 1:10. Components were identified by comparing their mass spectra with Wiley and NIST library data and standards of the main components. Quantification of components was obtained by integrating the peak area of the chromatogram.

2.5. Antifungal assay

Leaf oil and its main constituents were dissolved in 150 μL of ethanol, respectively, and then added into 15 ml PDA to obtain the different final concentrations. Mycelial plugs (5 mm in diameter) from the edges of each culture were incubated in the center of each PDA plate (90 mm diameter). Cultures were incubated in the dark at 26 °C and 70% RH for ca. 8–14 days. Tests were repeated in triplicate. Antifungal index was calculated as the following: Antifungal index (%) = (1 − Da/Db) × 100, where Da: the diameter of growth zone in the test plate. Db: the diameter of growth zone in the control plate. IC_{50} (concentration that produces a 50% inhibitory effect) values of constituents were graphically obtained from the dose-response curves based on measurement at five different concentrations.

2.6. Statistical analysis

Mean values and standard deviations were calculated for all tests. The data were grouped by Scheffe's test of the SAS system (at a level of significance of $p < 0.05$).

3. Results and discussion

3.1. Antifungal activity of C. macrolepis var. formosana leaf oil

The antifungal effects of leaf oil of *C. macrolepis* var. *formosana* against plant pathogenic fungi are shown in Fig. 1. The antifungal indexes of leaf oil against damping-off pathogens, *Fusarium oxysporum* (*F.o.*) and *Rhizoctonia solani* (*R.s.*), were 15.0% and 33.1% at a 2 mg ml^{-1} concentration. As for the pathogens which caused leaf infections of plants, the antifungal indexes of the oil were 65.0% and 16.7% against *Pestalotiopsis funerea* (*P.f.*) and *Colletotrichum gloeosporioides* (*C.g.*), respectively. The antifungal indexes of the oil against root rot pathogens, *Ganoderma australe* (*G.a.*) and *Fusarium solani* (*F.s.*), were 22.5% and 52.1%, respectively. These results showed that leaf oil was highly inhibitory to mycelial growth of *P. funerea* and *F. solani* among the pathogens tested.

3.2. Chemical component analysis and antifungal activities of the constituents against damping-off pathogens

The chemical characterization of the leaf oil by GC/MS previously allowed identification of α-pinene (44.2%), limonene (21.6%), β-myrcene (8.9%), β-caryophyllene (8.2%), caryophyllene oxide (2.4%), α-cadinol (1.6%), β-pinene (1.2%) and T-muurolol (1.1%) as main components (Cheng et al., 2004). It also contains some other constituents in minor level (<1%).

Fig. 2 shows the antifungal activities of leaf oil constituents against damping-off pathogens, *R. solani* and *F. oxysporum*. Antifungal activities of α-pinene and β-pinene were less than 10% at a concentration of 200 μg ml^{-1} (data are not shown in Fig. 2). Limonene and β-myrcene also showed the weak antifungal activities. Results of these monoterpenoids are consistent with the data reported by Cakir et al. (2004) against *R. solani* and *F. oxysporum*. Sesquiterpenoids, β-caryophyllene, caryophyllene oxide,

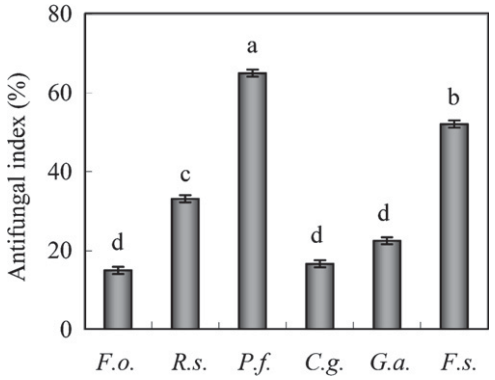

Fig. 1. Antifungal activities of the leaf oil (2 mg ml^{-1}) from *Calocedrus macrolepis* var. *formosana* against the plant pathogenic fungi. Bars with different letters (a–d) are statistically different at $p < 0.05$ according to the Scheffe's test.

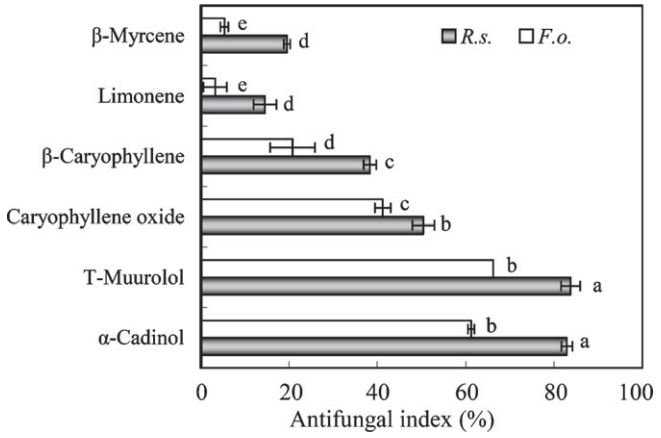

Fig. 2. Antifungal activities of the constituents (200 μg ml^{-1}) of *Calocedrus macrolepis* var. *formosana* leaf oil against the seedling pathogenic fungi. Bars with different letters (a–e) are statistically different at $p < 0.05$ according to the Scheffe's test.

T-muurolol and α-cadinol, exhibited better activities among leaf oil constituents. T-muurolol and α-cadinol exhibited strong activity against *R. solani* and *F. oxysporum* with the highest antifungal indexes ranging from 60% to 85%. IC_{50} values of T-muurolol against *R. solani* and *F. oxysporum* were 15.2 and 34.2 and α-cadinol had IC_{50} values of 21.0 and 30.1 μg ml^{-1} against *R. solani* and *F. oxysporum* (Table 1). Muller-Riebau and coauthors (1995) found that inhibition values of thymol, a fungicide, were 34.2% against *R. solani* at a concentration of 50 μg ml^{-1}. It revealed that α-cadinol would be more effective compared to thymol. Among the antifungal constituents isolated from *Taiwania cryptomerioides* heartwood extract (Chang et al., 2000), it was reported that α-cadinol completely inhibited the growth of wood-rot fungi, *Coriolus versicolor* and *Laetiporus sulphureus*, at a concentration of 100 μg ml^{-1}. These results manifested that α-cadinol has a wide spectrum of antifungal activity.

3.3. Antifungal activities of the oil constituents against pathogens causing leaf diseases

C. gloeosporioides is one of the important pathogens of plants which cause leaf spotting, dieback of twigs, death

Table 1
IC_{50} values of sesquiterpenoid constituents of *Calocedrus macrolepis* var. *formosana* leaf oil against plant pathogenic fungi

Constituents	Plant pathogenic fungi					
	R.s.	*F.o.*	*C.g.*	*P.f.*	*F.s.*	*G.a.*
β-Caryophyllene	–	–	57.9	–	92.3	–
Caryophyllene oxide	125.9	164.0	66.1	–	70.2	–
T-Muurolol	15.2	34.2	17.1	87.3	17.3	93.0
α-Cadinol	21.0	30.1	11.7	51.9	36.5	44.3

R.s.: *Rhizoctonia solani*; *F.o.*: *Fusarium oxysporum*; *C.g.*: *Colletotrichum gloeosporioides*; *P.f.*: *Pestalotiopsis funerea*; *G.a.*: *Ganoderma australe*; *F.s.*: *Fusarium solani*; Unit: μg ml^{-1}; -: IC_{50} value > 200 μg ml^{-1}.

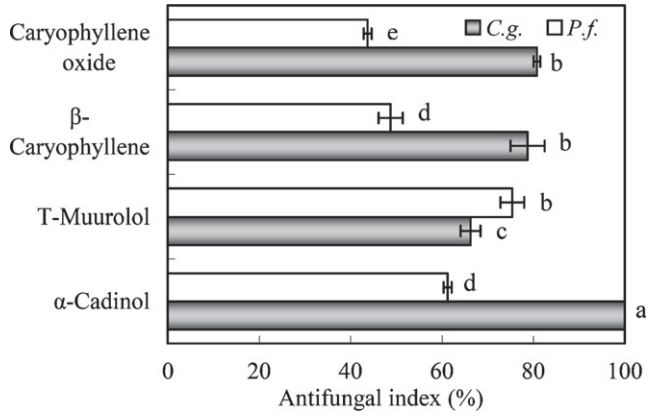

Fig. 3. Antifungal activities of sesquiterpenoid constituents (200 μg ml^{-1}) of *Calocedrus macrolepis* var. *formosana* leaf oil against the leaf pathogenic fungi. Bars with different letters (a–e) are statistically different at $p < 0.05$ according to the Scheffe's test.

of foliage, and post-harvest problems (Chang et al., 1997; Almada-Ruiz et al., 2003). Among leaf oil constituents, α-pinene, β-pinene, β-myrcene and limonene cannot inhibit the growth of *C. gloeosporioides* at a concentration of 200 μg ml^{-1} (data are not shown in Fig. 3). The better antifungal activities were found for sesquiterpenoid constituents. It is obvious that α-cadinol exhibited the highest antifungal activity completely inhibiting the growth of *C. gloeosporioides*. IC_{50} values of β-caryophyllene, caryophyllene oxide, T-muurolol, and α-cadinol were found as 57.9, 66.1, 17.1, and 11.7 μg ml^{-1} (Table 1), respectively. Antifungal indexes of four sesquiterpenoid against *P. funerea* ranged from 40% to 80% (Fig. 3). Only T-muurolol and α-cadinol can effectively inhibit the mycelial growth of *P. funerea* with IC_{50} values of 87.3 and 51.9 μg ml^{-1}, respectively.

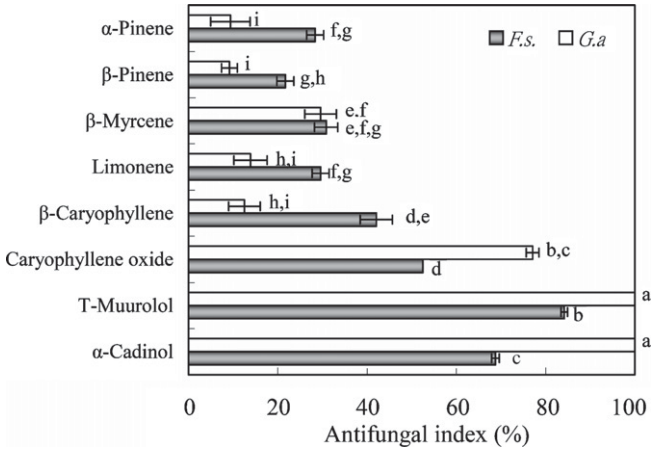

Fig. 4. Antifungal activities of the constituents (200 μg ml^{-1}) of *Calocedrus macrolepis* var. *formosana* leaf oil against the root pathogenic fungi. Bars with different letters (a–i) are statistically different at $p < 0.05$ according to the Scheffe's test.

3.4. Antifungal activities of the oil constituents against root rot pathogens

As shown in Fig. 4, caryophyllene oxide, T-muurolol, and α-cadinol possessed significant antifungal activities (antifungal index > 50%) against *F. solani* at a concentration of 200 μg ml^{-1}. T-Muurolol also showed noticeable activity with an IC_{50} value, 17.3 μg ml^{-1} (Table 1). Antifungal activities of leaf oil constituents against *G. australe* are presented in Fig. 4. Mycelial growth of *G. australe* was completely inhibited by T-muurolol and α-cadinol at a concentration of 200 μg ml^{-1}, whereas α-cadinol had a lowest IC_{50} value (44.3 μg ml^{-1}) against *G. australe*.

4. Conclusions

The present results show that, among the tested pathogenic fungi, the antifungal activities of leaf oil against *F. solani* and *P. funerea* were greater than that of the other fungi. Antifungal activities of sesquiterpenoid constituents were superior to those of its monoterpenoid constituents. Among active sesquiterpenoids, T-muurolol and α-cadinol possess the best activity against plant pathogenic fungi. Results suggest that *C. macrolepis* var. *formosana* leaf oil, T-muurolol and α-cadinol could be used as potential natural fungicide for controlling fungal pathogens and worth further investigation.

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