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Potential source of environmentally benign antifungal agents from *Cinnamomum osmophloeum* leaves against *Phellinus noxius*

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Abstract: The antifungal activity of leaf oils from different provenances of *Cinnamomum osmophloeum* was evaluated against *Phellinus noxius* and their chemical polymorphism. *C. osmophloeum* leaf oils of 15 provenances and their relative contents were classified into eight chemotypes, namely cinnamaldehyde, cinnamaldehyde/linalool, linalool, cinnamaldehyde/cinnamyl acetate, linalool/camphor, camphor/bornyl acetate, 1,8-cineole/*p*-cymene, and mixed types according to GC-MS, CA, and PCA. It was found that leaf oils of both cinnamaldehyde and cinnamaldehyde/cinnamyl acetate types had excellent inhibitory effects against *P. noxius*, and their IC_{50} values were 119.5 and 154.1 μg/ml, respectively. Furthermore, *trans*-cinnamaldehyde possessed the strongest antifungal activity among the constituents against *P. noxius*, and its IC_{50} values were 116.0 μg/ml.

Keywords: antifungal activity; genus *Cinnamomum*; *trans*-cinnamaldehyde; leaf oil; brown root rot disease

Fomes noxius was first described in Singapore by Corner in 1932 (Corner 1932) and reclassified by Cunningham in 1965 as *Phellinus noxius* (Corner) G. Cunn (Cunningham 1965). It is widespread in Australia, Africa, Central America, and in the Pacific Islands (Larsen & Cobb-Poule 1990; Chang & Yang 1998; Ann *et al*. 2002), where it causes the brown root rot disease affecting a great variety of important agricultural and forest plant species (Hodges & Tenorio 1984; Neil 1986; Nandris *et al*. 1987; Chang 1995a; Ann *et al*. 1999; Sahashi *et al*. 2007, 2010; Wu *et al*. 2011). *P. noxius* has a very wide host range; with more than 200 woody plant species representing 59 families currently reported as host plants worldwide (Ann *et al*. 2002). In addition, *P. noxius* poses a serious threat to forests and fruit trees in central and southern Taiwan at elevations below 800 m (Chang 1995b). The most practical approaches to the brown root rot disease control on plants include destroying inoculums through fumigating infested soils with ammonia (Chang & Chang 1999; Ann *et al*. 2002) or dazomet (Fu *et al*. 2012), and chemical control using several fungicides such as propiconazole or triadimefon (Tsai *et al*. 2005) for the brown root rot disease control on plants. Although effective, repeated use of synthetic fungicides in agriculture and public health programs have caused multifarious problems, including toxic hazards to human and non-target organisms, destabilisation of the ecosystem and environmental pollution (Ishii 2006). Hence, there is a need to discover and develop alternative strategies using environmentally safe, eco-friendly, biodegradable, and low-cost natural products as antifungal agents.

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The genus *Cinnamomum* Schaeffer belongs to the family Lauraceae which includes 250 species. It is a complex and economically significant genus distributed mainly in tropical and subtropical regions of Asia-Pacific and South America (Jayaprakasha *et al*. 2003). Many *Cinnamomum* plants show interesting bioactivities and have been applied as folk medicines. As an indigenous tree species in Taiwan, *Cinnamomum osmophloeum* Kanehira (Lauraceae) has many commercial applications (Hu *et al*. 1985). The chemical constituents of its leaf oil are similar to those of *C. cassia* bark oil (Chang *et al*. 2001), which is primarily used in the flavour and fragrance industries for imparting a cinnamon flavour and/or fragrance to various types of foods, beverages, perfumes, and medical products (Cheng *et al*. 2004). *C. osmophloeum* leaf exhibits considerable biological activities against fungi (Wang *et al*. 2005; Cheng *et al*. 2006, 2008), pathogens (Lee *et al*. 2005; Cheng *et al*. 2011), mildew (Chen & Chang 2002), bacteria (Chang *et al*. 2001, 2008), termites (Chang & Cheng 2002), red imported fire ants (Cheng *et al*. 2007), mosquito larvae (Cheng *et al*. 2004, 2009; Mdoe *et al*. 2014), mites (Chen *et al*. 2002), oxidants (Chua *et al*. 2008; Hsu *et al*. 2012; Wu *et al*. 2013), inflammation (Chao *et al*. 2005; Tung *et al*. 2008; Lin & Chang 2012), cancer (Fang *et al*. 2004; Huang *et al*. 2007), hyperuricemia (Wang *et al*. 2008), diabetes (Lee *et al*. 2009), and dyslipidemia (Lin *et al*. 2011). However, to the best of our knowledge there is no literature on the antifungal activity of *C. osmophloeum* leaf oil against *P. noxius*. To make up for such deficiency, this study examined the chemical polymorphism of leaf oils from different provenances of *C. osmophloeum*. The differences in antifungal activity against *P. noxius* among varieties of indigenous cinnamon leaf oils were also investigated.

MATERIAL AND METHODS

Plant materials. Fresh mature leaves of 15 *Cinnamomum osmophloeum* Kanehira provenances, in the family Lauraceae, were collected in January, 2013. Mature leaves from 12-year-old five specimens (JC-A, JC-B, JC-C, JC-D, and JC-E) were collected from Jhuci (23°30'28.79''N, 120°29'32.46''E) in Chiayi County, and those from 21-year-old trees of ten specimens (LL, P1, G2, D4, T3, G23, SP1, B1, T1-1, and S1-1) were collected from Lien Hua-Chin Research Center (23°55'21.95''N, 120°53'13.22''E) located in Nantou County in central Taiwan. Voucher specimens of each sample have been deposited with the wood chemistry laboratory at the School of Forestry and Resource Conservation, National Taiwan University. These species were identified by Mr. Yen-Ray Hsui (Taiwan Forestry Research Institute).

Chemicals. Camphene was obtained from ICN (Birsfelden, Switzerland). α-Pinene, β-pinene, benzaldehyde, *p*-cymene, 3-phenylpropionaldehyde, limonene, (–)-terpinen-4-ol, cinnamyl acetate, α-terpineol, coumarin, L-bornyl acetate, eugenol, *trans*-cinnamaldehyde, caryophyllene oxide, camphor, globulol, citral, geranyl acetate, and geraniol were purchased from Acros (Geel, Belgium). β-Myrcene, α-humulene, 3-methyl benzofuran, and fenchyl alcohol were obtained from Sigma (St. Louis, USA). α-Phellandrene, salicyaldehyde, 1,8-cineole, terpinolene, β-caryophyllene, 4-allylanisole, and linalyl acetate were purchased from TCI (Portland, USA). Sabinene was purchased from Chromdex (Shanghai, China). Alloaromadendrene, linalool, α-copaene, and guaiol were purchased from Fluka (Buchs, Switzerland). Neryl acetate was obtained from Alfa Aesar (USA). *epi*-Cubenol, α-cadinol, δ-cadinol, T-cadinol, and T-muurolol were isolated and purified in our laboratory.

Leaf oil preparation. About 150 g of mature *C. osmophloeum* leaves were hydrodistilled for 6 h in a Clevenger-type apparatus (Cheng *et al*. 2004). The leaf oils were kept in dark flasks at 4°C prior to analysis. The contents of leaf oils were analysed in triplicate and then averaged.

Gas chromatography (GC)-flame ionisation detection (FID). Analyses were performed on a Thermo Trace GC Ultra with an FID and 30 m × 0.25 mm × 0.25 mm DB-5 column (J & W Scientific, Folsom, USA). The injector temperature was 250°C and 1 µl of sample was injected at a split ratio of 1 : 10. The initial oven temperature was 60°C for 1 min, then increased to 220°C at a rate of 4°C/min and held for 2 min, and increased finally to 250°C at a rate of 20°C/min and held for 3 minutes. The flow rate of helium (the carrier gas) was 1 ml/minute.

GC-mass spectrometry. *C. osmophloeum* leaf oils were analysed on a Finnigan Trace GC-Polaris Q mass instrument (Finnigan-Spectronex, Waltham, USA), equipped with a fused silica column (30 m × 0.25 mm i.d.) and coated with DB-5ms (df = 0.25 µm). The mass spectrometer was recorded from 40 to 450 m/z at 1 scan/s, with 70 eV ionisation voltage and an ion source temperature of 230°C. The carrier

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gas was also helium at a flow rate of 1 ml/minute. The injector temperature was 250°C. The initial oven temperatures were programmed like in the GC-FID analysis. The injected samples were 1.0 µl (1 : 100, v/v, in ethyl acetate) in the manual split mode with a split ratio of 1 : 10.

Identification of constituents. The Kovats retention indices were calculated for all volatile constituents using a homologous series of C_9–C_{19} *n*-alkanes on DB-5ms column. Quantification was performed by the calculation of percentage peak areas determined using the GC-FID, and identification of the major components of *C. osmophloeum* leaf essential oils was confirmed by comparison with standards, by spiking, and on the basis of their mass spectral fragmentation using the NIST MS Search 2.0 and Wiley/NBS Registry of Mass Spectral Database 7.0, as outlined in the literature (Adams 2001, 2007). The contents of each compound were quantified according to the peak area integrated by the analysis program.

Fungal strain. The *Phellinus noxius* (Corner) Cunningham (BCRC35252) was purchased from the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute. The culture of the fungus was maintained on a potato dextrose agar (PDA) medium and stored at 4°C.

Antifungal assay. The antifungal assay was performed on solid media using the agar dilution method (Cheng *et al*. 2006). The leaf oils and their main constituents were dissolved in 150 µl of 99.5% EtOH and added into 15-ml sterilised PDA in 9-cm Petri dishes. EtOH was applied as the control test. The mycelium plug of the *P. noxius* strain was transferred to PDA media and then incubated at 26 ± 2°C and 70% RH until the mycelia of control dishes (without adding essential oils or constituents) reached the border of Petri dish, the antifungal index was then calculated. Triadimefon, a commercially available fungicide, was used as the positive control. Each test was repeated in triplicate and then averaged. The half-maximal inhibitory concentration (IC_{50}) value was calculated from a curve plotted between treated dose (µg/ml) and antifungal index (%), and expressed as 50% inhibition of fungal growth. The formula of the antifungal index is as follows:

Antifungal index (%) = {1 – diameter of the growth zone in the experimental dish (cm)/diameter of the growth zone in the control dish (cm)} × 100

Statistical analysis. Collected data were subjected to analysis of variance (ANOVA) and the means were separated by Scheffe's test at a 5% significance level. Assays were performed in triplicate and results were expressed as mean ± SD. The results were further processed with a discriminative study by CA and PCA to evaluate the similarity of leaf oils extracted from 15 *C. osmophloeum* provenances. CA and PCA were performed using multivariate statistical package software (MVSP for Windows v3.1.), Kovach Computing Services (Lee *et al*. 2003; Ko *et al*. 2006).

RESULTS

Contents of leaf oils. The leaf oil contents (calculated on dry-weight basis) from 15 sources of *C. osmophloeum* varied in the range of 0.06–3.03% (Table 1). The content of JC-B was recorded lowest (0.06%) while the highest oil content was found in LL (3.03%).

Table 1. Contents of different provenances from *C. osmophloeum* leaf oils

Specimens	Yield		Specimens	Yield	
	(ml/kg)	(%)		(ml/kg)	(%)
JC-A	6.98 ± 0.12^{G}	0.34 ± 0.12^{def}	D4	15.71 ± 0.93^{D}	1.09 ± 0.07^{c}
JC-B	1.87 ± 0.57^{H}	0.06 ± 0.01^{f}	T3	24.55 ± 0.50^{C}	1.80 ± 0.06^{b}
JC-C	7.15 ± 0.10^{G}	0.47 ± 0.06^{de}	G23	8.16 ± 1.10^{FG}	0.50 ± 0.11^{de}
JC-D	12.07 ± 1.02^{E}	0.68 ± 0.15^{d}	SP1	2.28 ± 0.35^{H}	0.07 ± 0.02^{f}
JC-E	12.17 ± 1.43^{E}	0.66 ± 0.17^{d}	B1	4.78 ± 0.56^{GH}	0.18 ± 0.02^{ef}
LL	33.20 ± 1.11^{B}	3.03 ± 0.07^{a}	T1-1	10.73 ± 0.89^{EF}	0.41 ± 0.09^{def}
P1	8.07 ± 0.73^{FG}	0.55 ± 0.02^{de}	S1-1	44.07 ± 0.67^{A}	2.79 ± 0.09^{a}
G2	17.78 ± 0.23^{D}	1.39 ± 0.11^{c}			

Results are mean ± SD (n = 3); different letters (A–H and a–f) are significantly different at the level of $P < 0.05$ according to Scheffe's test

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Table 2. Percentage compositions of 15 provenances from *C. osmophloeum* leaf oils

RT	KI	Component	Relative contents (%)														Identification
			JC-A	JC-D	JC-E	LL	P1	G2	JC-C	D4	T3	G23	SP1	JC-B	B1	T1-1	S1-1	
5.35	930	α-thujene	1.20								0.15	0.11						MS, KI
5.55	938	α-pinene	5.03				0.19	0.34	1.12	1.50	2.78	0.82			1.78	0.30		MS, KI, CO
5.96	955	camphene						0.21	0.77	1.12	1.47	0.42			1.09	0.23		MS, KI, CO
6.25	966	benzaldehyde		1.53	1.40		0.14	1.41	1.23	1.78		1.80		0.27	1.75		0.31	MS, KI, CO
6.52	976	sabinene	2.34															MS, KI, CO
6.68	981	β-pinene	3.62					0.17	0.50	0.76	0.95	0.26			0.27	0.14		MS, KI, CO
6.93	990	β-myrcene	2.94			0.34	0.36				1.07	0.23			0.25	0.12		MS, KI, CO
7.44	1008	α-phellandrene	3.25								0.29	0.17						MS, KI, CO
7.97	1027	*p*-cymene	20.29								0.53	3.06	12.85			0.37		MS, KI, CO
8.10	1031	limonene	2.53			0.30	0.46	0.26	0.53	0.59	3.69	0.96	1.08	0.20	0.92	0.77	0.03	MS, KI, CO
8.21	1035	1,8-cineole	38.86					0.28										MS, KI, CO
8.56	1047	salicyaldehyde											1.03	0.39	1.60	0.27		MS, KI, CO
9.86	1087	terpinolene														0.06		MS, KI, CO
10.29	1099	linalool				96.10	92.11	0.09			44.27	28.50	0.32		0.17	0.10		MS, KI, CO
10.98	1122	fenchyl alcohol														0.02		MS, KI, CO
11.88	1150	camphor									36.49					44.71	0.06	MS, KI, CO
12.35	1163	3-phenylpropionaldehyde		3.06	3.08			2.33	2.51	2.00		2.97	0.42	0.87	2.68	0.09	0.87	MS, KI, CO
12.78	1176	3-methyl benzofuran											2.24	1.32		1.80	0.10	MS
12.98	1181	terpinen-4-ol	4.30								0.28	1.29	7.26			1.30		MS, KI, CO
13.48	1195	α-terpineol	8.88			0.23	0.52		0.11	0.18	0.41	0.41		0.16	0.40	1.58	0.04	MS, KI, CO
13.57	1197	4-allylanisole		1.11	1.17	0.34	0.23	0.76	0.79	1.09	0.10	1.35	4.30	1.09	2.64		0.17	MS, CO
14.93	1240	*Z*-citral														0.12		MS, CO
15.29	1251	linalyl acetate					0.19			0.13		0.12	0.17		1.05			MS, KI, CO
15.33	1252	geraniol														0.23		MS, KI, CO
15.92	1269	L-citral														0.16		MS, CO
16.07	1273	*trans*-cinnamaldehyde		92.48	92.38	1.22	1.07	90.81	77.61	78.57		46.32	1.23	8.08	20.63	1.51	59.71	MS, KI, CO
16.46	1284	F-bornyl acetate		0.19	0.18		0.20	0.70	2.78	3.66	4.26	1.86	6.53	7.98	3.94	29.57	0.55	MS, KI, CO
18.63	1352	eugenol		0.52	0.51		0.18	0.26	0.43	0.66	0.22	1.09	0.45		0.29	1.18	0.28	MS, KI, CO
18.93	1360	neryl acetate														0.08		MS, KI, CO
19.44	1377	α-copaene						0.11	0.57	0.37		0.31	1.67	1.42	1.04	0.28	0.03	MS, KI, CO
19.57	1379	geranyl acetate														0.82		MS, KI, CO

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Table 2 to be continued

RT	KI	Component	Relative contents (%)														Identification
			JC-A	JC-D	JC-E	LL	P1	G2	JC-C	D4	T3	G23	SP1	JC-B	B1	T1-1	S1-1	
20.69	1412	*cis*-α-bergamotene														0.03		MS, KI
20.84	1419	β-caryophyllene	6.15		0.09	0.59	1.73	0.53	3.36	3.39	0.71	1.48	5.78	2.92	8.69	1.24	0.18	MS, KI, CO
21.26	1433	coumarin				0.39						0.15	0.39	1.02	0.26	0.64		MS, KI, CO
21.64	1444	cinnamyl acetate												1.89	0.20	0.40	36.93	MS, KI, CO
21.96	1454	α-humulene					0.19		0.39	0.40		0.17		0.50	1.21		0.03	MS, KI, CO
22.09	1457	*allo*-aromadendrene											0.29	0.72	1.47			MS, KI, CO
22.62	1474	γ-muurolene														0.08		MS, KI
23.27	1494	*epi*-cubebol														0.06		MS, CO
23.35	1496	α-muurolene														0.15		MS, KI
23.73	1506	γ-cadinene					0.22		0.35				0.61	0.69	1.50	0.23		MS, KI, CO
23.90	1511	δ-cadinene			0.09			0.25	0.42	0.26	0.14	0.67		1.41	5.98	1.07	0.05	MS, KI, CO
24.64	1540	α-calacorene														0.07		MS, KI, CO
25.79	1576	caryophyllene oxide		0.19	0.19		0.44	0.24	1.02	1.37		1.11	12.45	3.69	3.39	1.80	0.12	MS, KI, CO
25.90	1580	globulol												0.97	1.53			MS
26.23	1591	guaiol					0.37		0.38	0.18		0.33	1.45	0.47	1.62	0.51	0.03	MS, KI, CO
27.14	1623	*epi*-cubenol											3.49		3.65			MS, KI
27.61	1639	T-muurolol + T-cadinol					0.26					0.41	13.22	2.03	8.79	2.54		MS, KI, CO
27.69	1643	δ-cadinol												0.39	1.40			MS, CO
27.94	1652	α-cadinol						0.11				0.61		2.22	7.70	1.90		MS, KI, CO
28.36	1667	14-hydroxy-9-*epi*-(*E*)-caryophyllene											1.88					MS
35.57	1939	cembrene												25.64				MS
All identified components (%)			99.38	99.08	99.12	99.51	98.87	98.88	94.86	98.00	97.81	96.96	79.11	66.35	87.90	96.53	99.51	
Monoterpene hydrocarbons (%)			38.67	0.00	0.00	0.34	0.55	0.72	2.38	3.37	7.24	5.06	12.85	0.00	3.40	1.23	0.00	
Oxygenated monoterpenes (%)			54.56	0.19	0.18	96.63	93.49	1.33	3.41	4.56	89.40	33.13	15.36	8.34	6.47	79.46	0.68	
Sesquiterpene hydrocarbons (%)			6.15	0.00	0.18	0.59	2.15	0.89	5.09	4.42	0.85	2.64	8.34	7.68	19.90	3.15	0.29	
Oxygenated sesquiterpenes (%)			0.00	0.19	0.19	0.00	1.07	0.35	1.40	1.55	0.00	2.46	32.50	9.77	28.08	6.80	0.16	
Diterpene hydrocarbons (%)			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	25.64	0.00	0.00	0.00	
Other (%)			0.00	98.69	98.56	1.95	1.62	95.57	82.58	84.10	0.32	53.67	10.06	14.93	30.05	5.89	98.38	

Retention time (min); KI – Kovats index on a DB-5ms column relative to the homologous series of *n*-alkanes (C_9–C_{24}); identification based on a comparison of the mass spectrum (MS), Kovats index (KI) on a DB-5ms column in reference (Adams 2001, 2007) and co-injection (CO) with authentic compounds

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Chemical analysis of leaf oils. The compositions of 15 leaf oils were analysed using both GC-FID and GC-MS. A total of 53 compounds, representing 66.35–99.51% of leaf oil compositions, were identified and separated into six categories on the basis of their chemical structures (Table 2). The six categories were monoterpene hydrocarbons (0.00–38.67%), oxygenated monoterpenes (0.18–96.63%), sesquiterpene hydrocarbons (0.00–19.90%), oxygenated sesquiterpenes (0.00–29.00%), diterpene hydrocarbons (0.00–25.64%), and others (0.00–98.69%). The leaf oils of provenances JC-C, JC-D, JC-E, G2, D4, G23, and S1-1 show the presence of 18, 7, 9, 17, 18, and 17 identified compounds representing 94.86, 99.08, 99.12, 98.88, 98.00, 96.96, and 99.51% of the total oil content, respectively. Among these compounds, *trans*-cinnamaldehyde was the major constituent accounting for 77.61, 92.48, 92.38, 90.81, 78.57, 46.32, and 59.71% of the total oil content, respectively.

Furthermore, leaf oils from provenances LL and P1 show the presence of 8 and 18 identified compounds accounting for 99.5 and 98.87% of the total oil content with linalool as the major constituent (96.10 and 92.11%), respectively. Provenance T1-1 leaf oil contained 39 identified compounds, accounting for 96.53% of the total oil content with camphor as the major component (44.71%), followed by L-bornyl acetate (29.57%). The leaf oil of provenance T3 contained 18 identified compounds, accounting for 98.02% of the total oil content with linalool (44.27%) and camphor (36.49%) as its main constituents. Provenance JC-A leaf oil contained 12 identified compounds, accounting for 99.38% of the total oil content with 1,8-cineole (38.86%) as its main constituent. The leaf oils of provenances JC-B, SP1, and B1 contained 25, 22, and 31 identified compounds, accounting for 66.35, 75.62, and 87.90% of the total oil content with cembrene (25.64%), *p*-cymene (12.85%), and *trans*-cinnamaldehyde (20.63%), respectively, as their main constituents.

Multivariate data analyses of leaf oils. To characterise and verify the variations in essential oils and to identify the differences between possible chemotypes of the 15 provenances of *C. osmophloeum*, cluster analysis was performed to determine their essential oil compositions (Figure 1). Following the results obtained herein and the previous classification by Hu *et al*. (1985), Lee *at al*. (2003), and Cheng *et al*. (2004, 2006), this study divided the 15 provenances into eight main types in Euclidean distance 40. The main constituent of cinnamaldehyde

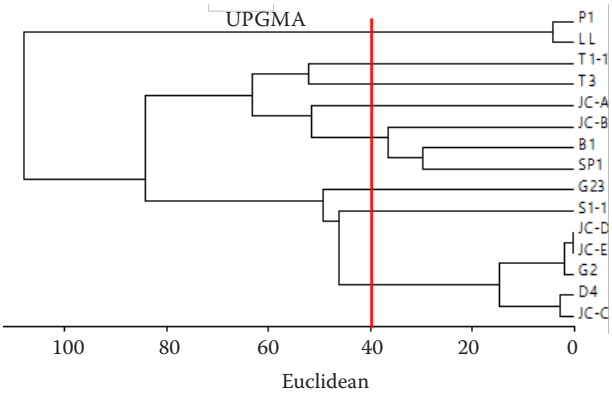

Figure 1. Dendrogram obtained by cluster analysis of the percentage composition of leaf oils from 15 provenances of *C. osmophloeum*

type was *trans*-cinnamaldehyde (77.61–92.48%) and comprised five provenances (JC-C, JC-D, JC-E, G2, and D4). The cinnamaldehyde/cinnamyl acetate type was composed of one provenance (S1-1) having an essential oil with *trans*-cinnamaldehyde (59.71%) and cinnamyl acetate (36.93%) as the major components. The cinnamaldehyde/linalool type comprised provenance G23 with essential oil containing *trans*-cinnamaldehyde (46.32%) and linalool (28.50%). The linalool type was composed of provenances LL and P1 characterised by oils with a high content of linalool (96.10 and 92.11%, respectively). T3 was the sole provenance of the linalool/camphor type containing mainly linalool (44.27%) and camphor (36.49%). The camphor/bornyl acetate type (found in oil from provenance T1-1) was characterised by a high content of camphor (44.71%) and L-bornyl acetate (29.57%). The 1,8-cineole/*p*-cymene type was composed of provenance JC-A having an essential oil with 1,8-cineole (38.86%) and *p*-cymene (20.29%) as the main constituents. The mixed type comprised three provenances (JC-B, SP1, and B1) having essential oils with cembrene (25.64%), *p*-cymene (12.85%), and *trans*-cinnamaldehyde (20.63%) as the major components. The essential oils from provenances JC-B, SP1, and B1 were classified as a mixed type owing to the absence of a dominant compound (> 30.0%).

In addition, PCA was employed to differentiate the data listed in Table 2. As indicated in the PCA score plot shown in Figure 2, PC1 and PC2 explained 61.0 and 24.0% of the total variance, respectively. The PCA results on the content of individual components for each provenance supported the differentiation of the samples obtained by CA analysis.

Antifungal activity of leaf oils from different chemotypes. A total of eight chemotypes of *C. os-*

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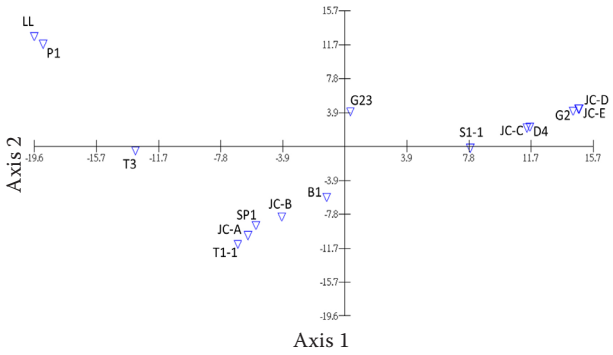

Figure 2. PCA analysis of the percentage composition of leaf oils from 15 provenances of *C. osmophloeum*

mophloeum leaf oil were investigated for their activity against *P. noxius*. Figure 3 shows antifungal activities of leaf oils from eight chemotypes of *C. osmophloeum* at concentrations of 200 and 400 µg/ml. As can be seen, the antifungal indices of the linalool, linalool/camphor, camphor/bornyl acetate, 1,8-cineole/*p*-cymene, mixed, cinnamaldehyde, cinnamaldehyde/cinnamyl acetate, and cinnamaldehyde/linalool types were 56, 17, 39, 65, 62, 100, 100, and 100% at a treatment concentration of 400 µg/ml, respectively (Figure 3). These results showed that leaf oils of cinnamaldehyde, cinnamaldehyde/cinnamyl acetate, and cinnamaldehyde/linalool types completely inhibited the growth of *P. noxius* at a concentration of 400 µg/ml. When the treatment concentration was decreased to 200 µg/ml, only leaf oils of cinnamaldehyde and cinnamaldehyde/cinnamyl acetate types still exhibited excellent activity against *P. noxius* with their antifungal indices being 100% (Figure 3).

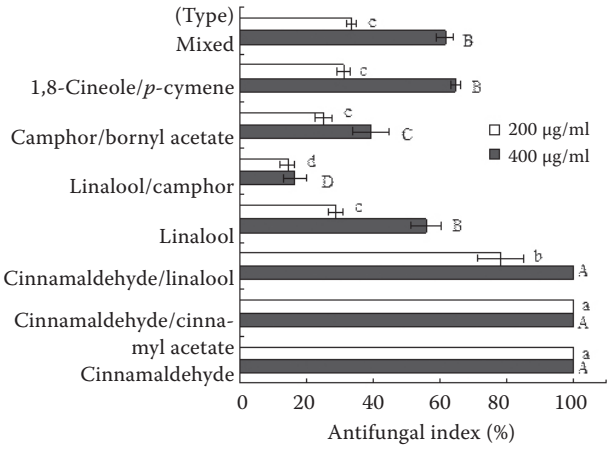

Figure 3. Antifungal activities of leaf oils from eight chemotypes of *C. osmophloeum* against *P. noxius*

Results are mean ± SD (n = 4); different letters (A–D, a–d) are significantly different at the level of $P < 0.05$ according to Scheffe's test

Table 3. IC_{50} values of leaf oils from different chemotypes of *C. osmophloeum* against *P. noxius*

Chemotypes	IC_{50} (µg/ml)	Chemotypes	IC_{50} (µg/ml)
Cinnamaldehyde (JC-E)	119.5	linalool/camphor (T3)	> 400.0
Cinnamaldehyde/cinnamyl acetate (S1-1)	154.1	camphor/bornyl acetate (T1-1)	> 400.0
Cinnamaldehyde/linalool (G23)	165.2	1,8-cineole/*p*-cymene (JC-A)	245.7
Linalool (LL)	342.6	mixed (JC-B)	298.6
Triadimefon*	6.4		

*positive control

Comparisons of IC_{50} values among eight chemotypes of *C. osmophloeum* leaf oil against *P. noxius* are given in Table 3. The IC_{50} values of leaf oils of linalool, linalool/camphor, camphor/bornyl acetate, 1,8-cineole/*p*-cymene, mixed, cinnamaldehyde, cinnamaldehyde/cinnamyl acetate, and cinnamaldehyde/linalool types against *P. noxius* were 342.6, > 400.0, > 400.0, 245.7, 298.6, 119.5, 154.1, and 165.2 µg/ml, respectively.

Antifungal activity of the constituents. To further understand the relative antifungal activity of the constituents in leaf oils of cinnamaldehyde and cinnamaldehyde/cinnamyl acetate types, the antifungal performance of 12 selected constituents (cinnamyl acetate, caryophyllene oxide, bornyl acetate, terpinen-4-ol, camphor, 1,8-cineole, linalool, β-caryophyllene,

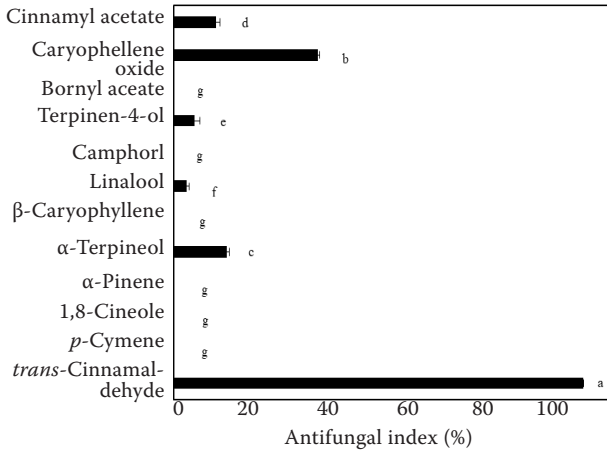

Figure 4. Antifungal activities of main constituents (200 µg per ml) of leaf oils from *C. osmophloeum* against *P. noxius*

Results are mean ± SD (n = 4); different letters (a–g) are significantly different at the level of $P < 0.05$ according to Scheffe's test

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α-pinene, α-terpineol, *p*-cymene, and *trans*-cinnamaldehyde) in *C. osmophloeum* was examined. As shown in Figure 4, *trans*-cinnamaldehyde exhibited the strongest activity against *P. noxius* among the 12 compounds tested. *trans*-Cinnamaldehyde inhibited totally the growth of *P. noxius* at a concentration of 200 μg/ml, and its IC_{50} value 116.0 μg/ml. However, the antifungal indices of the other 11 compounds against *P. noxius* did not exceed 35.0% (IC_{50} = > 200.0 μg/ml), indicating that cinnamyl acetate, caryophyllene oxide, bornyl acetate, terpinen-4-ol, camphor, linalool, β-caryophyllene, α-pinene, α-terpineol, and *p*-cymene could not inhibit the fungal growth of *P. noxius*.

DISCUSSION

In general, analytical methods, environment (climatic, seasonal, and geographical), and sample genotype are factors contributing to variations in the content and chemical composition of essential oils. Thus, the contents and chemical polymorphism of leaf oils from different provenances of *C. osmophloeum* were examined in this study. According to the results obtained, *C. osmophloeum* leaf oil (LL provenances) was the highest oil content (3.03%). These results are similar to previous findings (Lee *et al*. 2003; Cheng *et al*. 2008). For example, Lee *et al.* (2003) reported that the Li-Lon-San provenance had the highest content (4.70%) and the Pu-Li-Tso-Zi-Lia provenance had the lowest content (0.13%). In addition, Lin *et al.* (2007) reported that the average yield of leaf essential oils in 92 *C. osmophloeum* trees from steam distillation was 0.54%, and ranged from 0.09% to 2.65%. Such discrepancies might be attributed to the different provenances of the species, environmental, genetic and seasonal factors and harvest time.

In general, plants are usually classified based on their characteristic properties, i.e. taxonomy. In addition, plants can be classified based on their difference in gene or chemical composition (Lin *et al.* 1994; Adams 1998). To well organize and explain the results from the analyses of gene or chemical composition, the multivariate data analyses [e.g. cluster analysis (CA) and principal component analysis (PCA)] allow a more objective interpretation (Angioni *et al.* 2004). Russo *et al.* (1998) used CA or PCA to study the variability or chemotaxonomy of plants by analysing their chemical composition of essential oils. Chen *et al*. (2011) studied the phylogenetic relationships of the genus *Chamaecyparis* using PCA and CA to determine the chemical composition of their leaf essential oils. *C. osmophloeum* leaf oils of 15 provenances and their relative contents were classified into eight chemotypes, namely cinnamaldehyde, cinnamaldehyde/linalool, linalool, cinnamaldehyde/cinnamyl acetate, linalool/camphor, camphor/bornyl acetate, 1,8-cineole/*p*-cymene, and mixed types according to GC-MS, CA, and PCA. The present results plus those obtained by Cheng *et al*. (2004, 2006) show similar chemotypes extracted from *C. osmophloeum* leaf oils, namely cinnamaldehyde, cinnamaldehyde/cinnamyl acetate, linalool, and mixed types. However, this study was the first to identify cinnamaldehyde/linalool, linalool/camphor, camphor/bornyl acetate, and 1,8-cineole/*p*-cymene types in *C. osmophloeum* leaf oils of Taiwan.

The antifungal activities of eight chemotypes of *C. osmophloeum* leaf oil against *P. noxius* were investigated. The aforesaid results show the leaf oils of both cinnamaldehyde and cinnamaldehyde/cinnamyl acetate types exhibited greater antifungal activity (Table 3) and contained a higher amount of *trans*-cinnamaldehyde (Table 2) compared with those of the other chemotypes. Hence, it is obvious that antifungal activities of these two leaf oils are directly affected by the *trans*-cinnamaldehyde content. Similar findings were also noted in previous studies on the antitermitic (Chang & Cheng 2002), antifungal (Wang *et al*. 2005; Cheng *et al*. 2006), and mosquito larvicidal activities (Cheng *et al*. 2004, 2009) of *C. osmophloeum* leaf oils. In contrast, Zahari *et al.* (2014) examined the antifungal activities of 12 Malaysian medicinal plant extracts against three root rot fungi (*P. noxius*, *Ganoderma philippii*, and *Rigidoporus microporus*) and found that none of the extracts could inhibit the growth of *P. noxius*. Cheng *et al.* (2010) reported that 400 μg/ml of leaf, twig, and heartwood essential oils from *Taiwania cryptomerioides* completely inhibited the growth of *P. noxius*. The results revealed that cinnamaldehyde and cinnamaldehyde/cinnamyl acetate types of *C. osmophloeum* leaf oil may have a good potential to be applied as an antifungal agent against the root rot fungus *P. noxius*.

To further understand the relative antifungal activity of the constituents in leaf oils of cinnamaldehyde and cinnamaldehyde/cinnamyl acetate types, the antifungal activity of 12 constituents in *C. osmophloeum* was examined. The results of this study showed that *trans*-cinnamaldehyde exhibited the strongest activity against *P. noxius* among the 12 compounds tested (Figure 4). Thus, *C. osmophloeum* leaf oils of

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both cinnamaldehyde and cinnamaldehyde/cinnamyl acetate types have an excellent antifungal activity attributed mainly to *trans*-cinnamaldehyde. A similar result was also noted in our previous studies on the antibacterial (Chang *et al*. 2001), antitermitic (Chang & Cheng 2002), anti-mosquito (Cheng *et al*. 2004, 2009), antipathogenic (Lee *et al*. 2005; Cheng *et al*. 2011), and antifungal (Wang *et al*. 2005; Cheng *et al*. 2006) activities of *C. osmophloeum*.

In conclusion, this study is the first investigation on antifungal activities of leaf essential oils from *C. osmophloeum* against *P. noxius*. *trans*-Cinnamaldehyde as well as leaf oils of both cinnamaldehyde and cinnamaldehyde/cinnamyl acetate types are renewable natural products that may be further explored as a potential source of environmentally benign constituents for the development of antifungal agents or fumigants.

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