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Nematicidal activity of monoterpenoids against the pine wood nematode (*Bursaphelenchus xylophilus*)

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Summary. The nematicidal activity of twenty-six naturally occurring monoterpenoids was evaluated with a 96-microwell plate bioassay against the pine wood nematode, *Bursaphelenchus xylophilus*. Responses varied with test compounds and dose. Good nematicidal activity against male, female and juvenile nematodes of *B. xylophilus* was achieved with carvacrol, thymol, geraniol, nerol, (-)-menthol, β-citronellol, (±)-citronellal and citral (mix. of cis and trans). Phenol, alcohol and aldehyde compounds were generally more toxic to *B. xylophilus*. Carvacrol and thymol showed the most potent nematicidal activity among test compounds. LC_{50} values of thymol and carvacrol against juvenile nematodes were 0.096 and 0.099 mg ml^{-1}, respectively. Citral and citronellol showed similar toxicity to juvenile nematodes. Citronellal was more toxic to juvenile nematodes (LC_{50} = 0.253 mg ml^{-1}) than geraniol (LC_{50} = 0.417 mg ml^{-1}). LC_{50} values of nerol and menthol against juvenile nematodes were 0.979 and 1.021 mg ml^{-1}, respectively. LC_{50} values of the other test compounds and trunk-injection nematicide, levamisole hydrochloride, were > 1.0 mg ml^{-1} within 4 hours after treatment.

Key words:, *Bursaphelenchus xylophilus*, carvacrol, monoterpenoids, nematicidal activity, pine wood nematode, thymol.

Pine wilt disease, caused by the pine wood nematode, *Bursaphelenchus xylophilus,* is the most serious problem in southern Korea forests. This disease was first reported in Busan, Gyeongsangnam-do province in 1988 (Yi *et al.*, 1989). Within a few years, *B. xylophilus* has become the most economically important pest of *Pinus* species (Chung, 2002; Anon., 2005). As *Pinus densiflora* and *P. thunbergii* are predominant tree species in Korean forests and are very susceptible to the pine wood nematode, ecological and economical damage is substantial (Park *et al.*, 2005).

Control of this disease depends primarily on fumigation of disease-infected trees with metham-sodium, aerial application of synthetic pesticides such as fenitrothion and thiacloprid against *Monochamus alternatus,* the insect vector of this nematode, or trunk injection of nematicides, such as morantel tartrate, emamectin benzoate and levamisole hydrochloride (Kishi, 1995; Anon, 2003; Lee *et al.,* 2003). However, there is environmental and human health concerns on synthetic pesticides or nematicides. Additionally, factors such as increased cost of pesticide, labour and pesticide application have made pine wilt disease control difficult. These problems have highlighted the need for the development of pine wood nematode control alternatives. Phytochemicals are good candidates because they can be developed for use as nematicides themselves, or they can serve as model compounds for the development of chemically synthesized derivatives with enhanced activity or environmental friendliness (Chitwood, 2002). Furthermore, many, but certainly not all, phytochemicals are safer to the environment or humans than traditional chemical nematicides.

This paper describes a laboratory study to assess the potential of naturally occurring monoterpenoids for use as commercial nematicides to control the pinewood nematode using micro-well assays.

MATERIAL AND METHODS

Collection of pine wood nematodes. *Bursa-phelenchus xylophilus* was isolated from chips of infected pine wood collected in the Haman area (on March 2004), Gyoungsangnam-do province,

Korea and extracted by Baermann funnel method. The pine wood nematode isolate was rinsed three times with sterile distilled water and reared on a lawn of *Botrytis cinerea* cultured on potato dextrose agar medium (PDA) in the dark at 28°C. Juveniles and mature nematodes were extracted from fungal cultures with sterile distilled water in shallow pans 10 to 12 days after inoculation. They were collected after 4 to 8 h and concentrated. The nematodes were rinsed from the filter disks with sterile distilled water and collected.

Chemicals. The monoterpenoids tested were purchased from Aldrich, Fluka, Sigma, TCI (Tokyo Chemical Industry Co., Ltd.) and Wako Pure Chemical Industires, Ltd. (Osaka, Japan), respectively (Table 1). Levamisole hydrochloride (purity, 99%) was purchased from TCI.

Nematicidal activity. Concentrations of monoterpenoids were prepared by serial dilution with distilled water containing Triton X-100 (5000 ppm). Test solutions were introduced in wells of 96-well plates (Falcon, USA). In each well, the concentration was about 300 nematodes (mixtures of juvenile and adult nematodes, male:female:juvenile approx. 1:1:2) per 100 μl of water. Controls received distilled water-Triton X-100 solution. Treated and control nematodes were held under the same conditions as used for colony maintenance. Mortality of male, female and juvenile (second- and third-stage) nematodes was recorded after 4 h under a microscope. Nematodes were defined as dead if their body was straight and they did not move, even after mechanical prodding. All treatments were replicated four times.

Statistical Analysis. The treatment means were subjected to probit analysis to estimate LC_{50} values (SAS, 1999).

RESULTS

When 26 monoterpenoids were bioassayed, toxicity varied according to compounds, dose, stage and sex of nematode (Table 2). Phenols such as carvacrol and thymol showed the most potent activity against pine wood nematode. LC_{50} values of carvacrol against male, female and juvenile nematode were 0.125, 0.097 and 0.099 mg ml^{-1}, respectively. Among the alcohol group, citronellol showed the most potent activity. Juveniles were more susceptible (LC_{50} = 0.169 mg ml^{-1}) than males (LC_{50} = 0.245 mg ml^{-1}) and females (LC_{50} = 0.235 mg ml^{-1}). Toxicity of geraniol was weaker than citronellol. LC_{50} values of geraniol against male, female and juveniles were 0.540, 0.415 and 0.417 mg ml^{-1}, respectively. Nerol and menthol have similar LC_{50} values. The other alcohols displayed weak activity. In the aldehyde group, citral showed the strongest activity. LC_{50} values of citral against male, female and juveniles were 0.187, 0.139 and 0.110 mg ml^{-1}, respectively. LC_{50} values of citronellal against male, female and juveniles were 0.321, 0.298 and 0.253 mg ml^{-1}, respectively. LC_{50} values of all hydrocarbons were >1.0 mg ml^{-1}. The trunk-injection nematicide, levamisole hydrochloride, also showed very weak activity within 4 h. LC_{50} value of levamisole hydrochloride was > 1.0 mg ml^{-1}.

DISCUSSION

Many naturally occurring compounds are known to possess nematicidal activity (Chitwood, 2002). Polythienyls in *Tagetes* spp. (Kyo *et al.*, 1990), isothiocyanates and glucosinolates from Brassicaceae (Brown & Morra, 1997), cyanogenic glycosides (Magalhaes *et al.*, 2000), polyacetylenes from Asteraceae (Kogiso *et al.*, 1976), alkaloids (Matsuda et al., 1989), fatty acids (Saleh *et al.*, 1987), terpenoids (Oka *et al.*, 2000), phenolics (Evans *et al.*, 1984) and cinnamates from *Kaempferia galanga* (Choi *et al.*, 2006) have been reported to show nematicidal activity. In our study, the nematicidal activities of twenty-six naturally occurring monoterpenoids were evaluated against the pine wood nematode, *Bursaphelenchus xylophilus*. Responses varied with test compounds and dosage. Good nematicidal activity against male, female and juveniles of *B. xylophilus* was achieved with carvacrol, thymol, geraniol, nerol, menthol, citronellol, citronellal and citral.

A large number of plant compounds called isoprenoids are formed by the condensation of five-carbon isoprene units. Among the simplest are 10-carbon compounds called monoterpenoids, which are major components of plant essential oils. Monoterpenoid compounds have been considered as potential pest control agents because they are acutely toxic to insects and possess repellent (Watanabe *et al.*, 1993).

Monoterpenoids were selected in this study because many plant essential oils have proved to be active against the pine wood nematode in a previous study (Park *et al.*, 2005). Furthermore, the chemical structures of monoterpenoids are very simple. A total of 26 monoterpenoids were selected to investigate the nematicidal activity against pine wood nematode. There was a significant difference in nematicidal activity among functional groups. Phenol, alcohol and aldehyde compounds were generally more toxic than other monoterpenoids groups such as ketones and hydrocarbons. Two phenols (carvacrol

Table 1. List of monoterpenoids tested.

Compounds	Chemical formula	Purity (%)	Company
Alcohols and Phenols			
Borneol	$C_{10}H_{18}O$	70	TCI
(-)-Carveol	$C_{10}H_{16}O$	97	Aldrich
Carvacrol	$C_{10}H_{14}O$	95	TCI
β-Citronellol	$C_{10}H_{20}O$	95	Aldrich
Geraniol	$C_{10}H_{18}O$	96	Fluka
Linalool	$C_{10}H_{18}O$	98	Wako
(-)-Menthol	$C_{10}H_{20}O$	99	Fluka
Nerol	$C_{10}H_{18}O$	97	Aldrich
α-Terpineol	$C_{10}H_{18}O$	95	TCI
Terpinen-4-ol	$C_{10}H_{18}O$	99	Fluka
Thymol	$C_{10}H_{14}O$	99	Fluka
(S)-cis-Verbenol	$C_{10}H_{16}O$	95	Aldrich
Aldehydes			
Citral (mix. of cis and trans)	$C_{10}H_{16}O$	95	Aldrich
(±)-Citronellal	$C_{10}H_{18}O$	85	Sigma
Ketones			
(+)-Carvone	$C_{10}H_{14}O$	96	Aldrich
(-)-Carvone	$C_{10}H_{14}O$	98	Aldrich
(+)-Fenchone	$C_{10}H_{16}O$	98	Fluka
(-)-Menthone	$C_{10}H_{18}O$	90	Aldrich
(+)-Pulegone	$C_{10}H_{16}O$	98	Aldrich
(-)-Pulegone	$C_{10}H_{16}O$	98	Aldrich
(-)-Verbenone	$C_{10}H_{14}O$	97	Fluka
Hydrocarbons			
(+)-Camphene	$C_{10}H_{16}$	80	Aldrich
(+)-Limonene	$C_{10}H_{16}$	95	TCI
Myrcene	$C_{10}H_{16}$	95	Fluka
α-Pinene	$C_{10}H_{16}$	95	TCI
β-Pinene	$C_{10}H_{16}$	94	TCI

Table 2. Nematicidal activity of monoterpenoids against the pine wood nematode.

Compounds[a]	LC_{50} (mg ml^{-1})		
	Male	*Female*	*Juvenile*
Carvacrol	0.125(0.096-0.145)[b]	0.097(0.067-0.121)	0.099(0.097-0.112)
Citronellol	0.245(0.199-0.289)	0.235(0.198-0.272)	0.169(0.138-0.198)
Geraniol	0.540(0.496-0.590)	0.415(0.330-0.492)	0.417(0.357-0.484)
Menthol	0.985(0.874-1.091)	0.894(0.757-1.012)	1.021(0.956-1.096)
Nerol	0.865(0.765-0.962)	0.926(0.853-1.029)	0.979(0.903-1.058)
Thymol	0.119(0.097-0.137)	0.110(0.090-0.127)	0.096(0.084-0.107)
Citral	0.187(0.113-0.236)	0.139(0.100-0.173)	0.110(0.093-0.125)
Citronellal	0.321(0.265-0.365)	0.298(0.240-0.342)	0.253(0.226-0.280)
Levamisole hydrochloride	>1.0	>1.0	>1.0

[a] Compounds with LC50 values < 1.0 mg ml^{-1} were reported.
[b] Values in parentheses indicate 95% confidence limit.

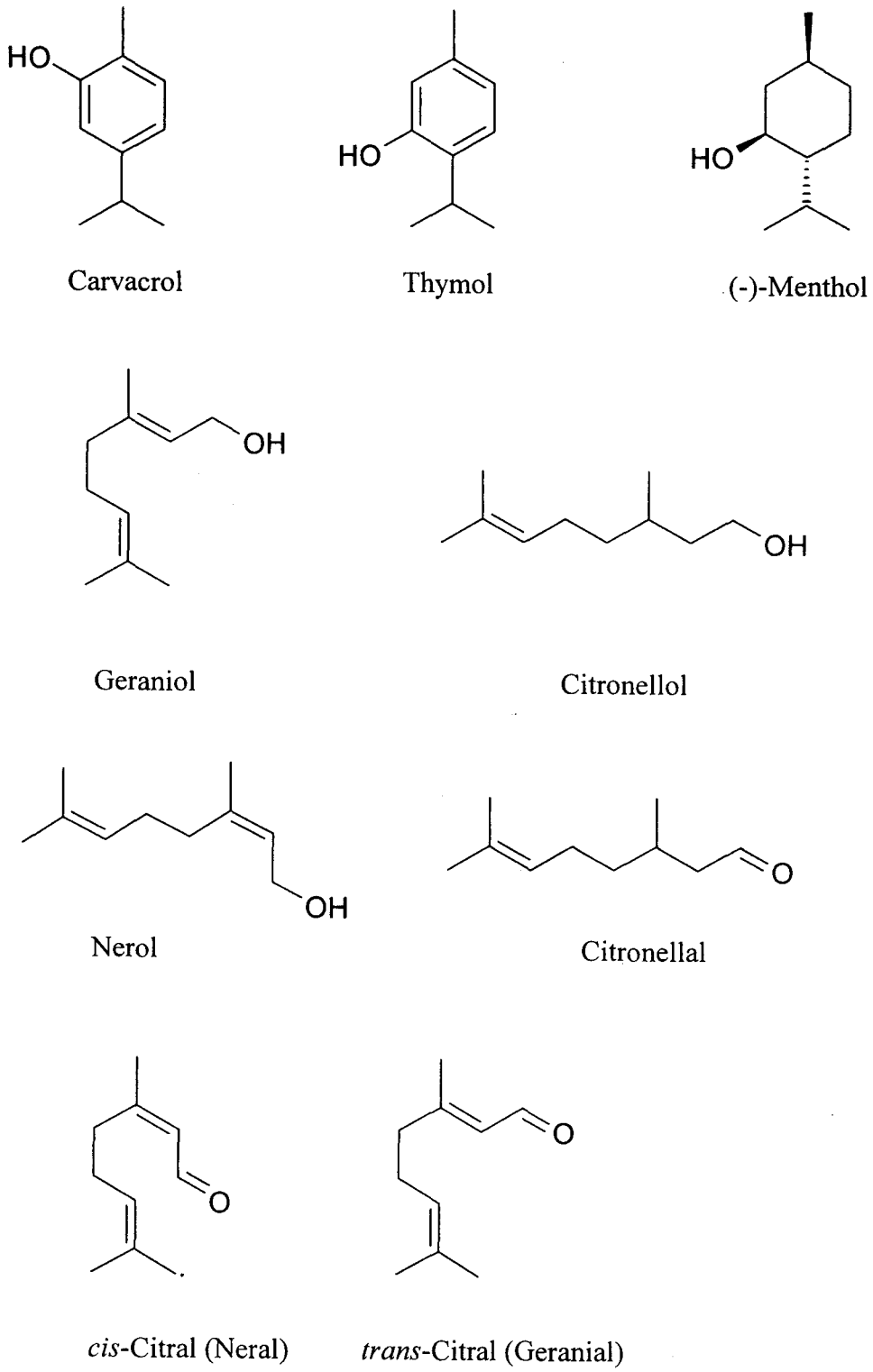

Fig. 1. Chemical structures of active monoterpenoids.

and thymol) showed the most potent nematicidal activity. Phenol is more acidic than alcohols. The acidity might be one reason for strong activity. Among the alcohol group, primary alcohols (citronellol, geraniol and nerol) were more active than secondary (menthol, carveol and verbenol) and tertiary alcohols (linalool, borneol and terpine-4-ol). This result means that the position of hydroxyl group is related to the nematicidal activity. The aldehyde group showed good nematicidal activity against the pine wood nematode. Aldehydes of essential oil components such as citral, (-)-perillaldehyde and trans-cinnamaldehyde have been reported to have nematicidal activity (Sangwan *et al.*, 1990; Tsao and Yu, 2000; Park *et al.*, 2005). These observations raise the possibility that the presence of the aldehyde functional group is related to toxicity against pine wood nematode. All ketones and hydrocarbons revealed weak or no activity in this study. Levamisole hydrochloride, commercial nematicides, was not effective at 1.0 mg ml^{-1} concentration with 4 h after treatment. However, the movement of pine wood nematode decreased compared to the movement of the control group.

To know the mode of action of compound is very important for the control of nematodes because it may provide useful information on the most appropriate formulation and delivery means. However, the mode of action of monoterpenoids against nematodes is unclear and should be further investigated. Park *et al.* (2005) and Oka *et al.* (2000) have reported a relationship between nematicidal and insecticidal activity.

Our results indicated that monoterpenoids could be useful as nematicides for use against the pine wood nematode. For the practical use of monoterpenoids as nematicides, further studies are necessary to develop formulations to improve their efficacy and stability.

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In-Ho Choi, Junheon Kim, Sang-Chul Shin, Il-Kwon Park. Нематицидная активность монотерпеноидов против нематоды древесины сосны (*Bursaphelenchus xylophilus).*

Резюме. В 96-и луночных планшетах проведена оценка воздействия 26 монотерпеноидов естественного происхождения на нематоду древесины сосны *Bursaphelenchus xylophilus*. Реакция варьировала в зависимости от химического состава и дозы. Хорошая нематицидная активность против самцов, самок и личинок *B. xylophilus* была достигнута при использовании карвакрола, тимола, гераниола, нерола, (-) ментола, β-цитронеллола, (±)-цитронеллолала и цитрала (смесь cis- и trans-форм). Фенол, спирты и альдегиды были обычно более токсичны для *B. xylophilus*. Карвакрол и тимол показали наиболее выраженные нематицидные свойства среди всех испытанных веществ. LC_{50} для тимола и карвакрола составили 0,096 и 0,099 мг мл$^{-1}$, соответственно. Цитрал и цитронеллол показали сходную токсичность для личиночных стадий нематод. Цитронеллал был более токсичным для личиночных стадий нематод (LC_{50} = 0.253 мг мл$^{-1}$), чем гераниол (LC_{50} = 0,417 мг мл$^{-1}$). Значения LC_{50} для нерола и ментола при их воздействии на личиночные стадии нематод составляли 0,979 и 1,021 мг мл$^{-1}$, соответственно. Значения LC_{50} для других соединений, а также инъекция в стволы деревьев гидрохлорида левамизола составляли более 1,0 мг мл$^{-1}$ в первые 4 часа после внесения вещества.