



Antifungal activities of β -thujaplicin originated in *Chamaecyparis obtusa*

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Abstract Environment-friendly, commercially-available agricultural products were investigated for antimicrobial activity against *Sclerotinia sclerotiorum*, as a pathogen of sclerotium disease. Then β -thujaplicin from *Chamaecyparis obtusa* was investigated for antifungal activity against six kinds of pathogenic fungi. It showed a statistically significant ($p < 0.001$) growth inhibition effect on *Sclerotinia sclerotiorum* as a pathogen of sclerotium disease, *Rhizoctonia solani* AG-4 as a pathogen of damping off, *Phytophthora capsici* as a pathogen of phytophthora blight, and *Colletotrichum coccodes* as a pathogen of anthracnose at a concentration of 50 ppm and on *Stemphylium solani* as a pathogen of spotting disease and *Alternaria alternata* as a pathogen of black mold at a concentration of 100 ppm. In conclusion, these results indicate that it may be possible to develop environment-friendly agricultural products using β -thujaplicin compounds.

Keywords Antifungal active substance · *Chamaecyparis obtusa* · Hinokitiol · *Sclerotinia sclerotiorum* · β -thujaplicin

Introduction

Sclerotium disease frequently develops in low temperature, humid environments and is more destructive in indoor cultivation than in

normal outdoor cultivation. In the Republic of Korea, 7 pathogens have been reported with *Sclerotinia sclerotiorum* and *S. minor* being the main causes of disease. *S. sclerotiorum* pathogens are fungi in the phylum Ascomycetes and form white hyphae and develop as white fungi with a snow-like appearance in disease affected areas. After white fungi form, they clump and form black, rat dropping-like sclerotia around the affected area, which helps easy identification from other blight. *Sclerotinia* pathogens cause cottony rot, white mold, soft rots, stem rot, canopy rot, and flower rot depending on the infected site of the host and environmental conditions. Sclerotium disease has a wide range of hosts and is known to have serious effects on various plants including vegetables and flowers. *S. sclerotiorum* pathogen is known to infect plants throughout the growth stages, such as seedling, mature plant, and harvest (Agrios 1998). Especially, sclerotium disease caused by the *S. sclerotiorum* pathogen mainly infects the Cucurbitaceae such as cucumber and pumpkin. Infection is gradually increasing in Korean melon, watermelon, and melon and also occurs in Brassicaceae crops such as napa cabbage and cabbage (NAAST 2000).

In lettuce, sclerotium pathogen forms sclerotia to survive for long time periods, and hyphae germinate directly from the sclerotia and infect plants by penetrating the structure of the lettuce root and aged leaves. This causes the lettuce leaf to rot and the white hyphae formed produce rat dropping-like sclerotia. Lettuce is a vegetable crop that is extensively cultivated in the Republic of Korea through indoor cultivation throughout the year. In 2014, the indoor lettuce cultivation area totaled 4,029 ha, which is the largest cultivation area of all leafy vegetables, accounting for 10% of the total (RDA 2015). Also 18 different kinds of lettuce disease are reported in the Republic of Korea (KSPP 2009). Among these diseases, *S. sclerotiorum* derived sclerotium disease occurs in the late fall through to spring, and it is an especially devastating disease in warehouse cultivation, which is a low temperature environment. Lettuce sclerotium disease was reported in the United States in 1890 (Subbarao 1998) and was first reported in the Republic of Korea in 1976 (Kim 1976). In the

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Table 1 Plant pathogenic fungal strains used in this study

Disease common name	Scientific name	Source
Sclerotinia rot	<i>Sclerotinia sclerotiorum</i> (Libert) de Bary	KACC NO. 40457
Damping off	<i>Rhizoctonia solani</i> AG-4 Kuhn	KACC NO. 40141
Phytophthora blight	<i>Phytophthora capsici</i> Leonian	KACC NO. 40157
Anthraco-nose	<i>Colletotrichum coccodes</i> (Wallroth) S. Hughes	KACC NO. 40011
Spotting disease	<i>Stemphylium solani</i> Weber	KACC NO. 40966
Black mold	<i>Alternaria alternata</i> (Fr. : Fr.) von Keissler	KACC NO. 43922

United States and Europe, it was reported that *S. sclerotiorum* is usually infected through members of the Ascomycota (Abawi and Grogan 1975; Whipps et al. 2002). Lettuce sclerotium disease influences lettuce production loss in areas such as the United States and Europe as well as in the Republic of Korea, and in the United States sclerotium disease is reported to cause losses of 1~75% of crop production (Purdy 1979).

Chemical control methods are used to control sclerotium disease. Protective fungicides such as geopan hydrating agent and geopan leafsol hydrating agent were used as soil treatments and were reported to control the sclerotium pathogen, thereby lowering disease rates (Hong et al. 2007); however, the indigenous pathogen in the soil was not fundamentally controlled. Also, these methods used penetrative disinfectants that may cause residue problems inside plants, and there is a need to develop eco-friendly agricultural materials for mitigating the problems of residue toxicity.

To verify the effect on growth inhibition of *S. sclerotiorum*, this experiment conducted research on 22 plant-derived chemical products available on the market, and the results confirmed the antifungal activation of β -thujaplicin that originated from *Chamaecyparis obtusa*. In order to verify the development possibility of a *Chamaecyparis obtusa*-derived β -thujaplicin chemical compound, antifungal activation toward 5 different kinds of fungi was reviewed.

Materials and Methods

Laboratory equipment

In this experiment, sclerotium disease pathogen was obtained from the Division of Biotechnology, Chonbuk National University (Prof. Kui-Jae Lee). Five kinds of other fungi excluding sclerotium disease pathogen, namely damping off, phytophthora blight, anthracnose, spotting disease, and black mold, were obtained from the National Agrobiodiversity Center. All pathogens were stored and cultured at the Natural Product Chemistry Lab of Plant Resources Environment Department, Graduate School of Ecology and Environmental System, Kyungpook National University (Table 1).

Plant originated bioactive substances geraniol (99.0%), limonene (99.0%), thymol (99.0%), myrcene (90.0%), nicotine (99.0%), α -pinene (98.5%), matrine (97.0%), α -terpinene (90.0%), γ -terpinene (98.5%), emodin (97.0%), resveratrol (99.0%), and cinnamaldehyde

(95.0%) were purchased through Sigma; tuberostemonine (98.0%) and quassin (96.0%) were purchased through Dayangchem; β -thujaplicin (99.0%) was purchased through Wako; and rotenone (95.0%), eugenol (99.0%), methyl palmitate (99.0%), methyl gallate (98.0%), emamectin benzoate (95.0%), and terthiophene (99.0%) were purchased through Aldrich.

Pathogen Culture Laboratory Condition

For the pathogens, culture medium was prepared in a petri dish with 1 L of potato dextrose agar (PDA) from BD Company. Fungi were collected with a cork borer (8 mm) and cultured in the middle of the medium, and then used after incubation at 18 °C.

Bioassay

To investigate antimicrobial activities, the method of Kang et al. (2013) was applied. Autoclaved PDA medium was dispensed in a petri dish (90×15 mm) and solidified to make flat agar plate. The

Table 2 Antifungal activities of chemical compounds against *Sclerotinia sclerotiorum*

Compound name	Activity*
β -Thujaplicin	+++
Quassin	++
Cinnamaldehyde	++
Terthiophene	++
α -Pinene	+
Limonene	-
γ -Terpinene	-
Thymol	-
Myrcene	-
Nicotine	-
Rotenone	-
Eugeol	-
Methyl palmitate	-
Geraniol	-
Matrine	-
α -Terpinene	-
Emodin	-
Methyl gallate	-
Resveratrol	-
Emmectin benzoate	-
Tuberstemonine	-

* +++ (very good effect), ++ (good effect), + (weak effect), - (non effect)

chemical substances were adjusted to 100 ppm by using methanol (MeOH) and 50 µL drops placed on a paper disc (8 mm). After MeOH was completely volatilized, the paper disc was placed in the edge of the PDA agar plate. Experimental strains were extracted with a cork borer (8 mm), placed in the middle of the medium, and incubated at 18 in an incubator for outgrowth of the experimental strains. Antimicrobial activity was investigated by formation of a clean zone after the outgrowth.

In order to investigate the antimicrobial activity of β-thujaplicin, β-thujaplicin was added in autoclaved PDA agar at concentrations of 0, 1, 5, 10, 50, 100, and 1,000 ppm to make agar plate in a petri dish (90×15 mm). After the agar plate became completely solidified, an experimental strain was placed in the middle of the agar plate by using a cork borer (8 mm). The agar plate was placed in an incubator at 18 and the antimicrobial activity investigated by measuring the growth rate of the strain.

Statistical Analysis

JMP 5.0.1 program was used to perform one-way Anova and find any statistically significant differences in the results of the experiment. SAS 9.4 program was used to obtain EC₅₀.

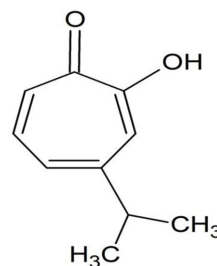


Fig. 1 Chemical structure of β-thujaplicin used in several antifungal bioassays. In the experiment, β-thujaplicin showed strong antimicrobial activity and restrained the growth of *S. sclerotiorum*, a sclerotium disease pathogen. Also, antifungal activity was investigated for damping off pathogen *R. AG-4*, phytophthora blight pathogen *P. capsici*, anthracnose pathogen *C. coccodes*, spotting disease pathogen *S. solani*, and black mold pathogen *A. alternata* (Fig. 2)

$$\% \text{ Inhibition of growth} = \frac{X - Y}{X} \times 100$$

where

X=Mycelial growth of pathogen in absence of antagonist
 Y=Mycelial growth of pathogen in presence of antagonist

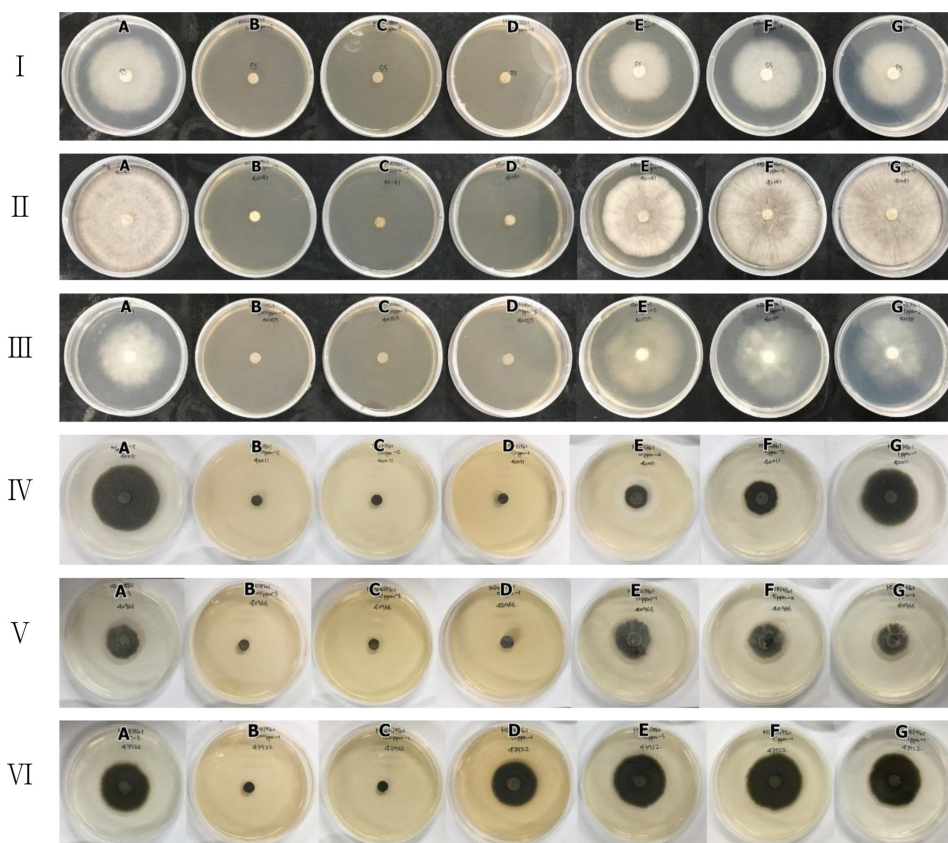


Fig. 2 Antibacterial activities of β-thujaplicin against *Sclerotinia sclerotiorum* (I), *Rhizoctonia solani* AG-4 (II), *Phytophthora capsici* (III), *Collectotrichum coccodes* (IV), *Stemphylium solani* (V), *Alternaria alternata* (VI). (A) Control; (B) 1,000 ppm β-thujaplicin; (C) 100 ppm β-thujaplicin; (D) 50 ppm β-thujaplicin; (E) 10 ppm β-thujaplicin; (F) 5 ppm β-thujaplicin; (G) 1 ppm β-thujaplicin. Six types of pathogen were cultured for a certain time period time in different concentrations of β-thujaplicin added to agar plate. The zone of inhibition was measured and the rate of inhibition was calculated, while statistically significant differences were determined through the JMP program (Table 3)

Table 3 Inhibition of growth rate by fungus in various concentrations of β -thujaplicin

Conc.	Stains	<i>Sclerotinia sclerotiorum</i>	<i>Rhizoctonia solani AG-4</i>	<i>Phytophthora capsici</i>	<i>Collectotrichum coccodes</i>	<i>Stemphylium solani</i>	<i>Alternaria alternata</i>
1 ppm		11.339 \pm 3.299 ^C	7.489 \pm 0.822 ^C	5.080 \pm 0.000 ^C	23.794 \pm 1.647 ^D	14.170 \pm 5.651 ^B	7.067 \pm 0.611 ^D
5 ppm		6.826 \pm 0.301 ^D	2.936 \pm 0.411 ^D	10.070 \pm 4.121 ^B	65.844 \pm 0.905 ^C	0.300 \pm 2.681 ^C	26.533 \pm 0.115 ^E
10 ppm		20.402 \pm 2.673 ^B	25.429 \pm 0.415 ^B	-19.980 \pm 2.374 ^D	89.638 \pm 0.164 ^B	-19.130 \pm 0.343 ^D	10.533 \pm 0.808 ^C
50 ppm		100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	97.720 \pm 0.171 ^A	13.733 \pm 1.006 ^B
100 ppm		100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A
1000 ppm		100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A	100.000 \pm 0.000 ^A

*The values represent mean \pm standard deviation for three independent experiments

^{A-E}Different superscripts indicate statistically significant difference by JMP at $p < 0.001$

For the results with a statistically significant difference ($p < 0.001$), 5 types of experimental pathogens, excluding *A. alternata*, showed complete growth inhibition with β -thujaplicin in concentrations of 50 ppm or higher, while *A. alternata* pathogen showed growth inhibition in concentrations of 100 ppm or higher.

EC₅₀ value was calculated by the SAS program with growth inhibition of the 6 pathogens being dependent on concentration (Table 4)

Table 4 EC₅₀ values in various concentrations of β -thujaplicin against several pathogens

EC ₅₀ Value (ppm)	<i>Sclerotinia sclerotiorum</i>	<i>Rhizoctonia solani AG-4</i>	<i>Phytophthora capsici</i>	<i>Collectotrichum coccodes</i>	<i>Stemphylium solani</i>	<i>Alternaria alternata</i>
	12.793	13.320	9.689	2.548	8.086	35.875

C. coccodes pathogen showed the lowest β -thujaplicin EC₅₀ value of 2.548 and *A. alternata* pathogen showed the highest of 35.875

Results and Discussion

An analysis of the antimicrobial activities of the plant originated chemicals against *S. sclerotiorum* showed that β -thujaplicin had the strongest activity; quassin, cinnamaldehyde, and terthiophene also showed strong activity; and α -pinene showed a little activity (Table 2).

Chemical pesticides, such as procymidone, benomyl have been used for control of soil-borne diseases including sclerotium disease that is problematic in lettuce cultivation. Geopan hydrating agent, and geopan leafsol hydrating agent, have been used for control sclerotium disease in fruit and vegetable; however, these fungicides are not effective in control of the sclerotium pathogen. In addition, these methods may also lead to mammalian toxicity, residues in plant because of use of a penetrative disinfectant, and environmental pollution. To resolve these problems, it is necessary to develop eco-friendly agricultural materials. For biological control, Budge and Whipps (2001) and Whipps and Gerlagh (1992) reported control of sclerotium disease with an antagonistic microbe, but there has been little progress in control with plant originated extracts.

Chamaecyparis obtusa (Gymnosperm, Coniferales, Cupressaceae), also known as cypress, is an evergreen tall-tree, that originated in Japan. It was introduced in Korea in 1904 and is known to grow naturally mainly in Jeju island and the southern part of the Korean peninsula. β -Thujaplicin, is a tropolene chemical with strong antibacterial and antimicrobial effects and is obtained from *Chamaecyparis obtusa*. It is known to act as a bioactive substance with effects on elimination of reactive oxygen species, maintenance of food production, and growth inhibition of plants (Trust and Coombs 1973; Fukuzawam et al. 1985; Inamori et al. 1991;

Arima et al. 1997; Koyama et al. 1997). Also, β -thujaplicin is known to be a nontoxic and stable substance that can be stored for long periods in conditions of heat, acid, alkali, and oxygen (Endo 1998).

This experiment on antimicrobial activity confirmed that β -thujaplicin inhibits fungi growth in *S. Sclerotiorum*. Furthermore, tests on antifungal with *R. solani AG-4*, *P. capsici*, *C. coccodes*, *Stemphylium solani*, and *A. alternata* showed absolute growth inhibition with β -thujaplicin in concentrations of over 100 ppm. Thus, it was concluded that further research should be conducted on β -thujaplicin as an eco-friendly agricultural material.

References

- Abawi GS, Grogan RG (1975) Source of primary inoculum and effects of temperature and moisture on infection of beans by *Whetzelinia sclerotiorum*. *Phytopathology* 65: 300–309
- Agrios GN (1998) *Plant pathology*. 4rd ed., Academic Press. Cambridge
- Arima Y, Hatanaka A, Tsykihara S, Fujimoto K, Fukuda K, Sakurai H (1997) Scavenging Activities of α -, β - and γ -Thujaplicins against Active Oxygen. *Chem Pharm Bull* 45: 1881–1886
- Budge SP, Whipps JM (2001) Potential for integrated control of *Sclerotinia sclerotiorum* in glasshouse lettuce using *Coniothyrium minitans* and reduced fungicide application. *Phytopathology* 91: 221–227
- Endo M, Mizutoni T, Matsumura M (1998) High-performance liquid chromatographic determination of hinokitiol in cosmetics by the formation of difluoroborane compounds. *J Chromatography* 455: 430–433
- Fukuzawam R, T Ito, T Matsuda (1985) *Jpn. Kokkyo Koho JP60141244*
- Hong SK, Kim WG, Sung GB, Nam SH, Kim JS (2007) Aspects of Popcorn Disease Occurrence on Mulberry Fruits in Korea. *Res Plant Dis* 13(3): 131–136
- Inamori Y, Nishiguchi K, Matsuo N, Tsujibo H, Baba K, Ishida N (1991)

- Phytogrowth-inhibitory activities of tropolone and hinokioil. *Chem Pharm Bull* 39: 2378–2381
- Kang JY, Kim DH, Lee DG, Kim IS, Jeon MG, Lee JD, Kim IH, Sanghyun Lee (2013) Screening of Antifungal Activities of Medicinal Plants for the Control of Turfgrass Fungal Disease. *Weed Turf Sci* 2(1): 70–75
- Kim KC (1976) The effect of ray on sclerotia formation of sclerotium disease. *Korean J. Plant Protect (In Korean)* 15: 223–243
- Koyama S, Yamaguchi Y, Tanaka S, Motoyoshiya J (1997) A new substance (Yoshixol) with an interesting antiviotic mechanism from wood oil of Japanese traditional tree (Kiso-Hinoki), *Chamaecyparis obtus*, *Gen Pharmacol* 28(5): 797
- National Institute of Agricultural Science and Technology (NAAST) (2000) Sclerotinia rot. Life and pesticides (*Agrochemical news magazine*) 21: 42–43
- Purdy LH (1979) *Sclerotinia sclerotiorum*: History, diseases and symptomatology, host range, geographic distribution, and impact. *Phytopathology* 69: 875–880
- Rural Development Administration (2015) The best friends of grilled or barbecued meat. RDA Interrobang, Jeonju
- Subbarao KV (1998) Progress toward integrated management of lettuce drop. *Plant Dis* 82: 1068–1078
- The Korean Society of Plant Pathology (2009) List of Plant Diseases in Korea, KSPP, Seoul
- Trust TJ, RW Coombs (1973) Antibacterial activity of beta-thujaplicin. *Can J Microbiol* 19: 1341–1346
- Whipps JM, Budge SP, McClement S, Pink DAC (2002) A glasshouse cropping method for screening lettuce lines for resistance to *Sclerotinia sclerotiorum*. *Eur J Plant Pathol* 108: 373–378
- Whipps JM, Gerlagh M (1992) Biology of *Coniothyrium minitans* and its potential for use in disease biocontrol. *Mycological Research* 96: 897–907