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Note

Nematicidal Activity of Bikaverin and Fusaric Acid Isolated from *Fusarium oxysporum* against Pine Wood Nematode, *Bursaphelenchus xylophilus*

Hyeok Ran Kwon[2], Seung Wan Son[1], Hye Rim Han[3], Gyung Ja Choi[1], Kyoung Soo Jang[1], Yong Ho Choi[1], Sunog Lee[1], Nack Do Sung[2] and Jin-Cheol Kim[1]*

[1]*Bio-Control Research Team, Korea Research Institute of Chemical Technology, Daejeon 305-600, Korea*

[2]*Department of Agricultural Chemistry, College of Agricultural and Life Sciences, Chungnam National University, Daejeon 305-764, Korea*

[3]*Division of Forest Diseases and Insect Pests, Korea Forest Research Institute, Seoul 130-012, Korea*

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Pine wood nematode, *Bursaphelenchus xylophilus*, causes pine wilt disease in a number of *Pinus* species, which is one of the most serious plant diseases in forest, Korea. In the course of a search for nematicidal substances from endophytic fungi, *Fusarium oxysporum* EF119 out of the 23 fungal strains tested showed the strongest activity to *B. xylophilus*. Two nematicidal substances were isolated and identified as bikaverin and fusaric acid. Fusaric acid showed somewhat higher nematicidal activity against *B. xylophilus* than bikaverin; fusaric acid and bikaverin, at 100 μg/ml, killed *B. xylophilus* with mortality values of 50% and 43%, respectively. In addition, both compounds acted synergistically. This is the first report on the nematicidal activity of bikaverin and fusaric acid.

Keywords **:** *Bursaphelenchus xylophilus*, bikaverin, fusaric acid, *Fusarium oxysporum*, nematicidal activity, pine wood nematode

Pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buherer) Nickle, causes pine wilt disease in a number of *Pinus* species (Tokushige and Kiyohara, 1969; Tokusige and Kiyohara, 1971; Mamiya and Kiyohara, 1972; Woo et al., 2007). Pine wilt disease was described as early as 1905 in Japan, and has occurred in the Far East areas such as Korea, Taiwan, China, and Japan. The plant disease causes severe economic losses of 1 billion US$ per year (Hajime et al., 2001). In Korea, this disease first occurred in Busan city in 1988 (Yi et al., 1989) and has been spread to *Pinus densiflora* and *P. thunbergii* in several southern and middle parts (Chung, 2002). In 2006, the natural infections of pine wood nematode in *P. koraensis* have been first occurred in Gyeonggi Province.

To control this disease, several methods have been used such as fumigation of diseased trees, aerial application of synthetic pesticides for the control of insect vector, *Monochamus alternatus*, and trunk injection with nematicides. In present, many synthetic pesticides have been using for plant protection, but the side effects of many synthetic pesticides such as environmental pollution, residual toxicity and resistance have caused many scientists to conduct research into natural substances, which can be used directly as natural pesticides or lead molecules for the development of novel pesticides (Cho et al., 2006).

Endophytic fungi are defined as fungi colonizing healthy plant tissue without causing overt symptoms in or apparent injury to the host (Bills, 1996). They within plants produce plant-growth regulatory, antimicrobial, antiviral or insecticidal substances to enhance the growth and competitiveness of the host in nature (Carroll, 1988; Wiyakrutta et al., 2004). Thus, they are a repository of novel metabolites of agricultural and/or pharmaceutical importance (Stierle et al., 1993; Strobel and Long, 1998; Tan and Zou, 2001).

In the previous study (Kim et al., 2007), we isolated endophytic fungi from vegetable plants and examined their *in vivo* anti-oomycete activity against *Phytophthora infestans* in tomato plants. Among 152 isolates, the fermentation broths of 23 isolates showed potent *in vivo* anti-oomycete activity. Generally, natural substances from plants, fungi, bacteria, etc. show various biological activities. In order to search natural nematicidal compounds from endophytic fungi, we tested *in vitro* nematicidal activity of the 23 isolates against *B. xylophilus* causing pine wilt disease. Then, we isolated two nematicidal compounds from the fermentation broth of *Fusarium oxysporum* EF119 and determined their chemical structures by instrumental analyses. In this study, we report the isolation and identification of nematicidal substances from *F. oxysporum* EF119.

B. xylophilus was isolated from chips of infected pine wood collected in Gangneung city, Gangwon Province in 2005 by Baermann funnel method (Chawla and Prasad, 1975; Viglierchio and Schmit, 1983). After rinsing three

*Corresponding author.
Phone) +82-42-860-7436, FAX) +82-42-861-4913
E-mail) kjinc@krict.re.kr

Table 1. Effect of the extracts of endophytic fungi on the pine wood nematode, *Bursaphelenchus xylophilus*

Strain	Species	Source[a] (plant/part)	Mortality (%)±SD		
			1,000 μg/ml	500 μg/ml	250 μg/ml
EF001	*Coniochaeta ligniaria*	Cs/r	20±4.2	–[b]	–
EF017	*Chaetomium* sp.	Ca/r	40±2.1	–	–
EF020	*Fusarium oxysporum*	Ca/r	10±1.4	–	–
EF021	*Chaetomium* sp.	Ca/r	53±1.4	38±1.4	31±8.5
EF037	ND[c]	Ca/r	57±2.1	48±5.7	38±7.8
EF038	*F. oxysporum*	Ca/r	2.4±4.2	–	–
EF039	*F. oxysporum*	Ca/r	15±4.2		
EF052	*F. oxysporum*	Ca/r	4.4±1.4	–	–
EF055	*Penicillium* sp.	Ca/r	58±12.0	21±4.2	19±7.8
EF061	*F. oxysporum*	Sl/r	33±0.7	–	–
EF088	*F. oxysporum*	Cs/r	41±9.9	–	–
EF090	*Fusarium* sp.	Cs/r	21±2.1	–	–
EF099	*F. oxysporum*	Sl/r	30±2.1	–	–
EF109	*F. oxysporum*	Cp/r	1.2±4.2	–	–
EF117	*Fusarium* sp.	Cp/l	51±4.2	–	–
EF119	*F. oxysporum*	Ca/r	62±4.9	47±2.1	22±3.5
EF129	*Fusarium* sp.	Bc/l	40±7.1	–	–
EF136	*F. oxysporum*	Bc/r	41±2.4	–	–
EF147	*F. oxysporum*	Cs/r	29±11	–	–
EF148	*Talaromyces* sp.	Cs/r	50±2.8	30±2.8	12±2.1
EF149	*Fusarium* sp.	Bc/r	32±5.5	–	–
EF150	*Colletotrichum* sp.	Bc/r	50±9.2	9.4±4.2	4.8±0.7
EF152	*Penicillium* sp.	Ca/s	44±2.1	–	–

[a]Cs, *Cucumis sativus*; Ca, *Capsicum annuum*; Sl, *Solanum lycopersicum*; Cp, *Cucurbita pepo*; Bc, *Brassica campestris* var. *pekinensis*; r, root; l, leaf; s, stem.
[b]–, not tested.
[c]ND, not determinable.

times with distilled water, the nematode strain was inoculated onto a lawn of *Botrytis cinerea* cultured on potato dextrose agar medium (PDA; Becton and Dickinson Co.) at 20°C for 7 days and then incubated at 28°C for 7 days.

The isolation and identification procedures of endophytic fungi used in this study were described in detail in the previous paper (Kim et al., 2007). Among the 23 isolates tested (Table 1), *F. oxysporum* EF119 was selected for the further study because the strain showed the strongest nematicidal activity. *F. oxysporum* EF119 isolated from a healthy root of red pepper (*Capsicum annuum* L.) was deposited with the Korean Collection for Type Cultures at the Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Daejon, Korea under accession No. KCTC10926BP.

Erlenmeyer flasks (500 ml) containing 200 ml of potato dextrose broth (PDB; Becton and Dickinson Co.) were autoclaved at 121°C for 15 min and then inoculated with mycelium plugs from the margins of actively growing cultures on PDA. The flasks were incubated for 2 weeks on a rotary shaker at 150 rpm and 25°C. The fermentation broths were filtered through two-fold cheese cloths. The filtrates were adjusted to pH 2.0 with concentrated HCl and then extracted with equal volumes of ethylacetate twice. Anhydrous sodium sulfate was added to the ethylacetate extracts to remove water. The organic solvent extracts were concentrated to dryness under reduced pressure and then stored at 4°C until used. The ethylacetate extracts of 23 fungal isolates were dissolved with methanol at a concentration of 50 mg/ml. Two μl aliquot of each stock solution was added to each well of 96-well plate (BD Biosciences, USA), which had already contained 98 μl nematode suspension. Juvenile and mature nematodes were harvested from the cultures with distilled water containing Triton X-100 (Sigma Chemical Co., USA) at a concentration of 100 μg/ml before use. The concentration of nematodes was about 150 nematodes per 100 μl. Controls were treated with 2 μl methanol alone. Morantel tartrate (10 μg/ml) (Sigma-Aldich Laborchemikalien GmbH, Germany) was used as a positive control. Each treatment was replicated three times. The well plates were incubated at 28°C for 2 days. Nematodes were defined as dead if they did not move on

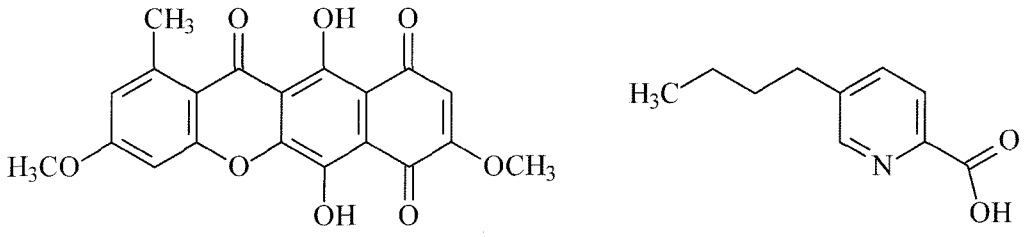

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Fig. 1. Chemical structures of bikaverin (**1**) and fusaric acid (**2**).

physical stimuli with a fine needle.

Among the 23 fungal strains tested, 6 strains (26%) exhibited *in vitro* nematicidal activity of more than 50% against *B. xylophilus* (Table 1). The 6 strains included *Chaetomium* sp. EF021, an unidentified strain EF037, *Penicillium* sp. EF055, *F. oxysporum* EF119, *Talaromyces* sp. EF148, and *Colletotrichum* sp. EF150. Most of the 15 strains of *Fusarium* species tested showed moderate nematicidal activity and only one strain EF119 isolated from *C. annuum* roots killed *B. xylophilus* over 50%. The 6 strains selected were tested for their nematicidal activity at lower concentrations. As the results, the ethylacetate extract of *F. oxysporum* EF119 showed the most potent activity.

Two nematicidal substances were isolated from the filtrate of fermentation broth of EF119 strain under the guidance of *in vitro* assay against *B. xylophilus* as reported previously (Son et al., 2007). Their chemical structures were determined to be bikaverin and fusaric acid by mass and NMR spectral data (Abraham and Hanssen, 1992; Kjaer et al., 1971) (Fig. 1).

The *in vitro* nematicidal activity of bikaverin and fusaric acid are shown in Table 2. Fusaric acid had somewhat stronger nematicidal activity against *B. xylophilus* than bikaverin. When both bikaverin and fusaric acid were applied together at a ratio of 1:1, the mixture showed more potent activity than either of the compounds alone at all concentrations tested. This suggests that both bikaverin and fusaric acid act synergistically on *B. xylophilus*.

Bikaverin, also known as lycopersin, is a wine-red pigment and produced by mainly *F. oxysporum* and *F. moniliforme*, showing various biological activities such as antiprotozoal activity (Balan et al., 1970), antitumor activity (Fuska et al., 1975), toxicity on rat mitochondria (Kitagawa et al., 1997), vacuolation of fungal hyphal tips (Cornforth et al., 1971), and anti-oomycete activity against *P. infestans* (Son et al., 2007). Fusaric acid is also one of representative secondary metabolites produced by many species of *Fusarium*. Fusaric acid have been known as an inhibitor of metal-containing oxidative enzymes (Jain, 1982), mycotoxin (Hidaka et al., 1969), and antibiotics (May et al., 2000; Son et al., 2007). It is reported to augment the overall toxicity of other mycotoxins; fusaric acid to animal toxicity acts synergistically with other naturally co-occurring mycotoxins (Bacon et al., 1995; Dowd, 1988; Smith and Sousadias, 1993). In this study, fusaric acid to the toxicity of *B. xylophilus* showed synergistic interaction with bikaverin. To our knowledge, this is the first report on the nematicidal activity of bikaverin and fusaric acid. This may provide a lead for an investigation of new nematicidal compounds. In the future, the nematicidal activity of the two metabolites isolated from *F. oxysporum* EF119 must be assessed against other phytopathogenic nematode species.

Table 2. Effect of bikaverin and fusaric acid from *Fusarium oxysporum* EF119 on the mortality (%) of pine wood nematode, *Bursaphelenchus xylophilus*

Compound	Concentration (μg/ml)	Mortality (%) ±SD
Bikaverin	100	43±4.3
	50	28±5.1
	25	11±2.3
Fusaric acid	100	50±8.7
	50	38±6.4
	25	21±2.1
Bikaverin+Fusaric acid (1:1, w/w)	100	68±7.3
	50	47±2.4
	25	32±2.5
Morantel tartrate	10	97±8.4

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References

Abraham, W.-R. and Hanssen, H.-P. 1992. Fusoxysporone – a new type of diterpene from *Fusarium oxysporum*. *Tetrahedron*

48:10559-10562.

Bacon, C. W., Porter, J. K. and Norred, W. P. 1995. Toxic interaction of fumonisin B_1 and fusaric acid measured by injection into fertile chicken egg. *Mycopathologia* 129:29-35.

Balan, J., Fusha, J., Kuhr, I. and Kuhrova, V. 1970. Bikaverin, an antibiotic from *Gibberella fujikuroi*, effective against *Leishmania brasiliensis*. *Folia Microbiol.* 15:479-484.

Bills, G. F. 1996. Isolation and analysis of endophytic fungal communities from woody plants. In: *Systematics, ecology and evolution of endophytic fungi in grasses and woody plants.* ed. by S. Redlin and L. M. Carris, pp. 31-65. APS Press, St. Paul, MN, USA.

Carroll, G. C. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology* 69:2-9.

Chawla, M. L. and Prasad, S. K. 1975. Techniques in nematology. II. Comparative efficiency of sampling tools and nematode extraction methods. *Indian J. Nematol.* 4:115-123.

Cho, J. Y., Choi, G. J., Lee, S.-W., Lim, H. K., Jang, K. S., Lim, C. H., Cho, K. Y. and Kim, J.-C. 2006. *In vivo* antifungal activity against various plant pathogenic fungi of curcuminoids isolated from the rhizomes of *Curcuma longa*. *Plant Pathol. J.* 22:94-96.

Chung, Y. J. 2002. Occurrence and spread of pine wilt disease in Korea. *Korea Tree Prot.* 7:1-7.

Cornforth, J. W., Ryback, G., Robinson, P. M. and Park, D. 1971. Isolation and characterization of a fungal vacuolation factor (bikaverin). *J. Chem. Soc.* 2786-2788.

Dowd, P. F. 1988. Toxicological and biochemical interactions of the fungal metabolites fusaric acid and kojic aicd with xenobiotics in *Heliothis zea* (F) and *Spodoptera frugiperda* (J.E. Smith). *Pestic. Biochem. Physiol.* 32:123-134.

Fuska, J., Proksa, B. and Fuskova, A. 1975. New potential cytotoxic and antitumor substances. I. *In vitro* effect of bikaverin and its derivatives on cells of certain tumors. *Neoplasma* 22:335-339.

Hajime, K., Tajuya, A. and Nobuo, O. 2001. Pine wilt disease caused by the pine wood nematode: the induced resistance of pine trees by the avirulent isolates of nematode. *Eur. J. Plant Pathol.* 107:667-675.

Hidaka, H., Nagatsu, T. and Takeya, K. 1969. Fusaric acid, a hypotensive agent produced by fungi. *J. Antibiot.* 22:228-230.

Jain, M. 1982. *Handbook of Enzyme Inhibitors*, pp. 1966-1967. New York: John Wiley & Sons.

Kim, H.-Y., Choi, G. J., Lee, H. B., Lee, S.-W., Lim, H. K., Jang, K. S., Son, S. W., Lee, S. O., Cho, K. Y., Sung, N. D. and Kim, J.-C. 2007. Some fungal endophytes from vegetable crops and their anti-oomycete acivities against tomato late blight. *Lett. Appl. Microbiol.* 44:332-337.

Kitagawa, A., Sugihara, Y., Okumura, M., Kawai, K. and Hamasaki, T. 1997. Reexamination of respiration-impairing effect of bikaverin on isolated mitochondria. *Cereal Res. Commun.* 25:451-452.

Kjaer, D., Kjaer, A., Pederson, C., Bulock, J. D. and Smith, J. R. 1971. Bikaverin and norbikaverin, benzoxanthentrione pigments of *Gibberella fujikuroi*. *J. Chem. Soc.* 2792-2797.

Mamiya, Y. and Kiyohara, T. 1972. Description of *Bursaphelenchus lignicolus* n sp. (Nematoda: Aphelenchoididae) from pine wood and histopathology of nematode-infested trees. *Nematologica* 18:120-124.

May, H. D., Wu, Q. and Blake, C. K. 2000. Effects of the *Fusarium* spp. mycotoxins fusaric acid and deoxynivalenol on the growth of *Ruminococcus albus* and *Methanobrevibacter ruminantium*. *Can. J. Microbiol.* 46:692-699.

Smith, T. K. and Sousadias, M. G. 1993. Fusaric acid content of swine feedstuffs. *J. Agric. Food Chem.* 41:2296-2298.

Son, S. W., Kim, H.-Y., Choi, G. J., Lim, H. K., Jang, K. S., Lee, S. O., Lee, S., Sung, N. D. and Kim, J.-C. 2007. Bikaverin and fusaric acid from *Fusarium oxysporum* show anti-oomycete activity against *Phytophthora infestnas*. *J. Appl. Microbiol.* DOI:10.111/j.1365-2672.2007.03581.

Stierle, A., Strobel, G. A. and Stierle, D. 1993. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science* 260:214-216.

Strobel, G. A. and Long, D. M. 1998. Endophytic microbes embody pharmaceutical potential. *ASM News* 64:263-268.

Tan, R. X. and Zou, W. X. 2001. Endophytes: a rich source of functional metabolites. *Nat. Prod. Rep.* 18:448-459.

Tokushige, Y. and Kiyohara, T. 1969. *Bursaphelenchus* sp. in the wood of dead pine trees. *J. Jap. Forest Soc.* 51:193-195.

Tokushige, Y. and Kiyohara, T. 1971. Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. *J. Jap. Forest Soc.* 53:210-218.

Viglierchio, D. R. and Schimit, R. V. 1983. On the methodology of nematode extraction from field samples: Baerman funnel modifications. *J. Nematol.* 15:438-444.

Woo, K.-S.; Kim, Y.-S.; Koo, Y.-B.; Yeo, J.-K. and Moon, Y.-S. 2007. Variation in susceptibility of pine species seedlings with the pine wood nematode, *Bursaphelenchus xylophilus*, in greenhouse. *Plant Pathol. J.* 23:70-75.

Yi, C. K., Byun, B. H., Park, J. D., Yang, S. I. and Chang, K. H. 1989. First finding of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle and its insect vector in Korea. *Res. Rep. Forestry Res. Ins.* 38:141-149.

Wiyakrutta, S., Sriubolmas, N., Panphut, W., Thongon, N., Danwisetkanjana, K., Ruangrungsi, N. and Meevootisom, V. 2004. Endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. *World J. Microbiol. Biotechnol.* 20:265-272.