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ORIGINAL ARTICLE

Bikaverin and fusaric acid from *Fusarium oxysporum* show antioomycete activity against *Phytophthora infestans*

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Abstract

Aims: To isolate and identify antioomycete substances from *Fusarium oxysporum* EF119 against *Phytophthora infestans* and to investigate their antimicrobial activities against various plant pathogenic bacteria, oomycetes and true fungi.
Methods and Results: Two antioomycete substances were isolated from liquid cultures of *F. oxysporum* EF119, which shows a potent disease control efficacy against tomato late blight caused by *P. infestans*. They were identified as bikaverin and fusaric acid by mass and nuclear magnetic resonance spectral analyses. They inhibited the mycelial growth of plant pathogenic oomycetes and fungi. Fusaric acid also effectively suppressed the cell growth of various plant pathogenic bacteria, but bikaverin was virtually inactive. Treatment with bikaverin at 300 μg ml^{-1} suppressed the development of tomato late blight by 71%. Fusaric acid provided effective control against tomato late blight and wheat leaf rust over 67% at concentrations more than 100 μg ml^{-1}.
Conclusions: Both bikaverin and fusaric acid showed *in vitro* and *in vivo* antioomycete activity against *P. infestans*.
Significance and Impact of the Study: *Fusarium oxysporum* EF119 producing both bikaverin and fusaric acid may be used as a biocontrol agent against tomato late blight caused by *P. infestans*.

Introduction

Phytophthora infestans belongs to the phylum Oomycota of the Kingdom Chromista (Kirk *et al.* 2001). Oomycetes, one of the divisions of Oomycota, comprises approximately 82 genera and more than 650 species. *Phytophthora infestans* is a foliar pathogen and causes serious losses of potato and tomato crops world-wide. It is probably the most important pathogen of both the plants today. Besides potato and tomato, *P. infestans* can infect only a few other, closely related plants. Occasionally, peppers and eggplants are mildly infected. The parasitic species within the Oomycetes, including *P. infestans* are particularly successful and have proved difficult to be controlled by synthetic fungicides because of high persistence, absence of target sites for many conventional fungicides, variability and increasing resistance to fungicide (Griffith *et al.* 1992; Gisi and Cohen 1996).

Metalaxyl is the best-known acylalanine and most widely studied among phenylamide fungicides. It has provided excellent control against species of *Phytophthora*, *Pythium*, *Pseudoperenospora*, *Plasmopara* and *Peronospora* (Schwinn and Staub 1995). Metalaxyl is translocated readily in the xylem and shows good curative activity in addition to protective activity. However, metalaxyl-resistant *Phytophthora* isolates have occurred rapidly and are widespread world-wide. The occurrence of metalaxyl-resistant *Phytophthora* species in potato fields has resulted in devastating late blight problems owing to the failure of disease control in most production areas (Deahl *et al.* 1993; Hwang and Kim 1995). The indiscriminate and excessive use of fungicides owing to the reduced efficacy

of fungicides has led to environmental pollution and residual toxicity. In addition, the demand for organic agricultural products cultivated without using any agricultural chemicals or chemical fertilizers is increasing. Biocontrol is regarded as an environment friendly alternative to synthetic fungicides for protection against late blight of tomato and potato.

Fusarium oxysporum is commonly found in soil throughout the world. Some strains, known as formae speciales, are pathogenic and are responsible for wilt on various plant species (Gordon and Martyn 1997) and other strains are nonpathogenic, which live in healthy plants and do not cause disease symptoms. Several nonpathogenic strains of *F. oxysporum* have been selected as potential biological control agents (Benhamou and Garand 2001; Shishido *et al.* 2005). For example, Fo47 and Fo-B2 have reduced disease severities of *Fusarium* wilt on various crops (Benhamou and Garand 2001; Shishido *et al.* 2005). Four mechanisms in the protection of crops against pathogenic *F. oxysporum* have been raised, including competition for nutrients (Couteaudier 1992), competition for infection sites and root colonization (Eparvier and Alabouvette 1994), induced resistance (Benhamou and Garand 2001) and antibiosis (Benhamou *et al.* 2002). As for antibiosis, Benhamou *et al.* (2002) reported that Fo47 caused the reduction of *Pythium ultimum* growth by the production of certain fungal inhibitors. However, they did not identify the antioomycete substance.

During a search for an antagonist of tomato late blight, we found that *F. oxysporum* EF119 isolated from healthy root tissue of red pepper plants showed potent *in vivo* antioomycete activity (Kim *et al.* 2007). The objective of this work was to purify and identify the antioomycete substances produced by this fungus and to examine their *in vitro* and *in vivo* antimicrobial activities against various plant pathogens.

Materials and methods

Fungal isolation

Fusarium oxysporum EF119 was isolated from a healthy root of red pepper (*Capsicum annuum* L.) and deposited with the Korean Collection for Type Cultures at the Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea under accession no. KCTC10926BP.

Isolation and identification of antioomycete metabolites from *Fusarium oxysporum*

Sterilized Erlenmeyer flasks (500 ml) containing 200 ml of potato dextrose broth (PDB; Becton and Dickinson Co., Boston, MA, USA) were inoculated with mycelium plugs from a 5-day-old culture of *F. oxysporum* EF119 on potato dextrose agar (PDA; Becton and Dickinson Co.) and then incubated for 5 days at 150 rev min^{-1} and 25°C. The fermentation broth in flasks was inoculated into sterilized PDB medium (3 l) in jar fermentor (5 l) at a rate of 1%. The jar fermentor was incubated for 5 days at 150 rev min^{-1} and 25°C, following which the fermentation broth (3 l) was filtered through Whatman No. 2 filter paper. The filtrate was extracted three times with equal volumes of chloroform until most of the red pigment was removed. The organic filtrates were combined and concentrated to dryness and then redissolved in a small amount of chloroform. The chloroform solution was diluted with diethyl ether, giving a crude precipitate. Recrystallization of the precipitate from chloroform yielded pure red pigment (**1**; 250 mg) as an antioomycete substance.

In order to isolate the other antioomycete substance, the culture filtrate was partitioned two times with equal volumes of butanol. The organic extract was evaporated under reduced pressure at 45°C. The residue (1·1 g) was loaded onto a silica gel column [150 g Kiesel gel 60 (70–230 mesh), 3·6 cm i.d. × 60 cm], which was then eluted with chloroform : methanol : water (60 : 20 : 2, v/v/v). The fractions were monitored by thin layer chromatography (TLC) (Kiegel gel GF 254, 0·25-mm film thickness; E. Merck, Darmstadt, Germany) and reduced to one active fraction, F1 (137 mg). The F1 fraction was further purified by preparative HPLC (Shimadzu Co., Tokyo, Japan) by using Capcell-Pak C_{18} (20 mm i.d. × 250 mm; Shiseido Co., Tokyo, Japan). The column was eluted with linear gradient solvent system from acetonitrile : water : trifluroacetic acid (20 : 80 : 0·5, v/v/v) to 100% acetonitrile with a flow rate of 10 ml min^{-1}. Preparative HPLC yielded a pure compound (**2**; 30 mg) as an antioomycete substance.

The structures of compounds **1** and **2** isolated from *F. oxysporum* EF119 were determined using spectroscopic analyses. Mass spectra were recorded on a double-focus high-resolution (HR) mass spectrometer (JMS-DX303; JEOL, Tokyo, Japan). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded in deuterochloroform on a Bruker AMX-500 (500 MHz) NMR spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany). The spectra were referenced to tetramethylsilane (TMS).

Growth inhibition activity

The growth inhibition activity of **1** and **2** were evaluated using two oomycete pathogens and six plant pathogenic fungi (Table 1). The two compounds were dissolved in

Table 1 The MIC and IC_{50} values of bikaverin and fusaric acid against the growth of various plant pathogenic fungi and bacteria

Species	Bikaverin		Fusaric acid	
	MIC (μg ml^{-1})	IC_{50} (μg ml^{-1})	MIC (μg ml^{-1})	IC_{50} (μg ml^{-1})
Alternaria brassicicola	>100	>100	>100	>100
Botrytis cinerea	>100	>100	>100	>100
Colletotrichum coccodes	>100	70	>100	81
Fusarium oxysporum	NI	NI	NI	NI
Magnaporthe grisea	>100	70	100	50
Phytophthora capsici	>100	10	33	0·36
Phytophthora infestans	>100	60	11	1
Rhizoctonia solani	>100	<1·2	>100	11
Acidovorax konjaci	>100	>100	100	0·2
Agrobacterium tumefaciens	>100	>100	11	3
Burkholderia glumae	>100	>100	33	1·7
Pectobacterium carotovora ssp. *carotovora*	>100	>100	33	12
Pseudomonas syringae pv. *lachrymans*	>100	>100	33	11
Xanthomonas euvesicatoria	>100	>100	100	0·2

*MIC, minimum inhibitory concentration; IC_{50}, concentration causing 50% growth inhibition; NI, no inhibition.

dimethylsulfoxide (DMSO) and then incorporated into sterile PDA medium for all test fungi except *P. infestans*, for which V-8 juice agar medium was used. Solvent concentration in the media was 1%. The media amending with compounds **1** and **2** were poured into sterile dishes (5 cm i.d.). Media containing **1** and **2** at concentrations of 100, 33, 11, 3·7, 1·2 and 0·41 μg ml^{-1} were inoculated at the centre of the dishes with agar discs of the pathogens. Control dishes were treated with DMSO (1%) alone, and five replicates for each pathogen were used. *Phytophthora infestans* and *Botrytis cinerea* were incubated at 20°C and the other pathogens were incubated at 25°C. When control pathogens grew almost to the margins of the dishes, radial growth was measured. This experiment was conducted twice and the activity was expressed as minimum inhibitory concentration (MIC) and IC_{50} (concentration causing 50% growth inhibition).

The MIC and IC_{50} values of purified compounds against six plant pathogenic bacteria were also determined in a 96-well microtitre plate (Table 1). Bacteria were cultured on a nutrient broth at 30°C and then diluted with 0·9% sterilized NaCl solution at 10^3 cells per ml. One microlitre of each inoculum suspension was then inoculated in each well containing 100 μl of nutrient broth medium. The two compounds dissolved in DMSO were treated at concentrations of 0·41, 1·2, 3·7, 11, 33 and 100 μg ml^{-1}. The control wells were treated with DMSO (10 μl ml^{-1}) alone, and three replicates of each concentration for each micro-organism were used. The inoculated well plates were incubated at 30°C for 1–4 days. The optical density of each well was determined at 600 nm and compared with those of the untreated control wells. This experiment was conducted twice and the activity was expressed as MIC and IC_{50} values.

In vivo antioomycete and antifungal activity

The *in vivo* antioomycete and antifungal activities of the fermentation broth of *F. oxysporum* EF119 and compounds **1** and **2** were performed against six plant pathogens as described (Kim *et al.* 2004): *Magnaporthe grisea* (a causal agent of rice blast); *Rhizoctonia solani* (a causal agent of rice sheath blight); *P. infestans* (a causal agent of tomato late blight); *B. cinerea* (a causal agent of tomato grey mould); *Puccinia recondita* (a causal agent of wheat leaf rust); and *Blumeria graminis* f. sp. *hordei* (a causal agent of barley powdery mildew). Compounds **1** and **2** were dissolved in 1% DMSO containing Tween 20 (250 μg ml^{-1}) as a surfactant. The control plants were treated with 1% DMSO containing Tween 20. Chlorothalonil (100 and 50 μg ml^{-1}) and flusilazole (10 and 2 μg ml^{-1}) were applied as positive controls for tomato late blight and wheat leaf rust, respectively, which were suppressed by bikaverin and fusaric acid. All experiments were repeated once, and the results were shown as mean values of six estimates with standard deviation.

Results

The fermentation broth of *F. oxysporum* EF119 effectively suppressed the development of only tomato late blight caused by *P. infestans* among the six plant diseases tested (Table 2). While it showed a control value of 93% at the application of 50-fold diluent of fermentation broth of

Table 2 Disease-control activities of the fermentation broth of *Fusarium oxysporum* EF119 against six plant diseases*

	Control value (%)†					
Dilution	RCB‡	RSB	TGM	TLB	WLR	BPM
Onefold	13 ± 10	12 ± 9·5	0	96 ± 1·2	53 ± 2·5	0
Threefold	0	0	0	96 ± 1·2	27 ± 4·9	0
Ninefold	0	0	0	93 ± 2·5	0	0
50-fold	0	0	0	90 ± 1·2	0	0

*Seedlings were inoculated with spores or mycelial suspensions of the test organisms 1 day after various dilutions of fermentation broth from *Fusarium oxysporum* EF119 were sprayed on the leaves to run off.

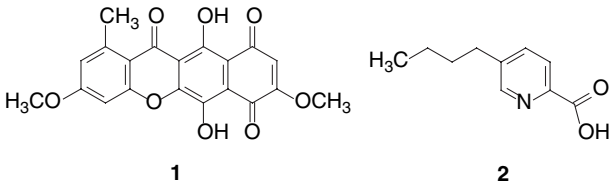
†Control value (%) = 100 × (disease severity of untreated plants – disease severity of treated plants) ÷ disease severity of untreated plants. Each value represents the mean of six estimates ± the standard deviation.

‡RCB, rice blast; RSB, rice sheath blight; TGM, tomato grey mould; TLB, tomato late blight; WLR, wheat leaf rust; BPM, barley powdery mildew.

F. oxysporum EF119, it had no *in vivo* antifungal activity against the other plant pathogens even at a onefold diluent of fermentation broth.

Two antioomycete substances were isolated from the filtrate of fermentation broth of EF119 strain under the guidance of *in vivo* assay against tomato late blight. Their chemical structures were determined by mass and NMR spectral analyses. Electron impact (EI) mass spectrum of compound **1** showed a molecular ion peak at *m/z* 382 (base peak) and fragment ion peaks at *m/z* 367, 339, 325, 311 and 283. Twelve protons attached to carbon atoms were present in the ^{1}H NMR spectrum of compound **1**; δ2·86 (s, 3H), δ3·93 (s, 3H), 3·95 (s, 3H), δ6·35 (s, 1H), δ6·77 (s, 1H) and δ6·92 (s, 1H). Mass and NMR spectral data were identical to those of bikaverin previously reported by Kjaer *et al.* (1971). Thus, compound **1** was identified as bikaverin (Fig. 1).

The EI mass spectrum of compound **2** displayed a molecular ion at *m/z* 179 and major fragment ions at *m/z* 135 (base peak), 119, 91, 77 and 65. ^{1}H NMR spectrum of compound **2** showed the presence of 12 protons attached to carbon atoms and one proton attached to oxygen; δ0·94 (t, 3H), δ1·37 (tq, 2H), δ1·66 (tt, 2H), δ2·74 (t, 2H), δ7·80 (d, 1H), δ8·19 (d, 1H), δ8·65 (s, 1H), δ12·0 (s, 1H). In addition, ^{13}C NMR spectrum of compound **2** displayed the presence of a carboxyl group (δ165·3, s) in the molecule. The interpretation of NMR data suggests that compound **2** is identical to fusaric acid, which was reported previously by Abraham and Hanssen (1992) (Fig. 1).



1 2

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

Both bikaverin and fusaric acid isolated from *F. oxysporum* EF119 inhibited, to varying degrees, the growth on amended PDA of mycelia of various plant pathogenic oomycetes and fungi (Table 1). Although fusaric acid suppressed more strongly than bikaverin, both chemicals showed similar antioomycete and antifungal spectra; *Colletotrichum coccodes* (a causal agent of red pepper anthracnose), *M. grisea, Phytophthora capsici* (a causal agent of red pepper blight), *P. infestans* and *R. solani* were relatively sensitive to the two substances, but the other fungi tested were relatively resistant or insensitive. In particular, fusaric acid effectively suppressed the mycelial growth of two oomycetes, such as *P. capsici* and *P. infestans* with IC_{50} values less than 1 μg ml^{-1}.

On the other hand, the two substances displayed different antibacterial spectra against plant pathogenic bacteria. While fusaric acid inhibited strongly the growth of the bacteria tested, bikaverin was virtually inactive (Table 1). Fusaric acid suppressed completely the growth of all bacteria tested at concentrations less than 100 μg ml^{-1}. The IC_{50} values ranged from 0·2 to 12 μg ml^{-1}.

The results of *in vivo* tests with bikaverin and fusaric acid are given Table 3. Of the six plant diseases, bikaverin suppressed relatively strongly the development of only tomato late blight caused by *P. infestans* and moderately the development of wheat leaf rust caused by *Pu. recondita*. Even though the red pigment inhibited the mycelial growth of some fungal species, it was virtually inactive against rice blast, rice sheath blight, tomato grey mould and barley powdery mildew. Fusaric acid was also effective in controlling both tomato late blight and wheat leaf rust. However, it showed little or no *in vivo* antifungal activity against other plant pathogenic fungi.

Discussion

In this study, *F. oxysporum* EF119, which was isolated from surface-sterilized healthy root tissues of red pepper, showed specifically potent antioomycete activity against tomato late blight caused by *P. infestans. Fusarium oxysporum* is commonly found in soil and certain strains are pathogenic, causing wilt disease in various plant species (Gordon and Martyn 1997). However, many strains are known to be nonpathogenic or endophytic, forming inconspicuous infections within tissues of healthy plants for all or nearly their life cycle (Rubini *et al.* 2005). *Fusa-*

Table 3 Disease-control activity of bikaverin and fusaric acid isolated from *Fusarium oxysporum* EF119 against six plant diseases*

Chemical	Conc. (μg ml^{-1})	Control value (%)†					
		RCB‡	RSB	TGM	TLB	WLR	BPM
Bikaverin	300	13 ± 2·5	12 ± 10	7 ± 12	71 ± 21	33 ± 2·5	8 ± 7·5
	100	13 ± 2·5	12 ± 9·5	0	29 ± 15	13 ± 4·5	0
	33·3	0	0	0	7 ± 6·3	0	0
Fusaric acid	300	0	15 ± 2·7	14 ± 3·2	96 ± 1·2	77 ± 15	0
	100	0	0	7 ± 5	68 ± 12	67 ± 13	0
	33·3	0	0	7 ± 12	29 ± 14	33 ± 10	0
Chlothalonil	100	–§	–	–	100	–	–
	50	–	–	–	90 ± 1·2	–	–
Flusilazole	10	–	–	–	–	100	–
	2	–	–	–	–	73 ± 5·7	–

*Seedlings were inoculated with spores or mycelial suspensions of the test organisms 1 day after chemical solutions were sprayed on the leaves to run-off.

†Control value (%) = 100 × {disease severity of untreated plants - disease severity of treated plants} ÷ disease severity of untreated plants. Each value represents the mean of six estimates ± the standard deviation.

‡RCB, rice blast; RSB, rice sheath blight; TGM, tomato grey mould; TLB, tomato late blight; WLR, wheat leaf rust; BPM, barley powdery mildew.

§–, not tested.

rium oxysporum EF119 was found to be nonpathogenic to various plants. Several nonpathogenic strains of *F. oxysporum* have been known to have antagonistic activity against several plant diseases (Couteaudier 1992; Eparvier and Alabouvette 1994; Benhamou and Garand 2001; Benhamou *et al.* 2002; Rodriguez *et al.* 2005; Shishido *et al.* 2005). *Fusarium oxysporum* Fo47 effectively suppressed the development of cucumber damping-off caused by *Py. ultimum* and Fusarium wilt on various crops caused by *F. oxysporum* (Benhamou *et al.* 2002). Diffusible metabolites produced by Fo47 strain inhibited the growth of *Py. ultimum*, but the metabolites were not determined.

Both bikaverin and fusaric acid were isolated from *F. oxysporum* EF119 as antioomycete substances against *P. infestans*. Bikaverin is a wine-red pigment and is produced mainly by *F. oxysporum* and *Gibberella fugikuroi* (teleomorph of *Fusarium moniliforme*). The same compound was reported previously under the name of lycopersin with an incorrect formula (Kreitman and Nord 1949). Bikaverin exhibits specific antiprotozoal activity against *Leishmania brasilensis* (Balan *et al.* 1970). It inhibits its DNA and protein biosynthesis in various tumour cells (Fuska *et al.* 1975) and succinate- and NAD-linked respiration in rat mitochondria and causes swelling of the mitochondria (Kitagawa *et al.* 1997). It is also known as a fungal vacuolation factor, which induced vacuolation, normally indicative of senescence, in hyphal tips of fungal cultures, such as *Aspergillus niger* at concentrations down to 0·01 μg ml^{-1} (Cornforth *et al.* 1971). However, antioomycete and antifungal activities against plant pathogens have not been reported yet.

Fusaric acid is produced by many species of *Fusarium*, some of which also produce trichothecene mycotoxins (Marasas *et al.* 1984). This compound was one of fungal metabolites implicated in the pathogenesis of tomato wilt symptoms caused by *F. oxysporum* f. sp. *lycopersici* (Gaumann 1957). Although fusaric acid is thought to be involved in the pathogenic process, this involvement has been disputed (Kuo and Scheffer 1964). Fusaric acid inhibits metal-containing oxidative enzymes (Jain 1982). It is mildly toxic to mice (Hidaka *et al.* 1969) and several pharmacological properties in the brain and pineal gland of rats (Porter *et al.* 1995). Fusaric acid is also known to have an antimicrobial activity against two rumen bacteria, such as *Ruminococcus albus* and *Methanobrevibacter ruminantium* (May *et al.* 2000). However, to our best knowledge, there has been no report on the antioomycete and antifungal activity of fusaric acid yet. This is the first report on the isolation of bikaverin and fusaric acid as antioomycete substances.

Among six plant diseases tested, bikaverin and fusaric acid were active to tomato late blight alone and tomato late blight and wheat leaf rust, respectively. The *in vivo* antioomycete and antifungal spectra of bikaverin and fusaric acid were similar to those of the fermentation broth of *F. oxysporum* EF119, indicating that the two substances were the main antioomycete and antifungal principles produced by *F. oxysporum* EF119.

In this study, bikaverin and fusaric acid showed inhibition of growth of various plant pathogenic oomycetes and fungi. The metabolites displayed the most potent *in vivo* antioomycete activity against tomato late blight among the six plant diseases tested. Bell *et al.* (2003) suggested that bikaverin could have a role in the pathogenesis of *F. oxysporum* f. sp. *vasinfectum* based on availability and toxicity. However, no effects of bikaverin on plant cells

have been reported. In addition, of the nine avirulent isolates of *F. oxysporum* from cotton roots, four isolates produced appreciable bikaverin (Bell *et al.* 2003). Although fusaric acid is known to play an important role in the plant disease process in at least two instances (Tamari and Kaji 1954; Gaumann 1957), no correlation between plant toxicity and the amount of fusaric acid produced by the infecting isolates has been made. Bacon *et al.* (1996) reported that no isolates of any of the *Fusarium* species examined were negative for the production of fusaric acid on autoclaved corn, and while several of the *Fusarium* species were strict plant pathogens, others were isolated from symptomless *F. moniliforme*-infected plants. *Fusarium oxysporum* EF119 used in this study was not pathogenic to various plants. Thus, fusaric acid seems not to be involved in the disease process. This suggests that nonpathogenic *F. oxysporum* EF119, which produces bikaverin and fusaric acid, could be a good candidate for the biocontrol of *P. infestans* in tomato and potato. However, both bikaverin and fusaric acid were known as mycotoxins (Hidaka *et al.* 1969; Kitagawa *et al.* 1997). Further research about safety would be necessary for the practical use of the two substances.

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