In vitro antimicrobial and *in vivo* antioomycete activities of the novel antibiotic thiobutacin

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Abstract

BACKGROUND: A number of synthetic fungicides are not effective when confronted by oomycete pathogens because many fungicide targets are absent from oomycetes. Moreover, resistance to fungicides has already arisen in oomycete species, and thus development of new, effective and safe compounds for use in oomycete disease control is necessary.

RESULTS: Zoospore lysis began at 10 μg mL^{-1} of thiobutacin, and most of the zoospores were collapsed at 50 μg mL^{-1}. Thiobutacin also revealed inhibitory activity against the cyst germination and hyphal growth of *Phytophthora capsici* at 50 μg mL^{-1}. Treatment with thiobutacin exhibited protective activity against development of Phytophthora disease on pepper plants.

CONCLUSION: The authors verified *in vitro* antioomycete activity of thiobutacin against *P. capsici* and its control efficacy against Phytophthora blight *in vivo*. This is the first report to demonstrate *in vivo* antioomycete activity of the novel antibiotic thiobutacin against *P. capsici* infection.

Keywords: thiobutacin; antimicrobial activity; antioomycete activity; Phytophthora blight

1 INTRODUCTION

Oomycetes have been classified in the kingdom Fungi because they apparently share morphological features, such as hyphae, appressoria, haustoria and spores, with some fungal plant pathogens. However, modern molecular and biochemical analyses suggest that oomycetes have little taxonomic affinity with filamentous fungi, but are more closely related to brown algae (heterokonts) and diatoms in the Stramenopiles, one of several major eukaryotic kingdoms.[1,2] Plant-pathogenic oomycetes cause devastating diseases in numerous crop, ornamental and native plants. Synthetic fungicides such as dimethomorph, ethaboxam, famoxadone, fosetyl-aluminium, metalaxyl and propomocarb have been developed and used practically for the control of plant diseases caused by oomycetes.[3–5] However, a number of synthetic fungicides are not effective when confronted by oomycete pathogens because many fungicide targets are absent from oomycetes, and present knowledge of their distinct physiology is limited.[6] Moreover, resistance to fungicides has already arisen in oomycete species,[7] and thus development of new, effective and safe compounds for use in oomycetes disease control is necessary.

More than 60 species of the genus *Phytophthora* are included in oomycetes, and they are arguably the most devastating pathogens of dicotyledonous plants.[8] The *Phytophthora* spp. cause enormous economic damage to important crop species such as potatoes, tomatoes, peppers, soybeans and alfalfa, as well as environmental damage in natural ecosystems.[9] Phytophthora blight, which is caused by *Phytophthora capsici* Leonian, is one of the most devastating soilborne diseases of pepper in Korea.[10] Intensive studies have been concentrated on the biology of *P. capsici*, evaluation of pepper germplasm for disease resistance, yield loss assessment and the testing of chemical, biological and cultural measures of control.[11]

In the authors' previous search programme for microorganisms producing antioomycete and antifungal compounds useful for the control of plant diseases, the actinomycete strain VK-A9, which showed substantial antagonistic activity against plant pathogens, was isolated from soils in Korea.[12] A novel antibiotic, thiobutacin [4-(2-aminophenyl)-4-oxo-2-methylthiobutanoic acid] (Fig. 1), was purified

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(*Received 4 November 2006; revised version received 27 June 2007; accepted 9 August 2007*)
Published online 10 December 2007; DOI: 10.1002/ps.1494

 Pest Manag Sci 1526–498X/2007/$30.00

and chemically characterized from the culture filtrate of the actinomycete strain VK-A9 by analysing NMR and MS spectral data.[13] Various antifungal compounds such as manumycin-type antibiotic SW-B,[14] fistupyrone,[15] phenazine-1-carboxylic acid,[16] aerugine,[17] A90931a[18] and staurosporine[19] isolated from soil microorganisms have been demonstrated to have potent antifungal or antioomycete activity for the control of economically important plant diseases.

In the present study, the antioomycete activity of thiobutacin was examined against *P. capsici* by evaluating zoospore lysis and inhibition of cyst germination and hyphal growth in addition to *in vitro* bioassay for antimicrobial activity. The authors also evaluated control efficacy of thiobutacin against Phytophthora blight of pepper plants by comparison with that of the commercial oomycete fungicide metalaxyl *in vivo*.

2 MATERIALS AND METHODS

2.1 Chemical materials

The antibiotic thiobutacin was isolated from cultures of the strain VK-A9 as described previously.[13] The oomycete fungicide metalaxyl [methyl *N*-(methoxyacetyl)-*N*-(2,6-xylyl)-DL-alaninate], which was obtained as technical-grade material (95% active ingredient) from Sungbo Chemical Co., Korea, was used to compare antioomycete activity with thiobutacin in the *in vitro* and *in vivo* assays. Both thiobutacin and metalaxyl were dissolved in methanol as stock solutions to use in this study.

2.2 Detection of *in vitro* antimicrobial activity

Minimum inhibitory concentrations (MICs) of thiobutacin against fungi and bacteria were determined in a 24-well microtitre dish (Cell Wells™; Corning Glass Works, Corning, NY) using a modified version of the method of Nair *et al*.[20] Mycelial suspension of *Rhizoctonia solani* Kühn, spore suspensions (10^5 spores mL^{-1}) of *Alternaria mali* Roberts, *Cladosporium cucumerinum* Ellis & Arthur, *Colletotrichum orbiculare* (Berk. & Mont.) Arx, *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *cucumerinum* JH Owen and *Magnaporthe grisea* (TT Hebert) ME Barr and cell suspensions (10^4 cfu mL^{-1}) of *Candida albicans* (CP Robin) Berkhout, *Cryptococcus neoformans* (Sanfelice) Vuillemin, *Bacillus subtilis* (Ehrenberg) Cohn subsp. *subtilis*, vancomycin-resistant *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (VRE), *Escherichia coli* (Migula) Castellani and Chalmers, *Pectobacterium carotovorum* subsp. *carotovorum* (Jones) Hauben *et al.*, *Ralstonia solanacearum* (Smith) Yabuuchi *et al.*, *Staphylococcus aureus* Rosenbach, methicillin-resistant *S. aureus* and *Xanthomonas vesicatoria* (ex Doidge) Vauterin *et al*. were prepared as inocula. A 100 µL suspension of each inoculum was added to each well containing 0.9 mL of potato dextrose broth for fungi and oomycetes, LB broth for VRE, *E. coli* and *S. aureus* and nutrient broth for other organisms. The antibiotic thiobutacin in methanol, ranging from 0 to 100 µg mL^{-1}, was dispensed into the microtitre plates. The antimicrobial activity of thiobutacin against the organisms was evaluated after incubation for 2–5 days. The plate inoculated with *C. cucumerinum* was incubated at 22 °C, plates inoculated with VRE, *E. coli, S. aureus* and methicillin-resistant *S. aureus* were incubated at 37 °C and those inoculated with other microorganisms were inoculated at 28 °C. A negative control containing culture broth and microorganisms without the antibiotic was included in this test. The lowest concentrations of thiobutacin where no growth of microorganisms was observed were considered to be MICs.

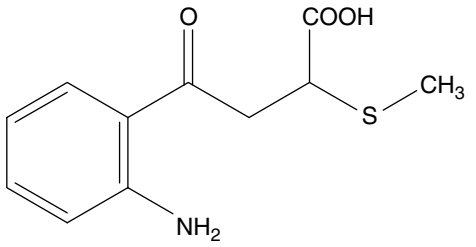

Figure 1. Structure of the antibiotic thiobutacin.

2.3 Zoospore lytic activity of thiobutacin

The lytic activity of thiobutacin against zoospores of *P. capsici* was evaluated by counting the number of zoospores collapsed after treatment with the antibiotic at various concentrations. Zoospore suspensions were prepared from *P. capsici* cultures grown on oatmeal agar for 10 days at 28 °C.[21] *Phytophthora capsici* isolate 87L19, which was isolated from pepper plants in Korea and is highly sensitive to metalaxyl, was used for the assay.[22] Aliquots (0.1 mL) of zoospore suspension (1×10^6 zoospores mL^{-1}) were mixed in microtubes with 0.4 mL of various concentrations of thiobutacin. The number of zoospores lysed at each concentration was determined under a light microscope after incubation for 5 min at 28 °C. The experiments were repeated 3 times with four replicates.

2.4 Inhibition of *Phytophthora capsici* cyst germination by thiobutacin

The encystment of zoospores of *P. capsici* isolate 87L19 was induced by vigorously shaking zoospore suspensions for 2 min at room temperature. An 0.1 mL aliquot of cyst suspension was dispensed in the microtube containing 0.4 mL of 0.5% potato dextrose broth amended with thiobutacin at each concentration. After incubation of cysts for 6 h at 28 °C, the cysts germinated were counted under a light microscope. The experiment was repeated 3 times with four replicates.

2.5 Effect of thiobutacin on hyphal growth

The suspension of zoospore cysts (0.1 mL) in potato dextrose broth was incubated in the microtubes at

28 °C, until the hyphae of the germlings had an average length of 30 μm. Thiobutacin or metalaxyl solution (0.4 mL) at various concentrations was added onto the germlings. The mixtures were then incubated at 28 °C until the control germlings attained an average length of 400 μm. The length of 50 individual hyphae was determined under a light microscope. Percentage inhibition of the hyphal growth was determined by comparison of the hyphal length of the germlings treated with thiobutacin or metalaxyl with that of control germlings. Experiments with four replicates were repeated 3 times with similar results.

2.6 *In vivo* evaluation of antioomycete activity of thiobutacin

The control efficacy of the antibiotic thiobutacin against *P. capsici* infection on pepper plants was evaluated in a growth room. Pepper seeds (*Capsicum annuum* L. cv. Hanbyul) were sown in a plastic tray (55 × 35 × 15 cm) containing steam-sterilized soil mix (peat moss + perlite + vermiculite, 5 + 3 + 2 by volume), sand and loam soil (1 + 1 + 1 by volume). Six seedling plants at the four-leaf stage were transplanted into a plastic pot (5 × 15 × 10 cm) containing the soil mix described above. Pepper plants were raised in a growth room at 28 ± 2 °C with approximately 80 μmol photons $m^{-2} s^{-1}$ (white fluorescent lamps) for 16 h day^{-1}. The commercial oomycete fungicide metalaxyl was used to compare the antifungal activity with thiobutacin. Solutions of thiobutacin and metalaxyl were prepared in methanol containing 0.05% Tween 20 at concentrations of 1, 10, 50, 100 and 500 μg mL^{-1}. Each solution was sprayed at 500 L ha^{-1} onto the surface of pepper plants at the first-branch stage 1 day before inoculation with *P. capsici* isolate 87L19. Control plants were sprayed with Tween 20 solution without chemical. The pepper plants treated with thiobutacin and metalaxyl were wounded by making 1 cm longitudinal slits on the stems 1 cm from the soil surface. The zoospore suspension was prepared as described previously.[21] Sterile cotton dipped in a zoospore suspension (10^5 zoospores mL^{-1}, prepared as described previously[21]) was placed on the wounded sites on the stem. The inoculated sites were covered with plastic tape to maintain a moist condition. Disease severity on pepper plants was rated daily after inoculation on the basis of a scale 0–5: 0 = no visible disease symptoms; 1 = leaves slightly wilted with brownish lesions beginning to appear on stems; 2 = 30–50% of entire plant diseased; 3 = 50–70% of entire plant diseased; 4 = 70–90% of entire plant diseased; 5 = plant dead. Data are the means of six plants per treatment.

3 RESULTS AND DISCUSSION

The novel antibiotic thiobutacin has been chemically characterized and demonstrated to have *in vitro* antioomycete activity against *P. capsici* in a previous study by the authors.[13] In the present study, assays were performed to evaluate the *in vitro* antimicrobial activity of thiobutacin against diverse fungi and bacteria. The antibiotic thiobutacin completely inhibited the mycelial growth of *C. cucumerinum* at 50 μg mL^{-1} (Table 1), as well as the oomycete pathogen *P. capsici* (MIC 10 μg mL^{-1}) and *B. cinerea* (MIC 50 μg mL^{-1}) as previously reported.[13] However, the growth of *A. mali, C. orbiculare, F. oxysporum* f. sp. *cucumerinum, M. grisea* and *R. solani* was not inhibited even at 100 μg mL^{-1}, and nor was the cell growth of *C. albicans* and *C. neoformans* affected at 100 μg mL^{-1}. Thiobutacin also showed antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus* with MICs of 25 and 50 μg mL^{-1} respectively. No other bacterial growth inhibition was observed at 100 μg mL^{-1}.

To evaluate zoospore lysis activity of the antibiotic thiobutacin, zoospore suspensions of *P. capsici* treated with thiobutacin were examined under a light microscope over a time sequence. Zoospores were rendered immotile within 10–15 s following exposure to thiobutacin (50 μg mL^{-1}), and subsequent lysis started to occur at 60 s (Fig. 2A). The zoospores of *P. capsici* began to swell and eventually ruptured at 60 s after treatment with thiobutacin, and complete lysis of zoospores occurred at 90 s (Fig. 2B). Compared with the untreated control, zoospore motility was distinctly

Table 1. Minimum inhibitory concentrations (MIC) of thiobutacin against various microorganisms as determined by the microtitre broth dilution method

Microorganism	MIC (μg mL^{-1})[a,b]	Reference
Fungus and oomycete		
Alternaria mali	>100	Present study
Botrytis cinerea	50	13
Cladosporium cucumerinum	50	Present study
Colletotrichum orbiculare	>100	Present study
Fusarium oxysporum f. sp. *cucumerinum*	>100	Present study
Magnaporthe grisea	>100	Present study
Phytophthora capsici	10	13
Rhizoctonia solani	>100	Present study
Candida albicans	>100	Present study
Cryptococcus neoformans	>100	Present study
Saccharomyces cerevisiae	30	13
Bacterium		
Bacillus subtilis subsp. *subtilis*	>100	Present study
Vancomycin-resistant *Enterococcus faecalis*	>100	Present study
Escherichia coli	>100	Present study
Pectobacterium carotovorum subsp. *carotovorum*	>100	Present study
Ralstonia solanacearum	>100	Present study
Staphylococcus aureus	25	Present study
Methicillin-resistant *Staphylococcus aureus*	50	Present study
Xanthomonas vesicatoria	>100	Present study

[a] The lowest concentration of thiobutacin required for complete inhibition of microbial growth.
[b] >100 indicates that growth of the microorganisms was not completely inhibited at 100 μg mL^{-1}.

Pest Manag Sci **64**:172–177 (2008)
DOI: 10.1002/ps

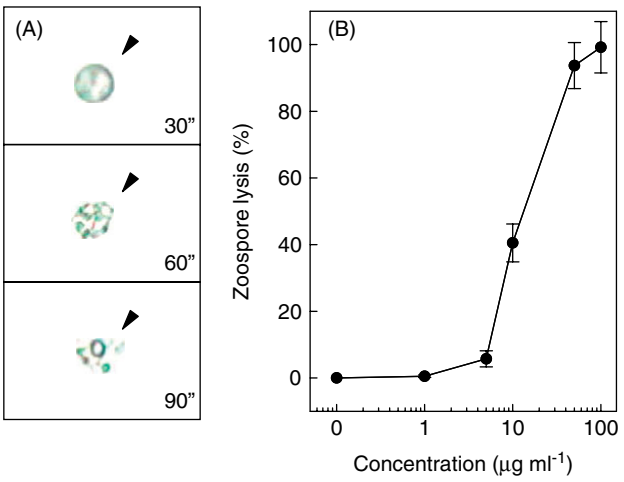

Figure 2. The lytic activity of thiobutacin against zoospores of *Phytophthora capsici.* (A) Time course of zoospore lysis after exposure to thiobutacin (50 μg mL^{-1}), examined under a light microscope (magnification, ×100). (B) The zoospore lysis activity of thiobutacin at various concentrations. The experiments were repeated 3 times with four replicates. Vertical bars represent standard errors.

reduced at 5 μg mL^{-1} of thiobutacin, although a weak zoospore lytic activity was observed (data not shown). Most zoospores were lysed at 100 μg mL^{-1} of thiobutacin.

Zoospores have been accepted as the principal dispersive agents of oomycetes. Therefore, zoospore movement and encystment are important factors in the pathogenicity of oomycete pathogens such as *Phytophthora* and *Pythium* spp.[23] The zoospore seems to be not only the most susceptible phase in the life cycle of zoosporogeneric fungi, but also the stage at which disease control could be efficient.[24] The zoospores may not be strong enough to bear osmotic pressure of water under conditions where active transport is inhibited, thus resulting in plasma membrane rupture, because zoospores do not have cell walls, only plasma membranes.[25] The authors previously demonstrated that rhamnolipid B isolated from *Pseudomonas aeruginosa* (Schroeter) Migula and phenylacetic acid produced by *Streptomyces humidus* Nakazawa and Shibata had lytic effects on zoospores as well as inhibitory activity against the cyst germination and hyphal growth of *P. capsici*.[26,27] Some natural products, such as anacardic acid from *Ginkgo biloba* L.[6] and phytuberin from potato tubers,[28] also exhibited antioomycete activity against *Phytophthora infestans* (Montagne) de Bary, resulting in the lysis of the zoospores. Cyst germination was completely inhibited after treatment with thiobutacin at 50 μg mL^{-1} (Fig. 3A). Thiobutacin strongly inhibited the hyphal growth of *P. capsici* at 50 and 100 μg mL^{-1} (Fig. 3B). The average hyphal length of germlings was reduced at 50 μg mL^{-1} to 58% of that in the untreated control.

In the present study it was shown that the antibiotic thiobutacin inhibited some stages of the life cycle of *P. capsici*, including zoospore motility, cyst germination and hyphal growth. It has been demonstrated that zoospore motility and release are affected by inhibition of the energy supply.[29] Energy generation-inhibitory molecules such as fluazinam and famoxadone inhibit zoospore motility and release in *P. infestans*,[30,31] while non-respiratory inhibitors, such as metalaxyl and dimethomorph, cause little or no inhibition of zoospore motility and release of *P. infestans*.[32,33] Further detailed studies are required to determine whether thiobutacin impairs the energy generation system in *P. capsici*.

In vivo control efficacy of thiobutacin for the suppression of Phytophthora blight on pepper plants was evaluated under greenhouse conditions (Fig. 4). As the concentration of the antibiotic thiobutacin and commercial fungicide metalaxyl increased, the development of Phytophthora blight was gradually inhibited on the pepper plants at the first-branch stage. Phytophthora disease symptoms were markedly reduced by treatment with 100 or 500 μg mL^{-1} of the two compounds. The control efficacy of thiobutacin against Phytophthora blight was in general less than

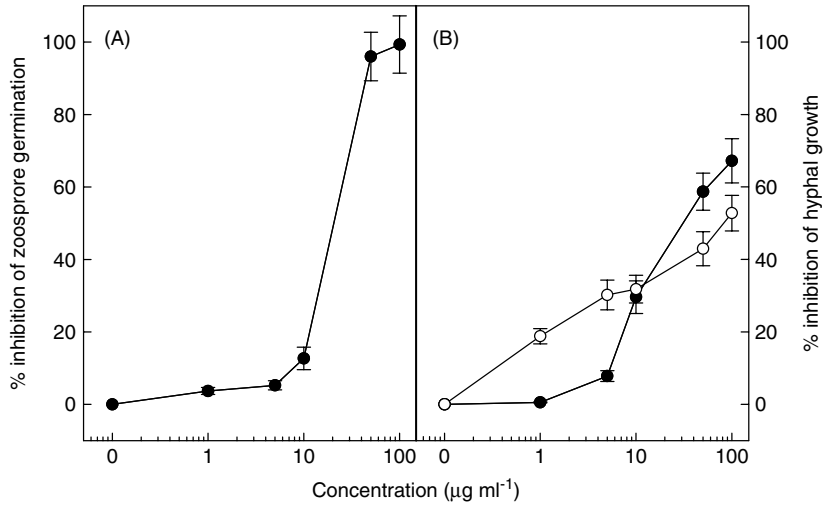

Figure 3. (A) Inhibitory effect of thiobutacin on cyst germination. (B) Inhibitory effects of (●) thiobutacin and (○) metalaxyl on hyphal growth of *Phytophthora capsici* at various concentrations. The experiments were repeated 3 times with four replicates. Vertical bars represent standard errors.

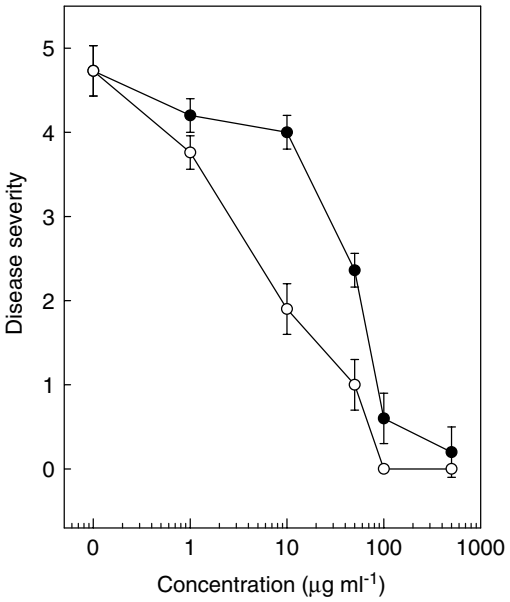

Figure 4. Effects of (●) thiobutacin and (○) metalaxyl on disease development in pepper plants inoculated with *Phytophthora capsici* at the first-branch stage. Disease severity is based on a 0–5 scale, where 0 = no visible symptom and 5 = plant dead. Data are the means of six plants per treatment. Vertical bars represent standard errors.

that of metalaxyl on pepper plants. However, *in vivo* efficacy of thiobutacin was not significantly different from that of metalaxyl at 500 μg mL^{-1}. Thiobutacin did not induce any phytotoxicity on pepper plants, even when treated with 500 μg mL^{-1} (data not shown).

In many cases, *in vivo* control efficacy of antibiotics against plant diseases correlates with their *in vitro* activity.[34] The present study ascertained not only the *in vitro* antimicrobial activity of thiobutacin but also its *in vivo* antioomycete activity in the host plants. These results suggest that the antibiotic thiobutacin may have a potent chemotherapy against *P. capsici* infection. To the authors' knowledge, this is the first report to demonstrate *in vivo* efficacy of thiobutacin for the control of Phytophthora blight in pepper plants. The finding that thiobutacin has antioomycete activity may enable it to be used as a lead for developing agricultural fungicides for the control of plant oomycete diseases such as Phytophthora blight.

In conclusion, the antibiotic thiobutacin has not only a potent *in vitro* antioomycete activity against *P. capsici* but also *in vivo* control efficacy against Phytophthora disease on pepper. Further studies on the mechanism of action of thiobutacin at the molecular level and field evaluation of its effectiveness for disease control and the design of applications for its practical use should be done.

ACKNOWLEDGEMENTS

This work was supported by a grant (CG1133) from the Crop Functional Genomics Centre of the Twenty-first Century Frontier Research Programme, a grant from the Centre for Plant Molecular Genetics and Breeding Research, Seoul National University, Korea, and a grant (20070401034028) from the BioGreen21 Programme, Rural Development Administration, Korea.

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