IN VITRO ANTIMICROBIAL ACTIVITY OF VOLATILE ORGANIC COMPOUNDS FROM *MUSCODOR CRISPANS* AGAINST THE PATHOGENIC OOMYCETE *PYTHIUM INSIDIOSUM*

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Abstract. *Pythium insidiosum* is an oomycete capable of causing a life-threatening disease in humans, called pythiosis. Conventional antifungal drugs are ineffective against *P. insidiosum* infection. A synthetic mixture of the volatile organic compounds (VOCs) from the endophytic fungus *Muscodor crispans* strain B23 demonstrates antimicrobial effects against a broad range of human and plant pathogens, including fungi, bacteria, and oomycetes. We studied the *in vitro* effects of B23 VOCs against 25 human, 1 animal, and 4 environmental isolates of *P. insidiosum*, compared with a no-drug control. The B23 synthetic mixture, at amounts as low as 2.5 l, significantly reduced growth of all *P. insidiosum* isolates by at least 80%. The inhibitory effect of the B23 VOCs was dose-dependent. The growth of all isolates was completely inhibited by a dose of 10.0 l of B23 VOCs, and all isolates were killed by a dose of 20.0 l. Synthetic B23 VOCs of *M. crispans* had inhibitory and lethal effects against all *P. insidiosum* isolates tested. Further studies are needed to evaluate this mixture for treatment of pythiosis.

Keywords: pythiosis, *Pythium insidiosum*, oomycete, *in vitro* susceptibility, *Muscodor crispans*, endophytic fungus

INTRODUCTION

Pythiosis is a life-threatening infectious disease caused by the pathogenic oomycete *Pythium insidiosum* (Mendoza *et al*, 1996). *P. insidiosum* belongs to the Stramenopiles of the supergroup Chromalveolates, and is the only *Pythium* species known to infect both humans and animals living in tropical and subtropical areas of the world (Mendoza *et al*, 1996; Kamoun, 2003; Keeling *et al*, 2005). The microorganism has two forms: branching hyphae and a mobile zoospore (Mendoza *et al*, 1993). The zoospore is an infective unit that invades host tissue. Although, *P. insidiosum* has similar morphological

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features to filamentous fungi, it is more phylogenically related to diatoms and algae than to true fungi (Kwon-Chung, 1994).

Pythiosis was first described in animals a century ago (Smith, 1884; de Haan and Hoogkamer, 1901). Its ability to cause disease in human was described in 1986 (Thianprasit, 1986). In the past decade, pythiosis has been increasingly diagnosed in humans and animals (Tabosa *et al*, 2004; Bosco *et al*, 2005; Mendoza and Newton, 2005; Rakich *et al*, 2005; Rivierre *et al*, 2005; Krajaejun *et al*, 2006; Rodrigues *et al*, 2006; Berryessa *et al*, 2008; Pesavento *et al*, 2008; White *et al*, 2008; Badenoch *et al*, 2009; Lekhanont *et al*, 2009; Neto *et al*, 2010; Pereira *et al*, 2010; Tanhehco *et al*, 2011; Videla *et al*, 2012). The disease has high morbidity and mortality rates. The clinical manifestations of pythiosis are similar to infections caused by some filamentous fungi (Mendoza *et al*, 2004). Under-recognition of pythiosis may due to clinicians, veterinarians and microbiologists being unfamiliar with the disease and its causative agent. Pythiosis is mostly diagnosed in horses and dogs (Mendoza *et al*, 1996). Affected animals usually present with cutaneous/subcutaneous infections or gastrointestinal tract infections. Animals with pythiosis die if left untreated. In humans, pythiosis has two common forms: 1. vascular pythiosis affecting medium-to-large size arteries resulting in arterial occlusion and 2. ocular pythiosis causing corneal ulcers or keratitis (Krajaejun *et al*, 2006). Definitive diagnosis of pythiosis is made by culture, which is an insensitive and time-consuming procedure. Serodiagnostic assays (immunodiffusion, ELISA, Western blot, immunochromatography, hemagglutination and immunohistological stain) and PCR-based assays have been devised to facilitate early detection of the disease (Pracharktam *et al*, 1991; Badenoch *et al*, 2001; Grooters and Gee, 2002; Krajaejun *et al*, 2002; Jindayok *et al*, 2009; Keeratijarut *et al*, 2009; Krajaejun *et al*, 2009; Supabandhu *et al*, 2009).

Conventional antifungal drugs are generally ineffective in eradicating the infection. An immunotherapy using the *P. insidiosum* protein extract demonstrated unfavorable treatment response, with disease clearance rates of ~50% for humans, ~30% for dogs, and ~60% for horses with pythiosis (Mendoza and Newton, 2005; Krajaejun *et al*, 2006). Therefore, radical excision of infected tissue or organ has become the main treatment option. Most vascular pythiosis patients undergo limb amputation, while the majority of ocular patients undergo infected eye removal. Post-operative relapse can occur and can cause further damage or death. Improving treatment outcomes for patients suffering from pythiosis is therefore the most important healthcare goal.

The ineffectiveness of conventional antifungal drugs against *P. insidiosum* may due to the lack of the antifungal drug-target: ergosterol biosynthetic pathway (Schlosser and Gottlieb, 1966; Krajaejun *et al*, 2006). *Pythium* species are classified as a unique group of microorganisms that differ phylogenetically and physiologically from almost all other fungal pathogens, which are the usual targets for conventional antifungal agents (Kwon-Chung, 1994). There is only one report of conventional antifungal drugs successfully treating human patient with pythiosis (Shenep *et al*, 1998). Many conventional antifungal drugs from different pharmacotherapeutic groups (itraconazole, posaconazole, voriconazole, terbinafine, caspofungin, amphotericin-B, and fluvastatin) have been tested *in vitro*, alone or in combination, against clinical isolates of

P. insidiosum (Sekhon *et al*, 1992; Pereira *et al*, 2007; Argenta *et al*, 2008; Cavalheiro *et al*, 2009a, b; Loreto *et al*, 2011; Argenta *et al*, 2012). Other agents (ibuprofen, diphenyl diselenide, rifampicin, metronidazole, macrolides, and tetracycline antibiotics) have been tested against the pathogen as well (Brown *et al*, 2008; Cavalheiro *et al*, 2009b; Loreto *et al*, 2011, 2012). These drugs have had no appreciable results, limited fungicidal activity, unachievable drug levels in tissue, or no *in vivo* evaluations. Mefenoxam has been shown to possess anti-oomycete activity (Brown *et al*, 2008; Hummel *et al*, 2011). However, mefenoxam is a fungicide developed for agricultural use and its toxicity in mammals, and especially humans, is unclear. There is no administrable formula of this drug for patients, making it inappropriate for use in the treatment of pythiosis. An antimicrobial agent clinically effective against *P. insidiosum* is still needed.

Some organic compounds extracted from endophytic fungi have been reported to have antimicrobial effects against a variety of pathogens (Strobel and Daisy, 2003; Ezra *et al*, 2004; Mitchell *et al*, 2010). Among these is a mixture of volatile organic compounds (VOCs) from the *Muscodor crispans* strain B23, a novel endophytic fungus of *Ananas ananassoides* (wild pineapple) growing in the Bolivian Amazon Basin (Mitchell *et al*, 2008, 2010). GC/MS analysis reveals the major components of this compound include: propanoic acid, 2-methyl-, methyl ester; propanoic acid, 2-methyl-; 1-butanol, 3-methyl-;1-butanol, 3-methyl-, acetate; propanoic acid, 2-methyl-, 2-methylbutyl ester; ethanol; and others. The VOCs of *M. crispans* demonstrate antimicrobial activity against various human and plant pathogens, including fungi, bacteria and oomycetes (*ie*, *Phytophthora* and *Pythium* species) (Mitchell *et al*, 2010). The use of synthetic B23 VOCs mimicking the components of the native *M. crispans* mixture revealed compatible and equivalent antimicrobial effects (Mitchell *et al*, 2010). The ingredients of the synthetic B23 VOCs are on the US Food and Drug Administration's GRAS list of harmless substances (http://www.fda.gov/), making it possible for *in vivo* therapeutic application in humans and animals. In the present study, we used a radial growth agar plate bioassay to test the antimicrobial effects of the synthetic B23 VOCs against 25 human, 1 animal, and 4 environmental isolates of *P. insidiosum*.

MATERIALS AND METHODS

Microorganisms

Thirty isolates of *P. insidiosum* were obtained from humans ($n = 25$; isolate ID, M01-25), the environment (agricultural fields in Thailand; $n = 4$; isolate ID, M26-29), and one animal (mosquito lava; $n = 1$; isolate ID, M30) (Table 1). The isolates were maintained by subculturing monthly on Sabouraud dextrose agar (SDA) containing 1% (wt/vol) bacto peptone (Becton Dickinson, Sparks, MD), 2% (wt/vol) dextrose (HiMedia, Mumbai, India) and 1.5% (wt/vol) agar (Becton Dickinson, Sparks, MD) and incubated at room temperature (~28°C).

M. crispans synthetic B23 VOCs

The synthetic B23 VOCs were prepared using the method of Strobel *et al* (2001) and Mitchell *et al* (2010). The components of the native B23 VOCs were analyzed using GC/MS techniques described elsewhere (Mitchell *et al*, 2010). The identified components were prepared by bacterial fermentation by Jeneil Biotech (Saukville, WI). The components were mixed in the same proportions as

they exist in the native mixture but some components, such as ethanol, acetaldehyde, and the cyclohexane, were omitted (Mitchell *et al*, 2010) with some modifications: substitution of 2-methyl-propanoic acid with propanoic acid. The mixture was stored in a sealed container in a freezer until used.

Radial growth assay

The radial growth assessment method used to analyze *P. insidiosum* in this study was modified from the methods of Brown *et al* (2008), Krajaejun *et al* (2010) and Strobel *et al* (2001). Briefly, *P. insidiosum* was subcultured on SDA at 37°C for 5 days. An agar plug (5-mm in diameter) with mycelium was cut from the margin of an actively-growing *P. insidiosum* colony. The SDA plate was divided into 4 quadrants and 4 agar plugs were tested simultaneously in one plate. A microtube cap was placed at the center of the plate to serve as a container of the B23 VOCs. To evaluate the inhibitory effect of the B23 VOCs, a *P. insidiosum*-attached agar plug was inoculated, with the mycelium in contact with the agar, in each quadrant of the SDA plate (Fig 1), in the presence of different volumes of B23 VOCs: 0.0 l (control), 2.5 l, 5.0 l, 10.0 l, 20.0 l and 40.0 l. The agar plate was then wrapped with a strip of parafilm and incubated at 37°C. The growth diameter of each *P. insidiosum* colony was measured daily for 3 days. Five millimeters (the diameter of the mycelial plug) was subtracted from the average colony diameter and then divided by 2 to obtain the mean radial growth. After 3 days incubation, each *P. insidiosum*-attached agar plug was transferred to a fresh SDA plate and incubated at 37°C for 3 more days without the B23 VOCs. Inhibitory and lethal effects of the B23 VOCs were then determined. All the assays carried out in duplicate.

Statistical analysis

The differences in radial growth between the groups of isolates growing in the presence of different volumes of the B23 VOCs (Group 2.5, Group 5.0, Group 10.0, Group 20.0 and Group 40.0) and the control were calculated using the Friedman test for one-way repeat measure analysis of variance by ranks. A p-value < 0.05 was considered significant. The Wilcoxon signed-rank test was used to compare the radial growth between the control and Group 2.5, between Group 2.5 and Group 5.0 and Group 5.0 and Group 10.0 using a Bonferroni-adjusted p-value of < 0.016.

RESULTS

Thirty isolates of *P. insidiosum* were tested *in vitro* against 6 different amounts of the synthetic B23 VOCs. The synthetic B23 VOCs evaporated in the closed space of the Petri dish and was absorbed directly by the growing *P. insidiosum* colonies. The inhibitory effects of the B23 VOCs against *P. insidiosum* were determined day 3 post-inoculation (Table 1). There was at least 80% reduction in growth in all studied groups compared to the control ($p < 0.001$). The higher the volume of B23 VOCs, the greater the growth reduction. Group 5.0 had greater reduction in growth than Group 2.5 ($p < 0.001$). The isolates in Group 10.0, Group 20.0 and Group 40.0 had an equivalent reduction in growth compared to Group 5.0 ($p < 0.001$). Eighty percent of isolates in Group 2.5 had at least 90% radial growth inhibition; of these, 27% had complete (100%) inhibition of growth (Table 2). All the isolates in Group 5.0 had at least 90% radial growth inhibition and 93% had complete (100%) inhibition of growth (Table 2). All the isolates in Group 10.0, Group 20.0 and Group 40.0 had complete (100%) inhibi-

Table 1
Percent growth inhibition (in relation to the non-drug control) and killing effect of synthetic B23 VOCs against *P. insidiosum* isolates.

ID	Site of infection	Group 2.5		Group 5.0		Group 10.0		Group 20.0		Group 40.0	
		% inhibition	Killed	% inhibition	Killed	% inhibition	Killed	% inhibition	Killed	% inhibition	Killed
M01	Artery	97.9	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M02	Artery	95.3	-	100.0	-	100.0	-	100.0	Yes	100.0	Yes
M03	Artery	98.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M04	Artery	100.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M05	Artery	100.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M06	Artery	94.4	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M07	Artery	100.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M08	Artery	92.9	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M09	Artery	96.3	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M10	Artery	100.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M11	Artery	89.7	-	100.0	-	100.0	-	100.0	Yes	100.0	Yes
M12	Artery	98.2	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M13	Artery	100.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M14	Artery	87.1	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M15	Artery	87.9	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M16	Cornea	100.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M17	Cornea	100.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M18	Cornea	86.4	-	100.0	-	100.0	-	100.0	Yes	100.0	Yes
M19	Cornea	96.1	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M20	Cornea	96.4	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M21	Cornea	81.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M22	Cornea	93.7	-	96.8	-	100.0	-	100.0	Yes	100.0	Yes
M23	Cutaneous	85.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M24	Brain	100.0	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M25	Gastrointestine	96.2	-	98.1	-	100.0	Yes	100.0	Yes	100.0	Yes
M26	Environment	97.4	-	100.0	Yes	100.0	Yes	100.0	Yes	100.0	Yes
M27	Environment	95.1	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes
M28	Environment	97.9	-	100.0	Yes	100.0	Yes	100.0	Yes	100.0	Yes
M29	Environment	98.1	-	100.0	-	100.0	-	100.0	Yes	100.0	Yes
M30	Mosquito larva	97.9	-	100.0	-	100.0	Yes	100.0	Yes	100.0	Yes

tion of growth. The minimal volume that completely inhibited growth in 90% of isolates was 5.0 l, and the minimal volume that completely inhibited growth of all isolates 10.0 l.

After exposure of *P. insidiosum* to the B23 VOCs for 3 days, the *P. insidiosum*-attached agar plugs were transferred to fresh SDA plates without the B23 VOCs and then incubated for 3 more days. An isolate that did not grow at this time was considered killed. One hundred percent of *P. insidiosum* isolates in Group 2.5 regenerated normally, and 100% of isolates

Table 2
Growth inhibition (in relation to the non-drug control) and killing rates of synthetic B23 VOCs against *P. insidiosum* isolates.

% growth inhibition	Group 2.5		Group 5.0		Group 10.0		Group 20.0		Group 40.0	
	Number of isolates	%	Number of isolates	%	Number of isolates	%	Number of isolates	%	Number of isolates	%
<80%	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
80-89%	6	20.0	0	0.0	0	0.0	0	0.0	0	0.0
90-99%	16	53.3	2	6.7	0	0.0	0	0.0	0	0.0
100%	8	27.0	28	93.0	30	100.0	30	100.0	30	100.0
Killed	0	0.0	2	6.7	25	83.3	30	100.0	30	100.0

in Group 20.0 and Group 40.0 had no growth, even after 2 more weeks incubation (Table 2). In Groups 5.0 and 10.0, 93.3% and 16.7% of isolates regenerated, respectively (Table 2). Minimal volume of B23 VOCs that killed 100% of isolates tested was 20.0 l.

DISCUSSION

Pythiosis is an infectious disease with high morbidity and mortality rates in humans, found especially in topical and subtropical countries. Treatment of pythiosis is problematic. Conventional antifungal drugs are ineffective against *P. insidiosum* infection, leaving radical, destructive surgery as the only option for disease control. The B23 VOCs from the endophytic fungus *M. crispans* strain B23 were recently reported to have broad spectrum antimicrobial activity (Mitchell *et al*, 2010). In this study, we explored the *in vitro* effect of these B23 VOCs against 30 isolates of *P. insidiosum*.

In the past, 3 methods for susceptibility testing with *P. insidiosum* have been reported: 1) use of a hyphae suspension for a broth dilution assay (Sekhon *et al*, 1992; Shenep *et al*, 1998; Brown *et al*, 2008); 2) use of a zoospore suspension for a broth dilution assay (Pereira *et al*, 2007; Argenta *et al*, 2008; Cavalheiro *et al*, 2009a, b); and 3) measurement of radial hyphal growth with an agar dilution assay (Brown *et al*, 2008). Because the B23 VOCs are volatile, we selected the radial hyphal growth agar plate assay to test for susceptibility testing. This agar-based method allows direct contact between the B23 VOCs and the test organism in a Petri dish and allows direct assessment of the growth of *P. insidiosum* hyphae, which is the pathogenic form of the organism. The radial hyphal growth agar plate assay has been demonstrated to provide clear and reproducible results for antimicrobial drugs against *P. insidiosum* isolates (Brown *et al*, 2008). In comparison with other susceptibility methods, the radial growth method is easier to prepare and use with dilution (Brown *et al*, 2008).

The B23 VOCs markedly reduced growth in all *P. insidiosum* isolates tested, even in amounts as little as 2.5 l (Tables 1 and 2). B23 VOCs inhibited *P. insidiosum* growth in dose-dependent manner. Our findings suggest B23 VOCs have inhibitory and lethal effect against *P. insidiosum* isolates. B23 VOCs have growth inhibition effect in other oomycetes, such

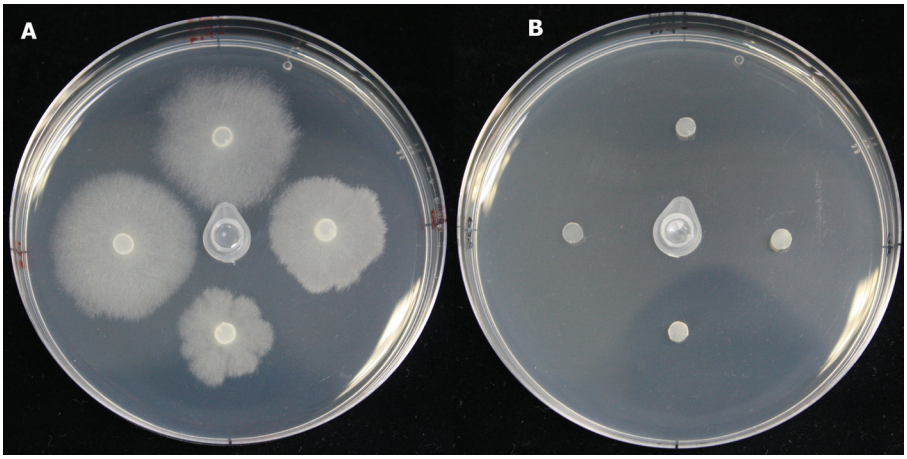

Fig 1–Radial growth agar plate assay used for *in vitro* susceptibility testing of synthetic B23 VOCs against *P. insidiosum*. A SDA plate was divided into 4 quadrants, and 4 agar plugs with actively-growing mycelium were tested simultaneously in one plate. A microtube cap placed at the center served as a container for various amounts of B23 VOCs. A, Agar containing *P. insidiosum* without B23 VOCs (control). B, Agar containing *P. insidiosum* inhibited by B23 VOCs.

as, *Phytophthora cinnamomi, Phytophthora palmivora, Pythium ultimum* (Mitchell *et al,* 2010).

M. crispans is not the only entophytic fungus of the genus *Muscodor* that produces antimicrobial VOCs. *Muscodor albus* is another *Muscodor* species that produces VOCs with broad-spectrum antibiotic activity (Strobel *et al,* 2001; Ezra *et al,* 2004; Strobel, 2006). The major components of *Muscodor* VOCs include alcohols, acids, esters, ketones, and lipids (Strobel, 2006). When evaluated individuals against pathogens, the various VOCs have limited inhibitory efficacy, but combination provides a synergistic antimicrobial effect (Strobel *et al,* 2001). A difference between the VOCs of *M. crispans* and other *Muscodor* species, including *M. albus*, is that *M. crispans* VOCs contain no naphthalene or azulene derivatives; all the ingredients of *M. crispans* VOCs are listed as harmless substances by the US Food and Drug Administration (Strobel, 2006; Mitchell *et al,* 2010). Thus, *M. crispans* B23 VOCs may be safe for agricultural, household, industrial or medicinal use. The mechanism of antimicrobial action of the B23 VOCs is unknown. Based on microarray analysis, it has been proposed the induction of pathogen DNA damage mediated by naphthalene derivatives is an underlying antimicrobial mechanism of the *M. albus* VOCs (Mitchell *et al,* 2010). However, this proposed mechanism is not compatible with naphthalene-lacking *Muscodor* species, such as *M. crispans*. An advantage of the B23 VOCs is they can be artificially synthesized by mimicking the components of the native *M. crispans* VOCs (Mitchell *et al,* 2010). This study and a previous study (Mitchell *et al,* 2010) (with various pathogens) found synthetic B23 VOCs have antimicrobial effects the same

as the original B23 VOCs. Synthetic B23 VOCs may be used in place of the original for the same antimicrobial purposes.

Although B23 VOCs have anti-*P. insidiosum* effects, clinical applications may be limited by the volatility of the substance. Repeatedly rinsing B23 VOCs through a *P. insidiosum*-infected lesion may be one technique of administering this substance to animal and humans patients. Placing gauze soaked with B23 VOCs over a lesion may be a way of using it clinically. Animal studies are needed before human studies can be carried out. In the United States, such studies are presently underway in dogs and other animals suffering pythiosis (Gary A Strobal, personal communication).

In this study, B23 VOCs inhibited and killed *P. insidiosum in vitro*. The components of B23 VOCs are listed as harmless substances by the FDA, opening the door to their possible clinical use. B23 VOCs can be artificially synthesized and have the same antimicrobial effects as the native compound. The antimicrobial properties of synthetic B23 VOCs are dose-dependent and effective against *P. insidiosum*. The B23 VOCs from *M. crispans* are a potential candidate for controlling infection caused by *P. insidiosum*. Case-control studies in animals with pythiosis are required to explore the *in vivo* therapeutic and adverse effects of B23 VOCs.

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