Isothiocyanates Produced by Brassicaceae Species as Inhibitors of *Fusarium oxysporum*

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ABSTRACT

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Glucosinolates contained in members of the Brassicaceae release isothiocyanates potentially useful in controlling *Fusarium oxysporum* pathogens in conifer seedling nursery soils. Our objective was to determine the toxicity of individual isothiocyanates to different growth stages of the fungus. Bioassays with four *F. oxysporum* isolates were conducted using sealed containers in which 0.3 µl of 2-propenyl, ethyl, buty, phenylethyl, benzyl, or phenyl isothiocyanate was allowed to volatilize. Propenyl and ethyl isothiocyanates were the most fungistatic of those compounds tested. The same concentrations of propenyl and ethyl isothiocyanates that inhibited mycelial growth completely suppressed conidial and chlamydospore germination of all isolates. Other isothiocyanates including ethyl, benzyl, and phenethyl were also fungitoxic to *F. oxysporum* conidia and chlamydospores. Reduction in pathogen populations resulting from a green-manure crop are likely achievable since chlamydospores are sensitive to isothiocyanate. Pathogenic *F. oxysporum* isolates infesting nursery soils would likely be most suppressed by species of plants such as *Brassica carinata*, *B. nigra*, and *B. juncea*, which contain glucosinolates that release high concentrations of propenyl isothiocyanate.

Additional keywords: forest nurseries, soil fumigation, soilborne fungi

Fusarium oxysporum Schlechtend. is a common soilborne fungus that causes damping-off, hypocotyl and root rot, and stunting or mortality of Douglas-fir nursery seedlings (3–5,22). These diseases, which occur most often in the first year of growth, are the primary causes of seedling mortality and can result in significant losses in forest nurseries (21). *F. oxysporum* is a soil inhabitant that infects plants through direct penetration or wounds in the roots. This fungus forms thick-walled resting structures called chlamydospores, thin-walled, sickle-shaped macroconidia, and smaller, one-celled microconidia, all of which act as potential sources of infection (6). The species also produces sclerotia that along with chlamydospores exist as resting structures in soil (35).

Fumigation of soil with compounds such as methyl bromide and chloropicrin reduces seedling losses (23). However, fumigant use is being reduced due to environmental and human health concerns and prohibitive costs of reregistration. For example, methyl bromide will not be produced in or imported into the United States after the year 2005 as directed by the EPA, the 1990 Clean Air Act, and subsequent rulings. Production of alternate fumigants, Telone and Vorlex, containing the active ingredients dichloropropene/dichloropropane and methyl isothiocyanate, has been canceled in California (50). Alternative control measures are thus necessary to replace synthetic organic pesticides.

Plants of the order Capparales, especially agriculturally important *Brassica* spp. of the Brassicaceae, contain allelochemicals that reduce numbers of certain soilborne pathogens (11,34,36,37,44). These toxic effects are linked to the biologically active degradation products of glucosinolates. Glucosinolates themselves possess limited biological activity; however, enzymatic degradation by myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) results in the formation of a number of allelochemicals (9,12,47). More than 20 different aliphatic and aromatic isothiocyanates together with other potential allelochemicals have been identified among degradation products of glucosinolates originating from *Brassica napus*, *B. hirta*, *B. campestris*, *B. juncea*, *and B. nigra* (7,8,10,45). Each species has a unique glucosinolate profile resulting in correspondingly different isothiocyanates.

Walker et al. (49) first described inhibition of fungi by pure isothiocyanates, and it has since been shown that isothiocyanates are general biocides that are highly toxic to some fungi (17,18). Inhibition of mycelial growth of *Gaeumannomyces graminis* (1), *Leptosphaeria maculans* (32), *Rhizoctonia solani*, *Fusarium graminearum*, *Bipolaris sorokiniana*, and *Pythium irregulare* (39) in the presence of isothiocyanates has been observed. Others have reported the effect of isothiocyanates on germination of *Botrytis cinerea* (16) and *Glomus mossae* (48) spores, and sporangia of *Peronospora parasitica* (20).

Toxicity or inhibition of fungal growth by Brassicaceae tissues has been demonstrated and linked to volatile glucosinolate hydrolysis products such as isothiocyanates (26,31,40,43,44). It has been determined that fungi respond differently to specific isothiocyanates (26,29,39,43), but regarding the differential sensitivity of *F. oxysporum* growth stages to individual compounds, little is known. This makes it difficult to select a suitable green-manure crop that might be used to reduce pathogen populations. The aims of this study were to (i) determine the sensitivity of four *F. oxysporum* strains to different isothiocyanates; and (ii) evaluate the stage in fungal development that is most sensitive to these compounds. Our overall goal was to use laboratory bioassays to predict the most promising green-manure crop to control diseases in forest nurseries caused by *F. oxysporum*.

MATERIALS AND METHODS

Fungal cultures. *F. oxysporum* isolates were obtained from forest tree nurseries in Idaho and Washington: (i) 9051C = container-grown white pine (*Pinus monticola*); (ii) 9243G = container-grown Douglas-fir (*Pseudotsuga menziesii* var. *glauca*); (iii) 9312F = bareroot nursery soil; and (iv) 9321A = bareroot nursery soil. All isolates were shown to be aggressive pathogens in laboratory tests on young Douglas-fir seedlings (24) and stored in sterile, silt-loam soil (13). Fungi were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI).

Isothiocyanates. Pure chemical compounds were used in all experiments. Pro-

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variable. In the second experiment, although phenethyl isothiocyanate decreased the number of germinating chlamydospores compared to the control, this inhibition was never complete. Phenyl and butyl isothiocyanate reduced chlamydospore germination of *F. oxysporum* ($P < 0.05$) by 30 to 90% depending on the isolate tested.

DISCUSSION

Inhibition of mycelial growth as a result of exposure to isothiocyanates has been observed for a variety of fungi (1,30,39). Our work showed that isothiocyanates inhibited mycelial growth of *F. oxysporum*, but at exposure levels to which they were subjected in this study, the effects were predominantly fungistatic, not fungitoxic. Fungitoxicity could be induced only by increasing the concentration and respective vapor pressure of the most inhibitory isothiocyanate, and even then the results varied with the isolate. Sarwar et al. (39) similarly determined that some isothiocyanates are fungitoxic to *F. graminearum* after a 24-h exposure period, whereas others are only fungistatic.

Additional investigations demonstrate that volatile compounds released from glucosinolate-containing plants (26,31) also inhibit mycelial growth. Ramirez-Villapudua and Munnecke (38) indicate that growth inhibition occurs when subjecting *F. oxysporum* f. sp. *conglutinans* to gases evolved from decomposing cabbage residues. Radial growth was inhibited when the fungus was grown on petri plates suspended above cabbage-amended soil placed in closed containers. Mycelial growth resumed on transfer of the fungus to fresh PDA. In other studies, fungitoxicity to *Verticillium dahliae*, *V. albo-atrum*, *Pythium ultimum*, *Colletotrichum coccodes*, and *R. solani* grown on V8 juice agar was observed in the presence of gases evolved from *B. juncea* (31). Direct comparison of bioassays conducted using *Brassica* tissues to those conducted with specific isothiocyanates must be approached cautiously since tissues produce a variety of volatile inhibitory compounds (2,7,8) that may express additive or synergistic effects on the bioassay organism. This has led Bending and Lincoln (2) to suggest that other volatile S compounds are likely to be as important as isothiocyanates in pest control. In addition, dose response comparisons are not possible without accurate measurement of gas phase concentrations of the responsible toxicants.

The extent of mycelial growth inhibition varied among isolates depending on the specific isothiocyanate to which the respective isolate was subjected. Differences in mycelial growth inhibition of different isolates of the same species in the presence of volatile compounds produced by cold-pressed *B. juncea* seed meal has been observed for *G. graminis* var. *tritici*, *R. solani*, *P. irregulare*, and *Bipolaris sorokiniana* (26). Variability in susceptibility to the isothiocyanates used here is especially important given the fact that all isolates where obtained from forest nurseries. This variable response must thus be considered when testing the efficacy of control strategies involving the use of isothiocyanate-releasing plant tissues.

Propenyl and ethyl isothiocyanates were the most inhibitory of all tested isothiocyanates to mycelial growth of the four *F. oxysporum* isolates. Correlations of reduced fungal growth with the release of propenyl isothiocyanate from *B. juncea* plant tissues have been observed (26,31). Inhibition of mycelial growth of *G. graminis* by propenyl isothiocyanate has also been reported (1). Other investigators have demonstrated the inhibitory nature of additional isothiocyanates including 2-phenylethyl (15) and those containing sulfur side chains such as 3-methyl-sulfinylpropyl, 3-methylsulfonylpropyl, and 4-methylthiobutyl isothiocyanates (29) to *Fusarium* species. However, incorporating isothiocyanates into the media as performed in the latter investigations makes comparisons with our bioassays conducted with only volatiles tenuous. Sarwar et al. (39) showed that aromatic isothiocyanates were more toxic than aliphatic isothiocyanates when incorporated in agar, but less toxic in volatile form as a result of lower vapor pressures. This work indicates that increased predictive capabilities concerning toxicities of individual isothiocyanates can only be achieved by considering their respective partitioning in a three-phase soil environment.

Mycelial growth was inhibited to a relatively small extent and thus represents a reasonably resistant stage in the life cycle of *F. oxysporum* isolates used in this work. Likewise, conidial formation was little affected by those isothiocyanates tested. In contrast, conidial and chlamydospore germination were highly susceptible to inactivation by isothiocyanates. Thus these two stages in the life cycle of *F. oxysporum* are the most susceptible to inhibition by a green-manure treatment. Propenyl, ethyl, benzyl, and in most cases, phenethyl isothiocyanates completely suppressed conidial and chlamydospore germination. In contrast, the toxic effects of phenyl and butyl isothiocyanates, although significant compared to the control, were almost always less than other isothiocyanates. These results indicate that fungicidal activity of isothiocyanates is strongly determined by the nature of the molecular structure, but that activity is not correlated with aliphatic or aromatic character.

These results are consistent with those of Smolinska and Horbowicz (43) in which the investigators measured the release of propenyl isothiocyanate from *B. juncea* tissues and determined that a correlation exists between the presence of this compound in volatile form and the inhibition of germination of chlamydospores of *F. oxysporum* var. *radicis* f. sp. *lycopersici*. As in our work, the effect was nonreversible, and thus propenyl isothiocyanate appeared to be fungitoxic. In addition, *B. juncea* tissues have been shown to reduce *F. oxysporum* f. sp. *lycopersici* chlamydospore viability and lessen wilt of tomato disease symptoms (42). These observations are inconsistent with speculation of Greenhalgh and Mitchell (20) that activity of propenyl isothiocyanate toward *Phytopthora parasitica* mycelium should be more deleterious than to sporangia, because the first is an active and the second is an inactive stage in fungal development.

From these results, we conclude that decreased pathogen populations resulting from the direct inhibition of *F. oxysporum* mycelial growth seem unlikely. Changes in populations of these organisms resulting from a green-manure crop will only be achievable if conidia and chlamydospores are targeted. This is a feasible approach since *F. oxysporum* may exist almost exclusively as chlamydospores until stimulated to germinate by the root tip of a susceptible host. Incorporation of a green-manure crop prior to sowing conifers may reduce the number of germinating chlamydospores, thereby decreasing the likelihood of seedling infection. Attempts at controlling growth stages of the fungus following chlamydospore germination will be less effective.

Pathogenic *F. oxysporum* isolates typically infesting nursery soils in western North America will likely be most suppressed by species of plants containing

Table 2. Germination of *Fusarium oxysporum* chlamydospores in the presence of isothiocyanates (ITC)

	Isolate			
Treatment	**9051C**	**9321A**	**9312F**	**9243G**
Control	169.7 a[y]	54.0 a[z]	63.0 a	123.7 a
Propenyl ITC	0.0 c	0.0 c	0.0 c	0.0 d
Ethyl ITC	0.0 c	0.0 c	0.0 c	0.0 d
Butyl ITC	16.3 c	21.3 b	38.3 b	31.7 b
Phenethyl ITC	0.0 c	0.0 c	32.0 b	0.0 d
Benzyl ITC	3.0 c	0.0 c	0.7 c	2.7 d
Phenyl ITC	70.0 b	19.3 b	44.0 b	20.3 c

[y] All values represent CFU. Each value is the mean of three replicates.

[z] Numbers in the same column followed by the same letter are not statistically different ($P > 0.05$) according to LSD analysis.

glucosinolates that release the highest concentrations of propenyl isothiocyanate. Other glucosinolates releasing ethyl, benzyl, and phenethyl isothiocyanates should also contribute to potential suppression of *F. oxysporum*. Propenyl glucosinolate is found in high concentrations in shoots of three mustard species (*B. carinata*, *B. nigra*, and *B. juncea*) and phenylethyl glucosinolate is most commonly found at high concentrations in the roots of rapeseed or *B. napus* cultivars (25). Several weed species contain high concentrations of benzyl glucosinolate (25), whereas *Lepidium menziesii* is the only species in which ethyl glucosinolate has been identified (27).

It is important to note that only a fraction of the isothiocyanate potentially available from the measured molar quantity of glucosinolate within the tissues is actually released and available for pest inhibition. In laboratory studies, leaf and stem tissues of *B. napus* have been shown to release only 8% of the butenyl isothiocyanate theoretically possible as calculated from the tissue glucosinolate concentration (8). Field investigations conducted by measuring allelochemicals released in soil after a *B. napus* green-manure crop was incorporated showed that compounds derived from root tissues dominated the profile of measured compounds and only traces of shoot glucosinolate products were detected (19). More recent investigations indicate that physical methods are required to maximize the rupture of plant cell membranes and release isothiocyanate (33). Ensuring efficient isothiocyanate release from a green-manure crop is critical since organic carbon inputs without effective *F. oxysporum* control may serve to increase pathogen populations.

The first step in the use of green-manure crops to control pathogenic *F. oxysporum* populations in forest nursery soils is thus to choose plants with high concentrations of the appropriate glucosinolates. Release of the most inhibitory isothiocyanates at a time appropriate to target a susceptible growth stage of the fungus will increase the likelihood of pest suppression. Our work indicates that chlamydospores may be inhibited prior to the introduction of conifer seedlings if the release of isothiocyanate from the selected green-manure crop is maximized.

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