Indian Mustard and Allyl Isothiocyanate Inhibit Sclerotium rolfsii

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Additional index words. Brassica, southern blight, soilborne pathogen, biofumigation

Abstract. Allyl isothiocyanate (AITC) is the predominant isothiocyanate produced by damaged tissues of Indian mustard (Brassica juncea (L.) Czerniak). This study investigated Indian mustard and AITC mediated suppression of mycelial growth and sclerotial germination of Sclerotium rolfsii Saccardo, a common soilborne pathogen. Indian mustard (IM) treatments of 0, 0.1, 0.2, 0.6, 1.0, 2.0, 4.1, 5.1, 10.2, 20.4, 40.8, 81.6, and 163.3 g·L⁻¹ (weight of reconstituted mustard per liter of air) were evaluated for suppression of mycelial growth. Treatment effect was evaluated by measuring the radial growth of mycelia. Sclerotia were placed in culture tubes containing 18 g autoclaved soil and covered with an additional 5 g soil. AITC at concentrations of 0, 4.0, 16.0, 64.0, 256.0, 1024.0, or 4096.0 µmol·L⁻¹ was injected into the tubes. Treated sclerotia were removed from tubes and plated on potato dextrose agar to determine viability. Mycelial growth was inhibited with IM treatments (P < 0.01). Inhibiting concentrations (IC) of IM for mycelial growth inhibition of 50% and 90% were 0.7 and 1.0 g·L⁻¹, respectively, with death resulting with >2 g·L⁻¹. Inhibition attributable to AITC alone was lower than that achieved by IM producing equivalent amounts of AITC. Germination of sclerotia was negatively correlated with AITC concentration (r = 0.96; P < 0.01). The IC₅₀ and IC₉₀ of AITC were 249.0 and 528.8 µmol·L⁻¹, respectively, at 42 hours. The lethal concentration for sclerotia was not reached; only suppression occurred at the highest treatment concentrations. Sclerotium rolfsii mycelia were sensitive to the IM volatiles and were suppressed at low concentrations. Sclerotia were more resistant than the mycelia and required higher concentrations of AITC to suppress germination.

By 2005, methyl bromide will be banned for agricultural use, in accordance with the U.S. Clean Air Act and the Montreal Protocol (U.S. Dept. Agr., 1999). As the predominant soil fumigant for broad-spectrum control of weeds, nematodes, and soilborne pathogens, the loss of methyl bromide will have a dramatic and immediate impact on agriculture. Although alternative chemical controls are available, none provide the broad-spectrum results achieved with methyl bromide. The need for alternative methods of controlling these pests and increased interest in reducing synthetic chemical inputs has prompted an increase in research on cultural and biological disease control. Glucosinolates (GSL), found in Brassica species, are of particular interest because their volatile degradation products have biocidal activity.

The genus Brassica L. contains >37 species, which include food crops such as turnip (B. campestris L. (Rapifera Group), black mustard (B. nigra (L.) W.D.J. Koch), white mustard (B. hirta Moench), Chinese cabbage [B. rapa L. (Pekinesis Group)], and the diverse structural variants of B. oleracea L. including cabbage (Capita Group), broccoli (Botrytis Group) and cauliflower (Botrytis Group) (Ludford and Isenberg, 1994). This genus and all tested members of the Brassicaceae family have been reported to produce GSL.

Glucosinolates are organic anions containing β-D-thioglucose and sulfonated oxime moieties (Brown et al., 1994) derived from methionine, tryptophan, and phenylalanine (Underhill et al., 1973). When hydrolyzed by the enzyme myrosinase, GSL produce D-glucose, sulfate, isothiocyanates (volatile mustard oils), thiocyanates, and nitriles (Larsen, 1981; Poulton and Moller, 1993). Both GSL and myrosinase occur in cells throughout the plant, but are isolated from each other. They mix and react when cells are damaged (Chew, 1988).

The breakdown products of GSL are toxic to fungi (Charron and Sams, 1999; Mayton et al., 1996; Sarwar et al., 1998) bacteria (Delaquis and Mazza, 1995), nematodes (Mojtahedi et al., 1991, 1993), insects (Noble et al., 1999) and some weed seeds in laboratory experiments (Al-Khatib et al., 1997). The fungicidal properties of isothiocyanates (ITC) (Jenkins and Averre, 1986). The organochlorine fungicide pentachloronitrobenzene is suggested for control of southern blight in heavily infested tomato fields (University of Tennessee...
Agricultural Extension Service, 2000). Several newer fungicides, tebuconazole [α-2-(4-chlorophenethyl)α-(1-1-dimethylthyl)-1H-1,2,4-triazole-1-ethanol], flutolanil [N-[3-(1-methylethoxy)phenyl]-2-(trifluoromethyl) benzamide], and fluazinam [3-chloro-1,2,4-triazole-1-ethanol], are also effective for control of southern blight (Hagan and Olive, 1999). However, these fungicides do not control nonfungal pests and all are relatively expensive. There is also growing consumer concern over the use of chemicals in fruit and vegetable production, which may make the future of all of these chemicals questionable. These factors have stimulated interest in biologically based pest management such as biofumigation using *Brassica* sp.

The objective of this research was to determine, in vitro, the toxicity of the Indian mustard (*Brassica juncea*) volatiles and allyl isothiocyanates (AITC) to *S. rolfsii*. Specifically, we examined (1) inhibition of mycelial growth by Indian mustard, (2) AITC released by Indian mustard, and (3) suppression of sclerotia germination by AITC.

Materials and Methods

**Plant material and general procedures.** During Fall 1998, Indian mustard (PI 458934; U.S. Dept. Agr./Agr. Res. Serv., Ames, Iowa) was grown at the University of Tennessee, Plant Science Unit of the Knoxville Experiment Station, Knoxville, Tenn. Whole Indian mustard plants were harvested at anthesis. Plants were lyophilized (Labconco, Kansas City, Mo.) and ground to pass a 20-mesh (0.84-mm) screen. Plant tissue was homogenized to reduce sampling error associated with the small amounts of residues needed for the experiments. Freeze-dried Indian mustard (FDM) was stored over silica desiccant until used. The volatiles of interest produced from FDM are similar to those produced by fresh Indian mustard (Price, 1999).

An isolate of *S. rolfsii* was collected from tomato (*Lycopersicon esculentum* L.) grown in Fall 1999 at the Knoxville (Tenn.) Experiment Station. The isolate was cultured on potato dextrose agar (PDA) and was maintained in the laboratory. Sclerotia used in this experiment were grown on PDA. Plugs from an actively growing culture were placed on fresh PDA petri plates and placed in an incubator (Kelvinator Commercial Products, Manitowoc, Wis.) at 30 °C. Plates were not sealed and sclerotial formation on PDA was triggered by desiccation at 20% relative humidity (RH). Formation occurred within 2 weeks and sclerotia were air dried 2 weeks at 30% RH. Sclerotia formed in this manner appeared more similar to soil formed sclerotia in size, texture, color, and germination rate than sclerotia formed on sealed plates (unpublished data).

**Inhibition of mycelial growth by Indian mustard.** Freeze-dried Indian mustard and water were mixed in 490-mL glass jars, which were then sealed with Teflon lids with septa. Treatments of 0.0, 0.05, 0.10, 0.30, 0.50, 1.0, 2.0, 2.5, and 5.0 g of reconstituted FDM were selected for analysis. AITC dilutions (in ethanol) for the standard curve were made from AITC standard (Sigma-Aldrich Corp, St. Louis). Jars were incubated at 30 °C for 15 min before sampling for 1 min with a 100 µm polydimethylsiloxane Solid Phase MicroExtraction (SPME) fiber (Supelco, St. Louis). The fiber was placed in the injection port of a Hewlett Packard 5890 gas chromatograph (GC) equipped with a Hewlett Packard 5972 mass selective (MS) detector to desorb for 1 min. The column was an Alltech EC Wax 30 × 0.25 × 0.25 (Alltech Associates, Inc., Deerfield, Ill.). The inlet and outlet temperatures were 200 and 280 °C, respectively. The oven parameters were programmed at 60 °C for 1 min, and then increased by 5 °C per min to a maximum of 150 °C. Detector response was quantified based on the equation for the AITC standard curve.

This experiment used a completely randomized design with four replications. Linear regression was used to produce the equations for quantifying AITC production and for describing the relationship between AITC release and FDM (Sigma Plot, 2000).

**Suppression of sclerotia germination by AITC.** Sclerotia were grown as described previously, collected, air-dried, and screened to uniformity (≥1 and <2 mm diameter). Five sclerotia were selected and placed on a 2-cm square of 0.2-mm polyester mesh. The mesh was then tied up around the five sclerotia. For each treatment, a healthy plug from FDM are similar to those produced by fresh Indian mustard (Price, 1999).

A completely randomized design was used for an experiment with four replications of the Indian mustard treatments and three replications of the AITC dilutions. To examine the dosage effect, a nonlinear regression of the percentage inhibition was performed. From the sigmoid equation $Y = a/(1 + e^{-B/C})$, the concentrations of FDM and AITC to produce 50% and 90% inhibition (IC50 and IC90) were calculated (SigmaPlot, 2000).

**Determination of AITC released by Indian mustard.** Freeze-dried Indian mustard and water were mixed in 490-mL glass jars, which were then sealed with Teflon lids with septa. Treatments of 0.0, 0.05, 0.10, 0.30, 0.50, 1.0, 2.0, 2.5, and 5.0 g of reconstituted FDM were selected for analysis. AITC dilutions (in ethanol) for the standard curve were made from AITC standard (Sigma-Aldrich Corp, St. Louis). Jars were incubated at 30 °C for 15 min before sampling for 1 min with a 100 µm polydimethylsiloxane Solid Phase MicroExtraction (SPME) fiber (Supelco, St. Louis). The fiber was placed in the injection port of a Hewlett Packard 5890 gas chromatograph (GC) equipped with a Hewlett Packard 5972 mass selective (MS) detector to desorb for 1 min. The column was an Alltech EC Wax 30 × 0.25 × 0.25 (Alltech Associates, Inc., Deerfield, Ill.). The inlet and outlet temperatures were 200 and 280 °C, respectively. The oven parameters were programmed at 60 °C for 1 min, and then increased by 5 °C per min to a maximum of 150 °C. Detector response was quantified based on the equation for the AITC standard curve.

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| Table 1. Radial growth ±SD and inhibition of *Sclerotium rolfsii* mycelia at 42 h after treatment with reconstituted Indian mustard residues. |
|-----------------|-----------------|-----------------|
| Indian mustard (g FW/L) | Radial growth of mycelia ±SD (mm) | Inhibition (%) |
| 0.00 | 21.4 ± 2.5 | 0.0 |
| 0.10 | 18.5 ± 2.8 | 12.4 |
| 0.20 | 17.0 ± 2.4 | 16.2 |
| 0.61 | 14.5 ± 6.0 | 29.6 |
| 1.02 | 1.4 ± 2.5 | 77.9 |
| >2.00 | 0.0 ± 0.0 | 100.0 |
| Nonlinear regression | ** | ** |

*Mean of four replications.*

**Analysis of dosage effect was performed using SigmaPlot nonlinear regression model ($Y = a/(1 + e^{-B/C})$). Dosages in excess of that needed to induce 100% inhibition were eliminated from the analysis.

**Significant at P ≤ 0.01.**
culture tube was packed with 15.60 ± 0.05 g of oven-dried clay loam soil (fine, kaolinitic, thermic, Typic Paleudult). The soil was brought to ∼60% field capacity with the addition of 2.4 g deionized water. Additional soil (≈5 g per tube) was weighed into a beaker and moistened similarly. The tubes, beaker, and soil therein were autoclaved to eliminate potential effects of antagonistic organisms.

Following autoclaving, sterile deionized water was added to the autoclaved soil (fine, kaolinitic, thermic, Typic Paleudult). The soil was brought to ≈field capacity. Additional soil (5 g per tube) was weighed into a beaker and moistened similarly. The tubes, beaker, and soil therein were autoclaved to eliminate potential effects of antagonistic organisms. Following autoclaving, sterile deionized water was added to the tubes and beaker to return them to their preautoclave weight (≈60% field capacity).

A mesh bag containing the sclerotia was placed on top of the soil in each tube and covered with 5 g of additional soil. Tubes were capped with septa. The treatments of AITC per headspace volume in each tube and covered with 5 g of additional soil. Tubes were incubated at 30 °C for 24 h and then checked for AITC concentration using GC–MS analysis was used to determine the concentration of AITC released from Indian mustard. A highly significant linear relationship existed (R² = 0.99; SE ± 0.11). The equation describing this relationship was: AITC (µmol·L⁻¹) = 0.41 × Indian mustard [g fresh weight (FW)/L] + 0.59.

Indian mustard treatments were more effective than equivalent AITC treatments (Fig. 3). AITC concentrations eliciting predicted levels of inhibition were translated to Indian mustard concentrations, based on the equations describing the relationships of AITC to mycelial inhibition and Indian mustard to AITC release. The second curve was constructed from this information to represent the inhibition of mycelial growth attributable to the AITC released by Indian mustard. A highly significant linear relationship existed (R² = 0.94; SE ± 0.63) produced the equation Y = 98.59/(1 + e⁻¹·(0.1034)/0.1034), from which an IC₅₀ and IC₉₀ of AITC at 1.6 and 4.5 µmol·L⁻¹, respectively, were calculated (Fig. 2).

Determination of AITC released by Indian mustard. Headspace sampling and GC–MS analysis was used to determine the concentration of AITC released from Indian mustard. A highly significant linear relationship existed (R² = 0.93; SE ± 0.01). The equation describing this relationship was: Indian mustard concentration (µmol·L⁻¹) = 0.41 × Indian mustard [g fresh weight (FW)/L] + 0.59.

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Suppression of sclerotia germination by AITC. The sclerotia of S. rolfsii were resistant to treatment with AITC. None of the
treatment levels was sufficient to kill sclerotia; however, suppression was achieved at high concentrations (Table 3). The equation, $Y = 95.54/(1 + e^{(0.5 - 3.589/103.81)})$, from the nonlinear regression of inhibition ($R^2 = 0.96; SE = 9.54$) was used to calculate the IC$_{50}$ and IC$_{90}$ of 249.0 and 528.8 $\mu$mol L$^{-1}$ AITC, respectively (Fig. 4). Suppressed sclerotia germinated 6 to 8 d after transfer to PDA.

**Discussion**

Volatile compounds released from 2.0 g Indian mustard into a headspace volume of 1 L (equaling AITC at 1.45 $\mu$mol L$^{-1}$) proved effective for lethal inhibition of *S. rolfsii* mycelial growth. Based on average soil porosity and Indian mustard production of leaf biomass at 12 t ha$^{-1}$ (Duke, 1984), the biomass needed for fumigation (approximately 1.68 t ha$^{-1}$) is achievable in field plantings. This is supported by the work of Chan and Close (1987) and Subbarao and Hubbard (1996). Their research with *Brassica* crop residues has demonstrated effective control of other pathogens in field settings.

Other application methods have also proven effective. Kirkegaard et al. (1996) were successful in suppressing soilborne cereal pathogens with mustard seed meal. The high glucosinolate meal was applied with wheat (*Triticum aestivum* L) seeds and fertilizer using a grain drill.

The predicted concentrations of Indian mustard necessary for inhibition of *S. rolfsii* mycelial growth were four times the concentrations actually observed, if AITC alone was responsible for the inhibition. This suggests that other chemicals released by the Indian mustard also play a role in its toxicity. AITC and other compounds acting together could be providing greater inhibition than either compound individually.

Use of AITC directly as a general soil fumigant has been suggested (Minuto et al., 1999). A similar compound, methyl isothiocyanates (MITC), is released from the commercial fumigant metam-sodium. AITC inhibited mycelial growth of *S. rolfsii* at concentrations in excess of 4.5 $\mu$mol L$^{-1}$. Inhibition of *S. rolfsii* sclerotial germination is more difficult to achieve with AITC than the inhibition of actively growing mycelia. At concentrations approaching 200 times those required to kill mycelia, sclerotia were only suppressed and recovered by day seven. MITC is sublethal at concentrations <620 $\mu$mol L$^{-1}$ soil airspace (based on 23 $\mu$g g$^{-1}$ soil (Hoynes et al., 1999) assuming an average soil bulk density of 1.5 g cm$^{-3}$). A higher concentration may prove toxic; however, applying higher rates is questionable, since the cost of application may negate any benefit due to increased yields. Because soil was sterilized for this study, the potential for antagonistic attack by other fungi or bacteria did not exist. In a natural soil environment, the AITC treatments might have weakened the sclerotia, making them more vulnerable to attack by antagonist (Lifshitz et al., 1983). This aspect will require further investigation. In addition, if sclerotia can be triggered to germinate before treatment, lower concentrations of AITC/mustard could provide adequate inhibition of both the saprophytic and newly emerged mycelium of *S. rolfsii*. Although not currently equivalent in total disease control, biofumigation offers growers an alternative to methyl bromide.

Other alternative methods for disease control have been successful under limited conditions. Fallow solarization is reported to reduce sclerotial survival and limit disease due to *S. rolfsii* (Chellem et al., 1997; Lewis and Fravel, 1996; Ristaino et al., 1996). The

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**Table 3. Radial growth ±SD and inhibition of *Sclerotium rolfsii* mycelia from germinating sclerotia at 42 h after treatment with different concentrations of allyl isothiocyanates (AITC).**

<table>
<thead>
<tr>
<th>AITC (µmol L$^{-1}$)</th>
<th>Radial growth of mycelia&lt;sup&gt;a&lt;/sup&gt; (mm)</th>
<th>Inhibition&lt;sup&gt;a&lt;/sup&gt; (%)</th>
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<tbody>
<tr>
<td>0.0</td>
<td>7.2 ± 2.0</td>
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</tr>
<tr>
<td>4.0</td>
<td>7.0 ± 2.7</td>
<td>6.4</td>
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<td>16.0</td>
<td>6.4 ± 2.6</td>
<td>26.9</td>
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<tr>
<td>64.0</td>
<td>7.5 ± 1.7</td>
<td>13.7</td>
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<tr>
<td>256.0</td>
<td>4.2 ± 3.8</td>
<td>50.0</td>
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<tr>
<td>1024.0</td>
<td>0.7 ± 0.7</td>
<td>91.5</td>
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<tr>
<td>4096.0</td>
<td>0.0 ± 0.0</td>
<td>100.0</td>
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<sup>a</sup>Means represent an average of four replications with five samples per replication.

<sup>b</sup>Analysis of dosage effect was performed using SigmaPlot nonlinear regression model ($Y = a/(1 + e^{(0.5 - 3.589/103.81)})$. Dosages in excess of that needed to induce 100% inhibition were eliminated from the analysis.

<sup>c</sup>Significant at P ≤ 0.01.

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**Fig. 4. Inhibition of mycelial growth from germinating sclerotia of *Sclerotium rolfsii* by known concentrations of allyl isothiocyanate (µmol L$^{-1}$).** The IC$_{50}$ and IC$_{90}$ (concentration resulting in 50% and 90% inhibition, respectively) were calculated from the equation produced by the nonlinear regression.
combination of solar heating and the introduction of antagonistic microbes, such as *Trichoderma harzianum* Rifai, resulted in less disease than either method alone (Lumsden and Papavizas, 1988; Ristaino et al., 1996). The efficacy of these methods is limited by climate, soil type, and pathogen isolate differences. If biofumigation is combined with other cultural practices, such as solarization, compost amendments and/or integrated use of pesticides, disease control may be further improved.

**Literature Cited**


