To the Graduate Council:

I am submitting herewith a dissertation written by Stephanie Gail Harvey entitled "Glucosinolates, Isothiocyanates and Biofumigation: a potential alternative method of controlling soilborne pests." I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plant and Soil Sciences.

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Glucosinolates, Isothiocyanates and Biofumigation: a potential alternative method of controlling soilborne pests

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for the Doctor of Philosophy Degree

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ABSTRACT

The systematic phase-out of methyl bromide in the United States has begun and will be complete by 2005. As the predominant soil fumigant, loss of MeBr will have a dramatic and immediate impact on agriculture. Viable alternative methods of controlling soilborne plant diseases are required. Glucosinolates (GS), found in *Brassica* spp. L. and other members of the Brassicaceae family, are naturally occurring secondary metabolites that exist in intact plant tissues. When tissue is damaged, myrosinase, normally physically isolated from GS, is released and catalyzes GS into the volatile isothiocyanates (ITC). These pungent chemicals have been shown to control some pests in laboratory experiments.

This study investigated, the application of *Brassica* spp. as a potential alternative for controlling soilborne pest. Three sets of experiments were conducted: 1) Indian mustard (*B. juncea*) and allyl ITC inhibition of *Sclerotium rolfsii* Sacc. mycecial growth were investigated., 2) In a three year study, *Brassica* spp. were used as cover crops and incorporated into soil (biofumigation) to determine effects on tomato fruit yield. 3) Intact GS from seed extracts were separated using capillary electrophoresis and individual ITC were assayed to determine their toxicity to *Botrytis cinerea* Pers.: Fr., *Penicillium expansum* Link, *S. rolfsii*, *Pythium myriotylum* Drechs., and *Agrobacterium tumefaciens* (Smith and Townsend) Conn.

The concentrations of Indian mustard needed to produce 50% and 90% inhibition (IC_{50} and IC_{90}) were 0.74 and 0.98 $g \cdot L^{-1}$ headspace volume. Allyl ITC

IC_{50} and IC_{90} were calculated at 1.6 and 4.5 $\mu mol \cdot L^{-1}$, respectively. The concentrations of Ally ITC produced by the Indian mustard were lower than could account for the total inhibition demonstrated by the Indian mustard.

For the 2000 crop, tomato plots biofumigated with fall raab cv. "Salade," demonstrated 40% increase in marketable fruit yield when compared to control plots ($P< 0.01$). Indian mustard biofumigated plots showed an 18.2 % increase in marketable fruit yield ($P< 0.01$).

The IC_{90} for methyl ITC, allyl ITC, phenethyl ITC, cheirolin, erucin and brassinin were calculated for each of the pathogens tested. Brassinin had the lowest IC_{90} value for *B. cinerea* at 26 μMoles mL^{-1}. Phenethyl ITC had the strongest level of inhibition against *P. myriotylum*, *S. rolfsii*, and *P. expansum* with IC_{90} values of 11-, 27-, and 22 26 μMoles mL^{-1}, respectively. *Agrobacterium tumefaciens* was less sensitive to the compounds tested. Erucin was projected to provide 90% inhibition at 126 26 μMoles mL^{-1}. However, only allyl ITC and phenethyl ITC achieved 90% inhibition within the tested concentration range, with IC_{90} values of 395- and 213 μMoles mL^{-1}, respectively.

While there is still a vast amount of research to be done in this area, the potential for *Brassica* spp. and other GS containing plants to be used to control soilborne pests is great. With the information discovered to date, biofumigation using these plants could provide organic and "green" growers with measurable control of some soil pests.

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Part I

INTRODUCTION

In 1994, under the province of the Clean Air Act, the Environmental Protection Agency (EPA) cited methyl bromide usage as a contributing factor to the depletion of the Earth's ozone layer. Immediate limits were placed on production and import of the chemical and provisions were made to halt all production and importation in 2001 (Ferguson and Padula, 1994). A brief reprieve was issued in 1998 when the ban was reset to coincide with a worldwide ban in 2005 (U.S. Department of Agriculture, 1999). As the predominant soil fumigant, loss of methyl bromide will have a dramatic and immediate impact on agriculture. The need for an alternative method of controlling soilborne plant pathogens has prompted an increase in research, much of which involves manipulation and application of biological mechanisms. Growing consumer concerns over chemical application in food crop production has added to the need for non-chemical alternatives that could be utilized in organic production.

Control of soilborne pathogens using non-pathogenic fungi and bacteria has produced favorable results in some applications (Gamliel and Katan, 1993; Quarles, 1993; Ristaino *et al.*, 1991). The limiting factor of many of these procedures is the inconsistency in which the antagonist becomes established. The reasons for the inconsistency are unknown, but are thought to be a combination of soil factors, including soil atmospheric composition, that vary from site to site (Kim and Misaghi, 1996).

Cultural practices also are being investigated. The importance of crop rotation is being re-emphasized as a mechanism for controlling inoculum densities. Selective crop rotation can deprive pathogens of a host and reduce inoculum potential. Solarization of soil also has been very effective against many pathogens. However, solarization has environmental limitations. Producers in areas with shorter growing seasons cannot afford to delay planting to implement this technique. In areas with low light intensity, soils do not reach temperatures necessary to eliminate pathogens (Himelrick and Dozier, 1991). Other physical controls have been studied but are impractical in most commercial settings (Baxter *et al.*, 1977). Grafting has been employed successfully in regions were the severity of bacterial wilt (*Ralstonia solanacearum*) would otherwise make tomato production impossible (Peregrine and Ahmad, 1982).

An area of growing interest is the utilization of glucosinolate degradation products such as isothiocyanates, as potential fumigants. Glucosinolates (GS), found in *Brassica* and other species, are naturally occurring secondary metabolites that exist in intact plant tissues. When tissue is damaged, myrosinase, normally isolated from GS, is released and catalyzes the conversion of GS into the volatile isothiocyanates. These pungent chemicals have been demonstrated to control fungi (Sarwar *et al.*, 1998; Mayton *et al.*, 1996), bacteria (Delaquis and Mazza, 1995), nematodes (Mojtahedi *et al.*, 1993 and 1991) and some weed seeds in laboratory experiments (Al-Khatib, *et al.*, 1997).

Tomato (*Lycopersicon esculentum* Mill.), an economically important crop in Tennessee, Florida, and South Carolina, depends heavily on methyl bromide. Often grown without rotations, tomato fields can develop high pathogen inoculum densities. Without chemical control of the pathogens, losses can be devastating. The ban of methyl bromide has been projected to produce an annual economic loss in excess of $350 million to tomato growers and consumers in the United States (Ferguson and Padula, 1994).

The objectives of this research were to determine the effectiveness of the application of *Brassica* species, as a green manure, under field conditions, in controlling the soilborne tomato pathogens, *Pythium ultimum* Trow and *Sclerotium rolfsii* Sacc. as well as its effect on the general microbial population. The effects of these treatments on disease ratings, fruit yield and quality will be evaluated. Treatment effectiveness on *S. rolfsii* will be evaluated in laboratory jar studies. In addition, individual glucosinolates will be isolated and assayed for lethality.

Part II

LITERATURE REVIEW

Brassicaceae

Brassicaceae, the mustard family, is large with over 3,000 species in about 350 genera and can be described taxonomically as follows: Kingdom Plantae; Subkingdom Tracheobionta (vascular plants); Superdivision Spermatophyta (seed plants); Division Magnoliophyta (flowering plants); Class Magnoliopsida (dicots); Subclass Dilleniidae; Order Capparales (USDA, NRCS, 2001). This herbaceous family includes annuals, perennials and biennials, ranging from important crop plants to weeds. The genus *Brassica* L. contains over 37 species, including 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. (Pekinensis 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). The genus also includes the rapeseed species, *B. napus* and *B. campestris,* grown for oil production (Downey and Robbelen, 1989). Other genera of agronomical and scientific importance include: *Raphanus* cultivated for its edible root, *Sinapis* grown for seeds used as condiments, leaves of *Eruca*, *Crambe Physorrhinchus* and some *Diplotaxis*, and used in salads in the Middle East and Pakistan, *Crambe strigosa* and *C. gigantean* cultivated as ornamentals, and lastly the vast number of weeds that belong to this family. This

genus and all tested members of the Brassicaceae family have been reported to produce GS.

Morphology and Morpho-taxonomy. Both inter- and intra-taxon variability in leaf shape has prevented its use as a taxonomic characteristic. Leaf morphology changes dramatically as plants move from early juvenile to adult stages. In general, the first two or three juvenile leaves are simple and very slightly lobed. The higher their position on the shoot, the more complicated the leaves become until mature foliage is reached. Leaf shape in Brassicaceae can be divided into four basic shapes: 1) simple, entire to shallowly lobed, 2) lobed to partite, with sinuses nor reaching the mid-vein, 3) divided, with sinuses reaching the mid-vein, and 4) lyrate, due to reduced number of segments. While many genera can be described as having one or two primary shapes, Brassica leaves exist in a vast array of shapes and sizes and thus provide little aid in classification. Trichomes, when present, are very simple and their absence or presence is a good taxonomic characteristic. For example, wild populations of *Brassica oleracea,* can be separated from *B. nigra* and *B. campestris* as the first is completely glabrous while the other two are hairy. Crosses between *B. oleracea* and hairy species produce slightly hair diploids.

Floral and fruiting structures of this large and diverse family are distinctive. The flowers are perfect and usually actinomorphic with the perianth consisting of a calyx of four distinct sepals and a corolla of four distinct petals (Fig. II-1). The

petals are typically positioned opposite one another and appear to form a cross from which the old family name Cruciferae or "cross-forming" was derived. The inflorescence is a standard raceme. The androecium consists of four long inner stamens and two short outer stamens and is referred to as tetradynamous. The gynoecium consists of two carpels that are generally separated by a persistent false partition called a replum. At maturity, the two valves of the fruit, called siliques, separate leaving the ovules attached to the persistent replum.

While the flower structure of Brassicaceae is distinct from other families, within the family it appears very homogeneous. However, close examination can reveal differences among genera and among species. Sepals may vary in color, size position with respect to the flower axis, pilosity and the presence or absence of basal, dorsal or apical swellings. Petal venation, color, size and shape may also vary.

The cotyledons of Brassicaceae seed are longitudinally folded around the radical in a position known as conduplicated or orthoploceae. This characteristic is often used in taxonomic identification. The average weight of the seeds of the Brassicaceae are approximately 1 mg, with the *Crambe* species having some of the largest at 10-15 mg and the *Diplotaxis* to no more than 0.05 or 0.1 mg. Most *Brassica* species range from one to five milligrams. The color of mature seeds varies from yellow to black. There appears to be a correlation between seed color and geographical position. Mediterranean species usually exhibit ochre or brown

colors while species from the Euro-Siberian region typically have dark brown to black seeds. When moistened, many Brassicaceae seed produce mucilage from the external cell layer of the seed coat. This may be an adaptation to endure temporal droughts during the early stages of germination. Mucilage production in dehiscent species may be of taxonomic interest due its genetic distribution. For example, it is absent in *Brassica nirgra, B. oleracea, B. rapa* and in all the components of the so-called "U-triangle" (a digram depicting the genomic relationships among some Brassica spp). In contrast, mucilage is produced in *B. fruticulosa, B. maurorum* and *B. spinescens* (Tsunoda *et al.*, 1980).

Glucosinolates and Isothiocyanates.

Glucosinolates (Fig. II-2) are organic anions containing ß-D-thioglucose and sulfonated oxime moieties (Brown *et al.*, 1994). They have been found in only 15 dicotyledonous families, with the largest groups being the Brassicaceae and Euphorbiaceae. Found in cell vacuoles throughout the plant, these compounds account for less than 0.1% of the fresh weight in intact plants. Glucosinolates are produced from amino acids (Fig. II-3).

Glucosinolate Biosynthesis. Biosynthesis of GS begins with the biosynthesis of the amino acids. Methionine, precursor for the straight chain GS, is synthesized from intermediates of the tricarboxylic acid cycle. Phenylalanine and tyrosine are utilized in the production of the aromatic GS, while tryptophan is the progenitor of

indole GS (Underhill *et al.*, 1973). These three amino acids are products of the shikimate pathway, using intermediates from glycolysis and the pentose phosphate pathway.

The production of GS can be divided into three stages: amino acid chain-elongation, synthesis of glucosinolate from the amino acid (or chain-elongated derivative), and side-chain modification. The chain elongation step was first proposed in the mid 1960's as a result of *in vivo* feeding studies (Chisholm and Wetter, 1964). While the side chain of many GS is structurally similar to amino acids, it was obvious that the pathway was not a direct incorporation. Many of the GS require the incorporation of amino acid derivates augmented with multiple methyl residues. The proposed pathway for this elongation begins with the deamination of methionine to produce an α-keto-acid (Fig. II-4). The condensation of an acetyl-CoA with 2-keto-acid adds two carbons to the skeleton to produce 2-alkylmalic acid, which subsequently undergoes an isomerization to produce 3-alkylmalic acid. One carbon is then lost through oxidative carboxylation to yield a new 2-keto-acid with an additional methylene group. Transamination can then occur to produce a chain-elongated amino acid or the elongation may continue to produce the skeletons that form the base structure of GS (Graser *et al.*, 2000).

Formation of the glucosinolate begins with the conversion of the chain-elongated amino acid to a corresponding oxime in a reaction catalyzed primarily

by a flavin-containing monooxygenase (Fig. II-5). The reactions from oxime to thiohydroximate are poorly understood but are thought to involve an intermediate of aci-nitro, which is then converted to S-alkythiohydroximate with an addition of cysteine. A CS-lyase may then cleave the S-alkythiohydroximate to yield the thiohydroximate. The UDPG:thiohydroximate glucosyltransferase is responsible for the glucosylation of the thiohydroximate to the desulfoglucosinolate. A sulfotransferase transfers sulfur from 2'phosphoadenosine 5-phosphosulfate to the desulfoglucosinolate to produce the glucosinolate.

Once formed, the side-chain of the GS can undergo modifications such as desaturation and hydroxylation. The initial oxidation of methylthioalkyl glucosinolate to methylsulphinaylalkyl is followed by the removal of the methylsulphinyl group and desaturation. This results in the alkenyl glucosinolates; a subsequent hydroxylation produces hydroxylalkenyl glucosinolates. This alone cannot account for the variety of GS seen in plants; within each step of this pathway, there are many opportunities for variation. While a few specific biochemical steps have been studied (Rossiter *et al.*, 1990), relatively little is known about these modifications.

Glucosinolate Location. Glucosinolates have been found in all plant tissues of the Brassicaceae. In broccoli, seed total GS concentrations may be in the range of 70-100 μmol g^{-1} (fresh weight), while the vegetative tissues of a mature plant may have concentrations as low as 1-4 μmol g/fresh weight (Fahey *et al.*, 1997).

In rapeseed, the floral components have a significantly higher concentration of GS than do the vegetative portions of the plant. The GS concentration in the seed is equally high. As mentioned earlier, the anti-nutritional aspects of GS in rapeseed meal, has limited its potential in animal fodders. After Lein's (1972) grafting experiment demonstrated that the siliques were the major site of GS synthesis, several research groups developed feeding studies to determine where GS synthesis was taking place. The majority of their work lead to the assumption that GS synthesis did take place in the sidewalls of the siliques and GS were then transported into the seeds. Further work by Du and Halkier (1998) however, demonstrated that isolated seed had the necessary enzymes to transform amino acids into GS. While rapeseed can biosynthesize GS, the amount is relatively small compared to the amount transported from siliques. Chen *et al.* (2001) reported a long-distance phloem transport of GS in arabidopsis observed using radiolabeled GS. Thus, glucosinolates synthesized in mature leaves are loaded into and transported by phloem by the osmotically driven translocation stream from the source to sink.

The location of myrosinase relative to GS has been debated for many years. Myrosinase was believed to be located in myrosin grains within myrosin cells, but this raised questions about the interaction between the enzyme and substrate. Antibodies against myrosinase and sinigrin (a GS) were used to determine their location. Recently, cells containing extraordinary high concentrations of

glucosinolates were found situated between the phloem of vascular bundle and the endodermis. Myrosin cells were detected adjacent to these cells, allowing for the enzyme's transport via plasmodesmata (Koroleva *et al.*, 2000).

Concentrations of GS vary with environmental factors. Soil nutrients such as nitrogen, sulfur (Josefsson, 1970), and boron (Kennedy, 1994) have been shown to affect GS levels. Water stress (Kocourkova, 1997), light intensity (Rosa and Rodrigues, 1998), and infection by pathogens (Siemens and Mitchell-Olds, 1998) also impact GS concentrations.

Glucosinolate Degradation. Glucosinolates are hydrolyzed by an enzyme, myrosinase (MYR) (β-thioglucosidase, EC 3.2.3.1), to produce D-glucose, sulfate, and one of several nitrogen containing compounds such as isothiocyanates (volatile mustard oils), thiocyanates and nitriles (Fig. II-6) (Larsen, 1981; Poulton and Moller, 1993; Wilkinson *et al.*, 1984). Both GS and MYR occur in cells throughout the plant, but are isolated from each other. Several theories exist on how this isolation is achieved. One theory suggests that the GS are restricted to the plant cell vacuoles and the MYR bound to cellular membranes. A second and less supported theory proposes a chemical separation in which the MYR it inactivated due to pH. The location of MYR has been debated also. Electron micrographs (Bones,1990) reveal the presence of grainy idioblasts within certain cells, called myrosin cells. Their enzymatic activity was demonstrated using immunocytochemical techniques (Thangstad *et al.*, 1990). In addition, activity has

been detected in the cytoplasm of other cells. Regardless of their reported localization, substrate and enzyme mix and react when tissues are damaged (Chew, 1988). The release of the biotoxic breakdown products in response to attack by bacterial and fungal pathogens as well as non-adapted insects and other herbivore grazing has bolstered the theory that GS/MYR system is a plant defense mechanism (Rask *et al.*, 2000; Siemens and Mitchell-Olds, 1998).

As reported earlier, MYR is a β-thioglucosidase. Its amino acid sequence strongly resembles that of the glycosylhydrolase family [EC 3.2.1±3.2.3]. A hydroxyl group at C-2 on the glucose moiety is required for glucosinolate/ enzyme binding. Following hydrolytic cleavage of the β-glucosyl moiety, there is a nonenzymatic release of sulfate to form an unstable intermediate. The intermediate spontaneously rearranges to form isothiocyanates, or other breakdown products depending upon the glucosinolate substrate and reaction conditions (Mithen *et al.*, 2000). Isothiocyanates are the predominate breakdown product under physiological conditions (pH >7). Nitriles will dominant in acidic conditions where pH <4 (Agerbirk *et al.*, 1998; Borek *et al.*, 1995). The substrates 2 propenyl-, benzyl- and 4-(methylthiol)butyl glucosinolate form thiocyanates, while substrates with a β-hydroxylated sidechain will result in oxazolidine-2thiones due to a spontaneous cyclization. Glucosinolates with a terminal double bond will result in epithionitriles if an epithiospecific protein and Fe^{2+} ions are present (Halkier and Du, 1997). The concentration of ITC produced also varies with environmental

factors. Soil texture, temperature, microbial community, and pH can significantly affect the conversion of GS to ITC (Price, 1999).

Myrosinase exists in multiple forms, with varying kinentics, even within the same plant (James and Rossiter, 1991). In addition to being found in plants, MYR is found in fungi (Ohtsuru and Hata, 1972) and bacteria. Myrosinase activity has been associated with some of the animal intestinal gut fauna. Large variations in MYR specific activity have been reported in various cruciferous plant sources based on its purification and characterization from several sources, including *Sinapis alba* (Palmieri *et al.*, 1986), *Lepidium sativum* (Durham and Poulton, 1989), *Brassica juncea* (Ohtsuru and Hata, 1972), *Brassica napus* (Lonnerda and Janson, 1973), and *Wasabia japonica* (Ohtsuru and Kawatani, 1979).

Isothiocyanates. Isothiocyanates (ITC) are one group of chemicals produced in the breakdown of GS. While the GS/MYR reaction requires the presence of water for the breakdown of GS, in aqueous solutions (pH $\geq$ 7) the ITC react with water molecules and OH^- ions. Decomposition in water is temperature dependent with rapid decomposition at 37 °C. As temperatures approach 0 °C decomposition slows and stops at –5 °C. Isothiocyanates are stable in most organic solvents such as hexane and are unaffected by light. Stability can be increased with the addition of citric acid, sugar esters, and salad oil. Mixed with medium chain oils, stability can be achieved at room temperature if oxygen is excluded. These volatile chemicals can permeate polyethylene and polypropylene

films and can deteriorate many softer plastics. Nylon, Teflon, and polyolefin resins are resistant to this corrosive activity (Depree *et al.*, 1999).

Medicinal properties of the Brassica species have been asserted for over 1000 years (Downey and Robbelen, 1989). Today, the potential health benefits and other pharmaceutical uses has become the focus of many research organizations. The majority of the activity associated with GS tissue is actually due to the production of ITC. Aggregation of platelet mediated by arachidonic acid, is inhibited by 6-methylsulphinylhexyl isothiocyanates (Kumagai *et al.*, 1994) through specific inhibition of the arachidonic acid cascade. Work with bronchial obstruction, indicates that isothiocyanates prevent these obstructions not by blocking the action of histamine or acetylcholine, but by inhibition of the inflammatory process at an early stage, such as the production of thromboxanes or prostaglandins.

In recent years, the chemoprotective and anticancer properties of GS and their corresponding ITC have received much interest (McDanell, *et al.*, 1988). Isothiocyanates activate phase II enzymes, which play a major role in protecting tissue from malignancy by destroying carcinogens or by making them inert (Fahey *et al.*, 1997). The ITC sulforaphane, extracted from broccoli, has been shown to induce several phase 2 enzymes and blocks dimethylbenz(a)anthracene-initiated mammary tumor formation in rats (Zhang *et al.*, 1994) and inhibits neoplastic nodule formation in mouse mammary glands (Gerhauser *et al.*, 1997). These

chemicals also have phytotoxic, nematocidal, fungicidal, and insecticidal properties that have focused research into other areas of science (Borek *et al.*, 1995; Charron and Sams, 2000; Delaquis and Mazza, 1995; Harvey *et al.*, 2002; Vaughn and Boydston, 1997).

The fungicidal properties of ITC have been reported since 1937 (Walker *et al.*, 1937). The chemical concentration necessary to demonstrate control of a given fungus varies with specific ITC. The type of ITC produced depends on the side chains of the glucosinolate from which it is derived. For example, methyl-ITC results from the hydrolysis of glucocapparin, while 2-propenyl forms result from sinigrin (Sarwar *et al.*, 1998).

Pathogens

Sclerotium rolfsii Saccardo. *Sclerotium rolfsii* is a soilborne pathogen with an extensive host range of more than 500 plant species, including both dicotyledons and monocotyledons (Singleton *et al.*, 1992). The fungus causes "Southern Blight" (seedling damping-off, blight and stem rot) throughout the tropics, subtropics and in areas of the southern and southeastern United States where high temperatures permit its growth and survival. Despite over a hundred years of near continuous research, *S. rolfsii* continues to trouble growers and cause extensive economic loss (Punja, 1985).

Sclerotium rolfsii is one of a number of plant pathogenic fungi that form resistant, over-wintering survival structures called sclerotia. Sclerotia are typically brown to tan in color and measure 0.5-mm to 2.0-mm in diameter. A sclerotium is comprised of a two to four cell-layers thick, melanized outer layer, a middle cortex and innermost medulla of loosely arranged filamentous hyphae (Chet *et al.*, 1969).

Infection of the host plants by *S. rolfsii* is preceded by an accumulation of mycelium at the host surface. Oxalic acid and polygalacturonases cause tissue death prior to hyphal penetration. The cell wall is weakened as oxalic acid sequesters calcium to produce calcium oxalates (Punja and Jenkins, 1984). In addition, this lowers the tissue pH to the optimum for endopolygalacturonase and cellulase activity. The necrotic and macerated appearance of infected tissues occurs as pectic substances in the cell walls are depleted in advance of the growing hyphae (Punja, 1985).

Temperature and moisture fluctuations may increase disease incidence due to *S. rolfsii*. Punja and Grogan (1981) reported that cycles of drying and wetting stimulate germination of sclerotia. Disease severity may also be enhanced by the presence of organic substrates for mycelial growth, (such as post-harvest litter or amendments), increasing host/inoculum contact by cultural practices (such as hilling soil around host) and decreasing populations of antagonistic soil microflora through pesticide use. Unlike many other diseases, stress factors do not appear to predispose plants to infection by *S. rolfsii* (Punja, 1985).

Control of *S. rolfsii* can be accomplished by soil fumigation with methyl bromide, chloropicrin and metham-sodium in preplant application (Jenkins and Averre, 1986). However, the future availability of all these chemicals is questionable. The use of ammonium fertilizers such as urea and ammonium bicarbonate lowers disease incidence and could be used to moderate the disease (Punja and Grogan, 1982; Thakur and Mukhopadhyay, 1972). The exact mechanism of action is not known but it is reported to directly inhibit sclerotial germination and retard mycelial growth. In addtion, increased calcium levels in plant tissue, following the application of calcium fertilizers such as calcium nitrate, may provide some level of control as host tissue cell walls have more calcium which may offset the oxalic acid effect (Punja *et al.*, 1986).

Current research is focused on alternative control measures. Solar heating of moistened soils under polyethylene tarp is reported to reduce both sclerotial number and limit disease due to *S. rolfsii* (Chellemi *et al.*, 1997; Lewis and Fravel, 1996). The combination of solar heating and the introduction of *Trichoderma harzianum* and *T. virens,* antagonistic fungi, resulted in less disease than either method alone. These methods tend to be limited in their use due to climatic, soil, and isolate differences.

Pythium spp. The genus *Pythium*, refers to fungal-like, oomycetous organisms, that are classified within the kingdom Chromista based on evolutionary lines and are more closely related to diatoms and brown algae than to fungi.

Many members of this genus are plant pathogens and can cause significant economic crop loss. The genus has a broad host range and a worldwide distribution (Plaats-Niterink, 1981).

The most common species of *Pythium* that cause agronomic problems are *P. myriotylum, P. aphanidermatum* and *P. ultimum* Trow. Both *P. myriotylum* and *P. aphanidermatum* dominate in the southern United States because they are adapted to higher soil temperatures. Their optimum temperature for growth and infection of plants ranges between 30 and 37 °C but they will occur from 5 to 40 °C. In contrast, *P. ultimum* prevails in cooler to cold soil with an optimum growth range of 77 to 86 °C.

Reproduction of *Pythium* spp. occurs through growth of hyphae and the production of four types of spores; sporangia, zoospores, oopsores and chlamydospores. Chlyamydospores form as thickened segments of hyphae, but they are not considered important to the disease cycle of most of the pathogenic species of *Pythium*. Sporangia germinate and form new hyphae but typically only affect the disease cycle by production of vesicles in which zoospores are formed.

Zoospores are short-lived flagellated spores produced in vesicles attached to sporangia. Water is necessary for production of sporangia, vesicles, and zoospores. Zoospores can swim for 20 to 30 hours and move three or more inches, against gravity, through soil. Moving water, such as rain runoff, can spread

the spores further. This is the most rapid method by which *Pythium* spp. spread throughout a field.

Oospores provide an effective survival mechanism for *Pythium* spp. because oospores typically have thickened walls and can survive in old crop debris such as undecomposed roots and stems. Because they are formed after mating of male and female portions of the thallus, they also provide the opportunity for genetic diversity. Oospores are extremely durable, surviving for more than 10 years in infested soil and passage through the intestinal tracts of some organisms (Hoope, 1966).

Pythium can cause both pre- and post-emergence damping-off. The taproots, root tips and feeder roots of mature plants may be attacked, stunting plants and reducing yields (Schlub and Lockwood, 1981). Zoospores are responsible for most infections and due to the extremely high numbers, at which they are produced, can quickly decimate a crop.

The quantity of propagules needed to produce disease incidence is generally thought to be less than 100 propagules per gram (ppg) of soil. Schlub and Lockwood (1981) found that as few as five ppg would significantly reduce seedling emergence and that no emergence occurred with 25 ppg. They also proposed that populations in natural soil must be approximately 100 times greater than in artificially infested soil in order to result in the same amount of disease. The existence of nonpathogenic isolates and the suppressive nature of some microbial

environments probably accounts for the need for increased inoculum concentrations.

Areas infested with *Pythium* are usually scattered throughout a field and do not form a pattern. *Pythium* can be found typically in the upper 30 cm of soil usually on old plant debris. The fungi commonly exist in ponds, lakes, and other sources of standing water and are frequently the sources of inocula for pathogenic species.

Infection occurs when germ tubes from zoospores penetrate the root or other tissues of a susceptible host. Colonization of plant tissue by *P. aphanidermatum* is assisted by the ability of this organism to produce enzymes that breakdown the pectins and cellulose of the host. A soft, mushy rot may develop in untreated seeds before the radical emerges (Jones *et al.*, 1991). Initial root symptoms develop in one to three weeks after planting as elongated water-soaked areas on the hypocotyl and roots. As the disease progresses the outer tissue of the stem becomes slimy. Roots will ultimately become mummified and turn a tan to brown color. Water logged regions on the infected seedlings or plants may extend several inches above the soil line with little if any visible fungal growth. Severely infected plants commonly wilt and die. Less severe root-attacks may result in stunted growth and reduced yield. Fruit that contacts moist soil may become infected and exhibit a watery soft rot and white fungal mycelia.

Inoculum density, soil water content, temperature, pH, light intensity, cation content and the presence of other microbes can affect infection rates (Hancock, 1981; Plaats-Niterink, 1981). Severity increases when conditions are less than ideal for the plant and more ideal for the fungus. Infection mostly takes place on the young roots where roots exudates can cause the accumulation of zoospores. Environmental factors can influence the development of Pythium diseases. As with most soilborne pathogens, soil conditions influence the aggressiveness of the pathogen. *Pythium* thrives in cool, moist soils at temperatures below 23°C. Soils with high organic content or high soil compaction also favor *Pythium*.

Residual populations of *Pythium* can be reduced by cultural practices such as three-year crop rotations with less susceptible crops including alfalfa, barley, wheat, oats, and corn. Fall plowing, verses disking, also reduces populations. Seeds should be planted in warm, well drained, but moist soils that promote rapid germination and emergence to minimize the window of susceptibility. The vigor and stress of a plant play a major role in its susceptibility to *Pythium*.

Deep placement of seeds should be avoided, minimizing seed germination time. Suppression of nematodes and insects pest in soils can also suppress disease by decreasing the number of entry points (wounds) for pathogens. High-density plantings should also be avoided, as they limit aeration and reduce drying within the canopy. This creates an ideal habitat for *Pythium* spp.

Chemicals are used in most agricultural systems to manage *Pythium*. , Telone C-17®, Vorlex®, Chloropicrin® and Vapam® are a few of the possible treatments for root rots and damping off. Seed treatments of Apron® and Ridomil® are effective as well (Averre, 2000). The effectiveness of these pesticides can be lost when environmental or cultural conditions become too adverse for proper plant development.

Beneficial Microbial Agents. The method of action for microbial biological controls can be direct antagonism, such as competition, antibiotic production, and mycoparasitism, or indirect inducement of plant resistance. A number of biocontrol products are commercially available for the control of plant pathogens. Bacterial organisms such as *Agrobacterium radiobacter* (Galltrol® and Nogall®), *Burkholderia cepacia* (Deny® and Intercept®) and some species of *Bacillus* (Companion®, Kodiak®, Serenade® and YieldShield®) and *Pseudomonas* (BioJect Spot-Less®, Bio-save®, BlightBan® and Cedomon®) are register as biocontrols. Several fungi also have been registered as biocontrols. *Trichoderma harzianum*, a biological-control fungus used for years, is one of the organisms in research. Development of new strains may solve problems associated with the establishment of the antagonist (Quarles, 1993). *Trichoderma* biocontrol agents for soil pathogens are one of the few biological controls commercially available to date (Binab-T® from Binab Bioinovation, Sweden and F-Stop® from G. Harman, Cornell University Agricultural Exp. Sta, Geneva, NY). *Trichoderma virens* has been closely examined as a

biological control agent for protection against damping-off caused by *Pythium* spp. and *Rhizoctonia solani* Kuhn. Other fungal biocontrols include *Ampelomyces quisaqualis* (AQ10), *Candida oleophila* (Aspire), and *Coniothyrium minitans* (Contans WG/Intercept WG) (McSpadden Gardener and Fravel, 2002).

Control may be achieved either directly or indirectly. Biocontrol agents may directly attack and kill pathogens or they may suppress disease by competing for limited resources. Solarization, in combination with these agents improves control. The high temperatures achieved with solarization kill many pathogens and appear to stimulate some beneficial microbes such as the fluorescent pseudomonads (Ristaino *et al.*, 1991). Additionally, it may quicken the decomposition of potential host debris in the soil, thus limiting survival of some pathogens.

Capillary Electorphoresis

Traditional methods for chemical separation of compounds have relied heavily on gas chromatograph (GC) and high performance liquid chromatograph (HPLC). With the limitations associated with GC analysis (e.g. volatility requirement) and the large solvent volume used in HPLC, other separation methods have been investigated. Electrophoresis, long associated with slab gels for separation of proteins and nucleic material, has evolved into an analytical tool with a very promising future.

Electrophoresis refers to the migration of charged electrical species in an electrolyte through which an electric current is passed. Anions are attracted toward the positively charged electrode (anode) and cations migrate toward the negatively charged electrode (cathode). Neutral solutes lack any inherent electorkinetic mobility as they are not attracted to either electrode. Traditional electrophoresis equipment requires the used of relatively low voltages due to excessive heat buildup, which leads to long analysis times. Another limitation was the lower level of automation. Following separation, detection of the separated bands required post-separation visualization techniques (Frazier *et al.*, 2000).

Capillary electrophoresis (CE) has several advantages that have lead to its adoption in many research and industry settings. Separations with CE can be accomplished with automated analytical equipment, with fast analysis times and on-line detection of the separated peaks. Unlike the slab gels and paper substrates used in conventional electrophoresis, the capillary effectively dissipates the heat generated under high voltage conditions and allows for rapid separation of components. In addition, the use of an in-line detector can allow on-capillary detection, thus eliminating additional visualization steps.

A CE system consists of a capillary, two buffer reservoirs, high voltage source, detector, and integrator (Fig. II-7). The capillary is typically uncoated fused silica, 25 to 100 cm in length and with an interior diameter of 0.50 to 0.75 μm. The exterior of the capillary is coated with polyimide protective coating for added

mechanical strength. The coating is removed at the ends of the capillary and at the detector.

The silanol groups (SiO^-) of the capillary produce a negatively charged surface within the capillary and are responsible for the electroosmotic flow (EOF) and the apparent mobility of the analytes. The EOF is highly dependent upon electrolyte pH as the zeta potential is largely governed by the ionization of the acidic silanols on the capillary wall. Below pH 4 the ionization is small and the EOF flow rate is therefore not significant, above pH 9 the silanols are fully ionized and EOF is strong. The EOF moves rapidly in the direction of the cathode and therefore, regardless of an analyte's charge, it will eventually be carried to the detector.

The EOF is generated by the entire length of the capillary and therefore produces constant flow rate at all distances along the capillary. The solutes are therefore, swept along at the same rate throughout their transport along the capillary and thus the flow profile is plug-like in nature with minimal sample dispersion. This is superior to the laminar flow profile produced in pumped systems such as HPLC (Fig. II-8). Laminar flow is caused as the solution is pushed from one end of the column. The solution at the edges of the column interacts with the column causing it to move slower than the solution in the middle of the column. This results in different solute speeds across the column and broadens the peaks more as they travel along the column.

Separation in CE is based on differences in the electrophoretic mobility of analytes. If the analytes to be separated have no charge or have limited differences in electrophoretic mobility, they can be separated using a CE mode called micellar electorkinetic capillary chromatography (MECC). The separation conditions involve use of a surfactant in the buffer phase. Surfactants are amphophilic molecules (i.e. molecule with both hydrophobic and hydrophilic parts) (Frazier *et al.*, 2000). Sodium dodecyl sulphate (SDS) is a common anionic surfactant. Above a specific surfactant concentration, the critical micelle concentration (CMC), the surfactant molecules begin to self-aggregate, forming micelles (Fig. II-9A) in which the hydrophilic head groups form an outer shell and the hydrophobic tail groups form a nonpolar core into which solutes can partition. Micelles of SDS have a negative charge and migrate against the EOF; however, the EOF is sufficiently strong to force the micelles to eventually pass through the detector. The micelles act similar to the stationary phase in HPLC, producing a pseudo-stationary phase. Differential partitioning between the buffered aqueous mobile phase and the pseudo-stationary phase is the sole basis for separation for neutral molecules. Sample species can partition into the interior of the micelle in a fashion similar to retention on a stationary phase in HPLC. Highly retained solutes will elute later, while solutes with limited interaction with the micelle will be eluted near the EOF front.

Others surfactants include cationic surfactants, such as cetyltrimethylammonium bromide (CTAB) and bile salts (an anionic surfactant).

Mixtures of surfactants can be used including neutral surfactants such as Tween and Brij. The selectivity of an MECC system is mainly controlled by the nature of the surfactant. Due to its cationic nature, CTAB greatly affects CE separation.

The use of CTAB and other cationic surfactants reverses the direction of the EOF (Fig II-9B). The pseudo-stationary phase is formed along the interior capillary walls in a double layer. This changes the net charge from negative to positive. The change in direction of the EOF has no direct effect on the separation principle in MEKC, however, it is usually necessary to switch the polarity of the electrodes (i.e., cathode at the injection point). Note that even though the micellar and EOF are in opposing directions, the net movement is still in the direction of the EOF due to its strength (Michaelsen *et al.*, 1992).

If species are charged then they will be separated in MEKC based on the sum of both their electrophoretic mobility and partitioning. Therefore, MEKC is useful for mixtures containing charged and uncharged components. The other feature of MEKC is that all components injected into the capillary, provided that they are sufficiently soluble in the electrolyte, will migrate between time-zero (EOF) and the time when micelles reach the outlet. This is unlike HPLC where some components may be irreversibly adsorbed onto the stationary phase.

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Appenix A

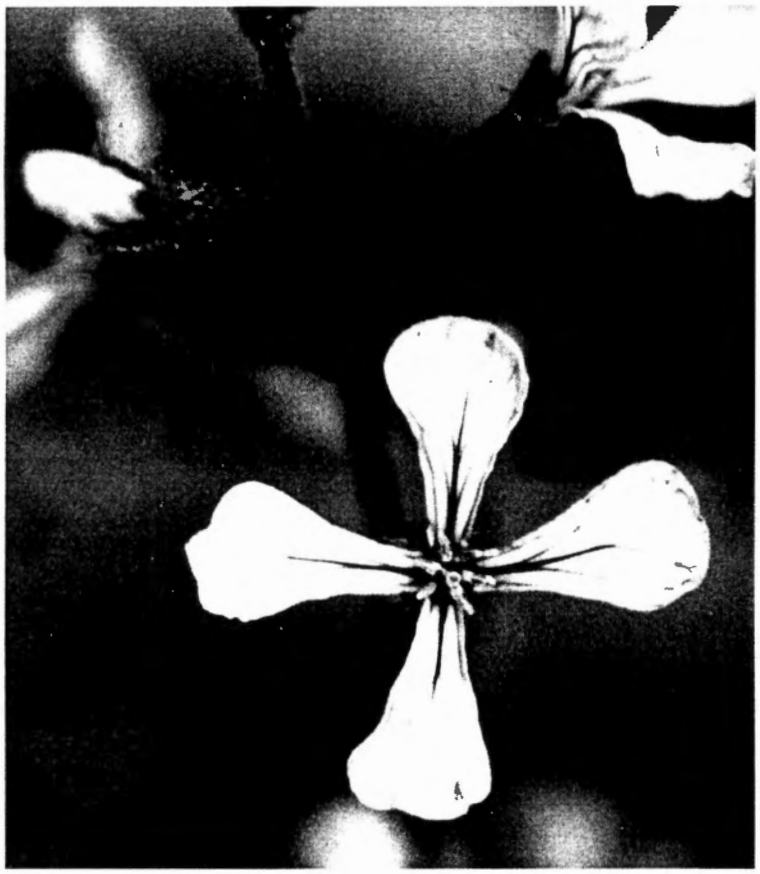

Fig. II-1. Flower Structure. Typical flower structure of Brassicaceae as exhibited by arrugula (*Eruca sativa* Mill.).

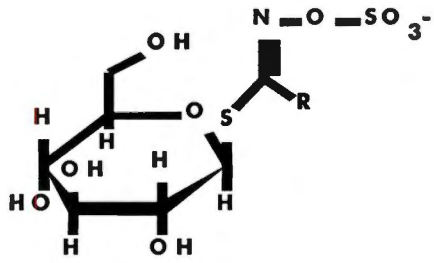

Fig. II-2. Glucosinolate Structure. The general structure of a glucosinolate is a thioglucose with a sulfonated oxime moiety.

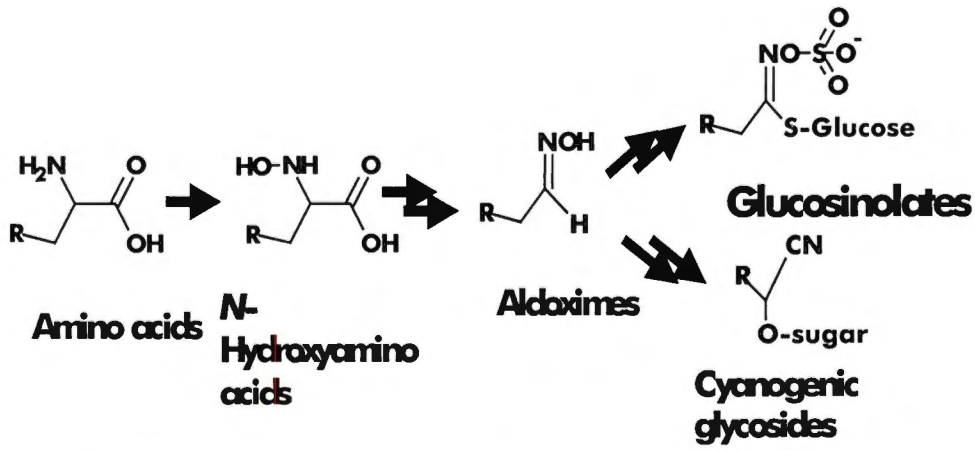

Fig. II-3. Glucosinolate Biosynthesis. Amino acids and their chain-elongated derivatives are the precursors for glucosinolates (Du *et al.*, 1995).

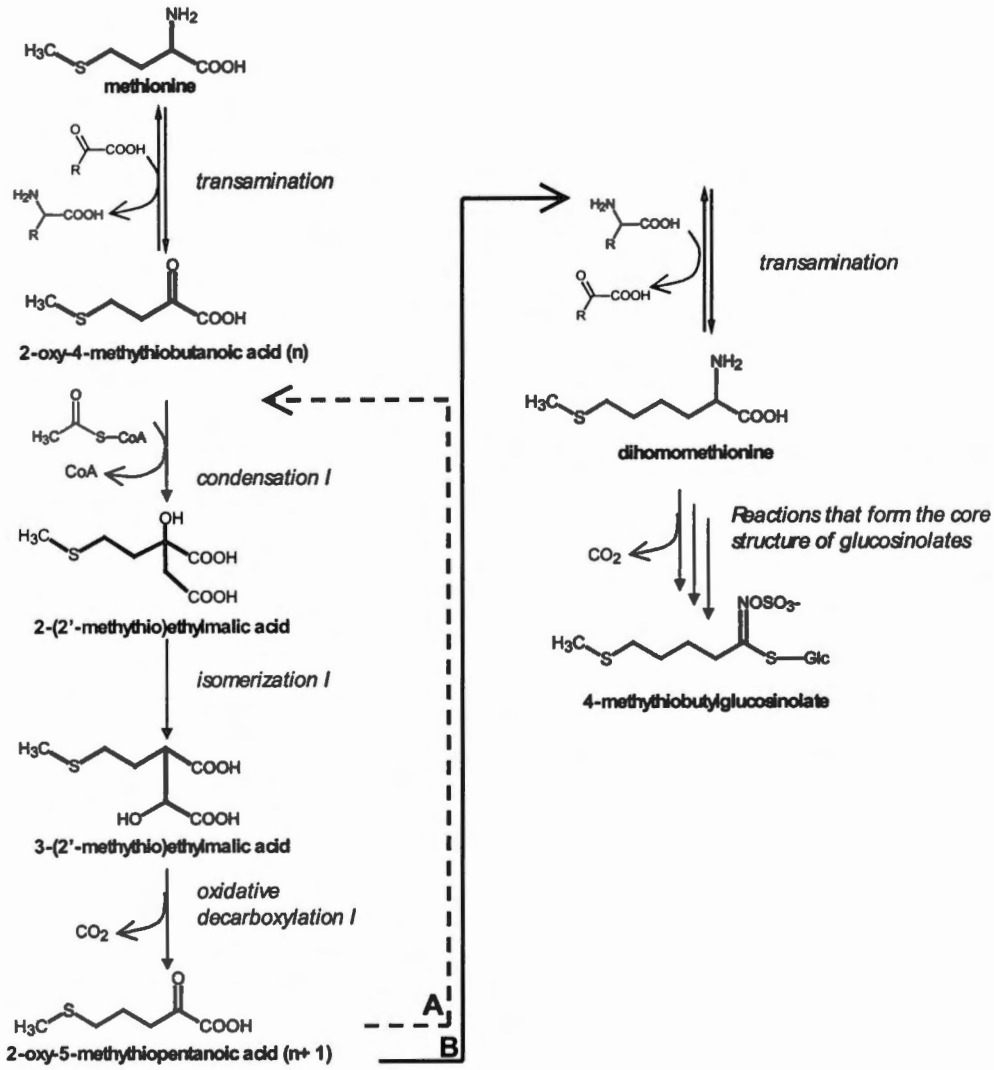

Fig. II-4. Amino acid chain elongation. A) Reaction product can undergo up to eight cycles of chain elongation or B) be transaminated to the corresponding methionine derivative (Modified from Graser et al., 2000).

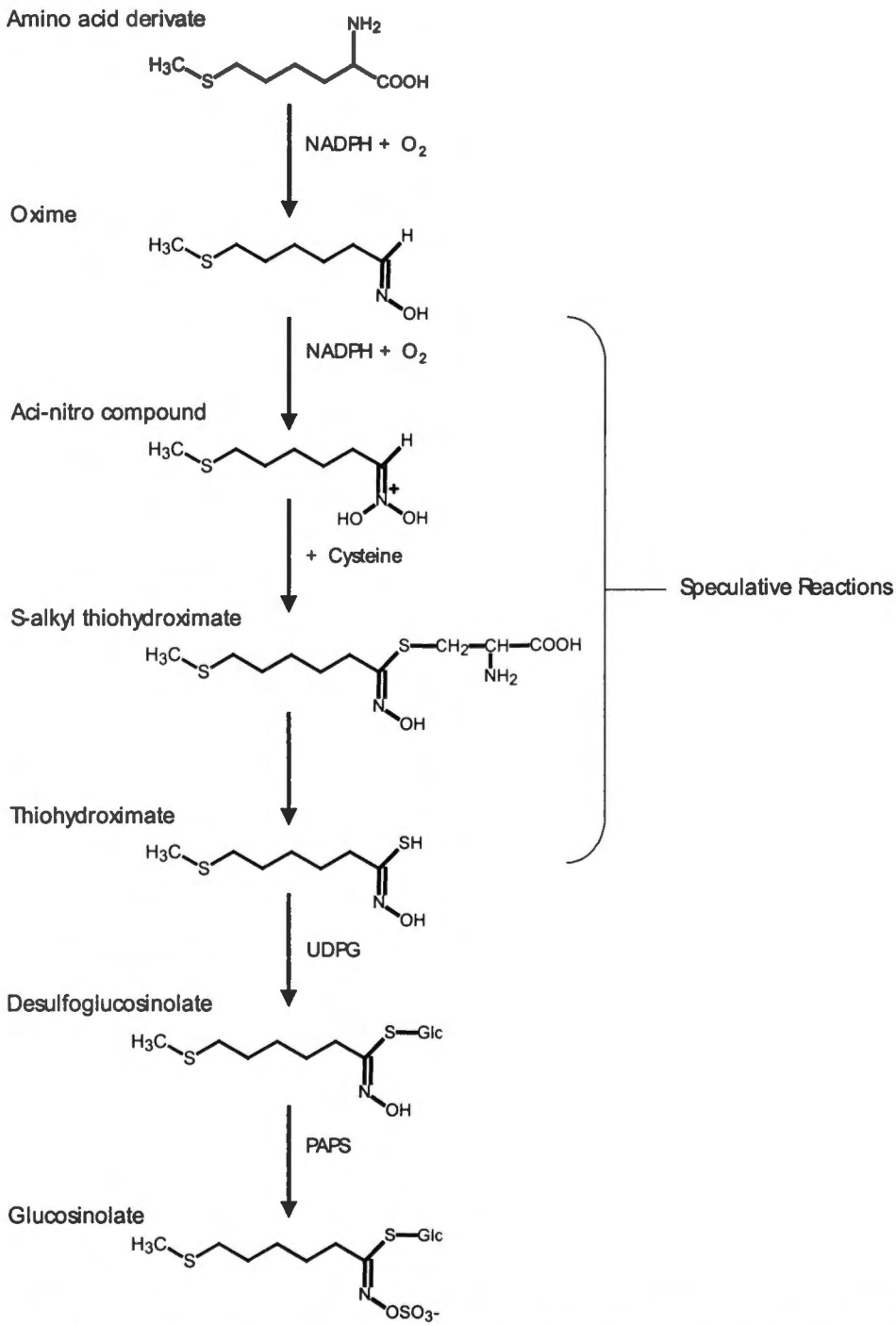

Fig. II-5. Oxime Formation. Conversion of chain-elongated amino acid to a corresponding oxime. (Modified from Graser et al., 2000).

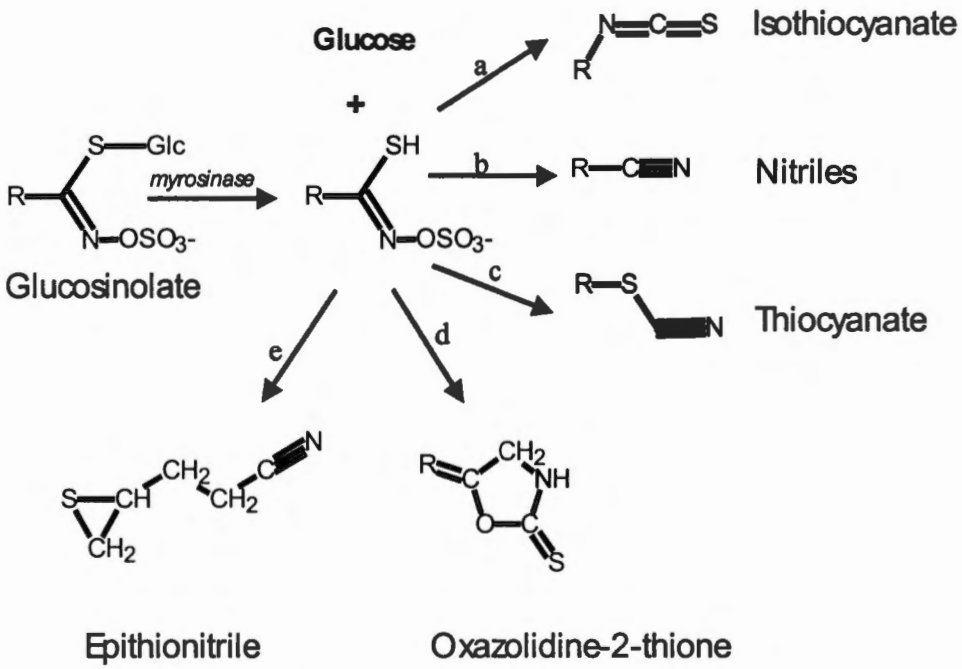

Fig. II-6. Glucosinolate Degradation. Hydrolysis of glucosinolate by myrosinase to one of several nitrogen containing compounds. (Modified from Larsen, 1981).

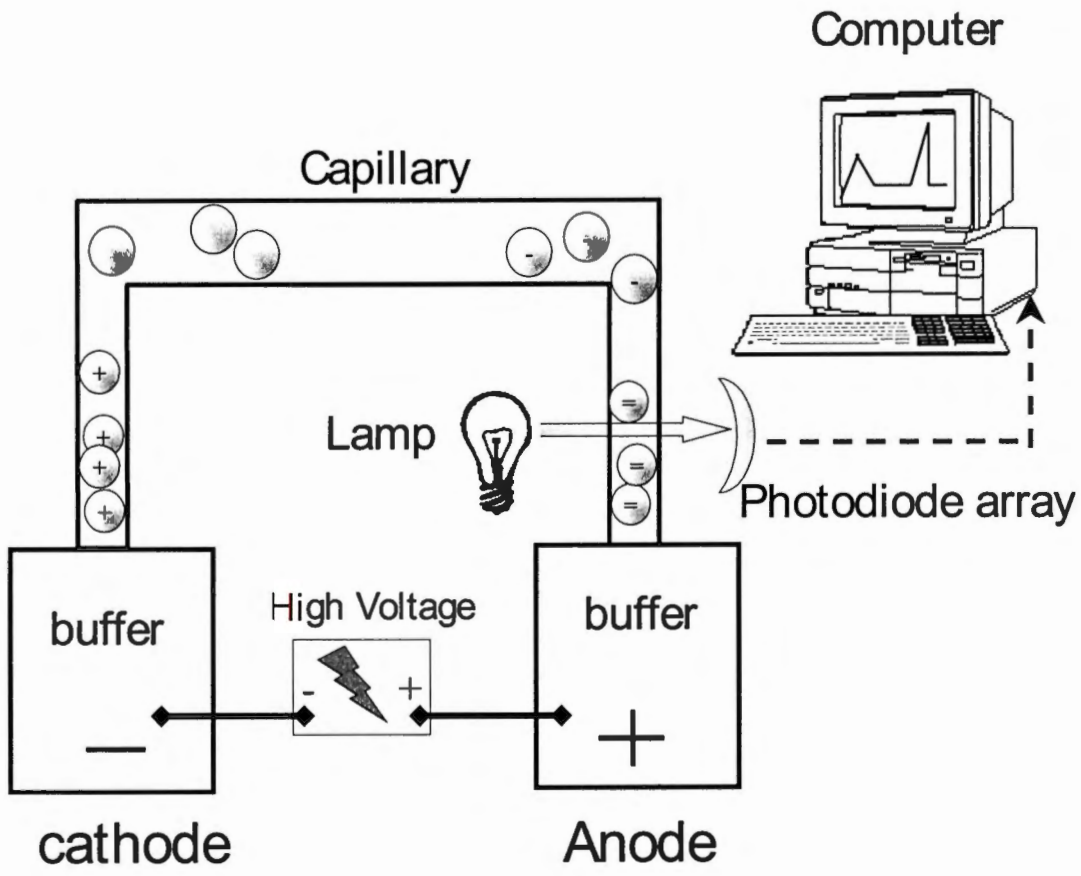

Fig. II-7. Capillary electrophoresis system. The primary components of a capillary electrophoresis system are the two buffer reservoirs, high voltage source, detector and integrator.

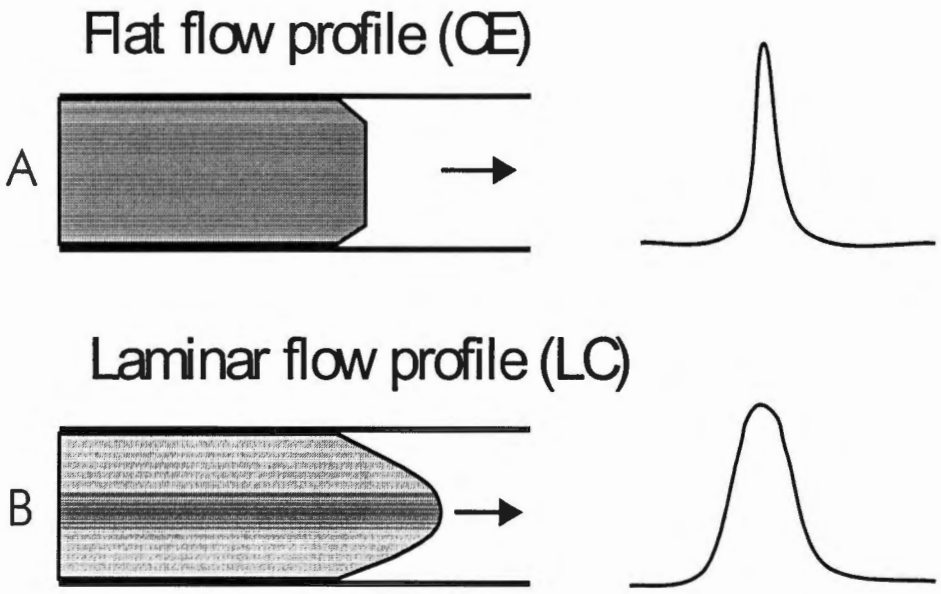

Fig. II-8. Flow Profiles. The plug-like flow (A) profile of capillary electrophoresis is superior to the laminar flow (B) of a pumped system.

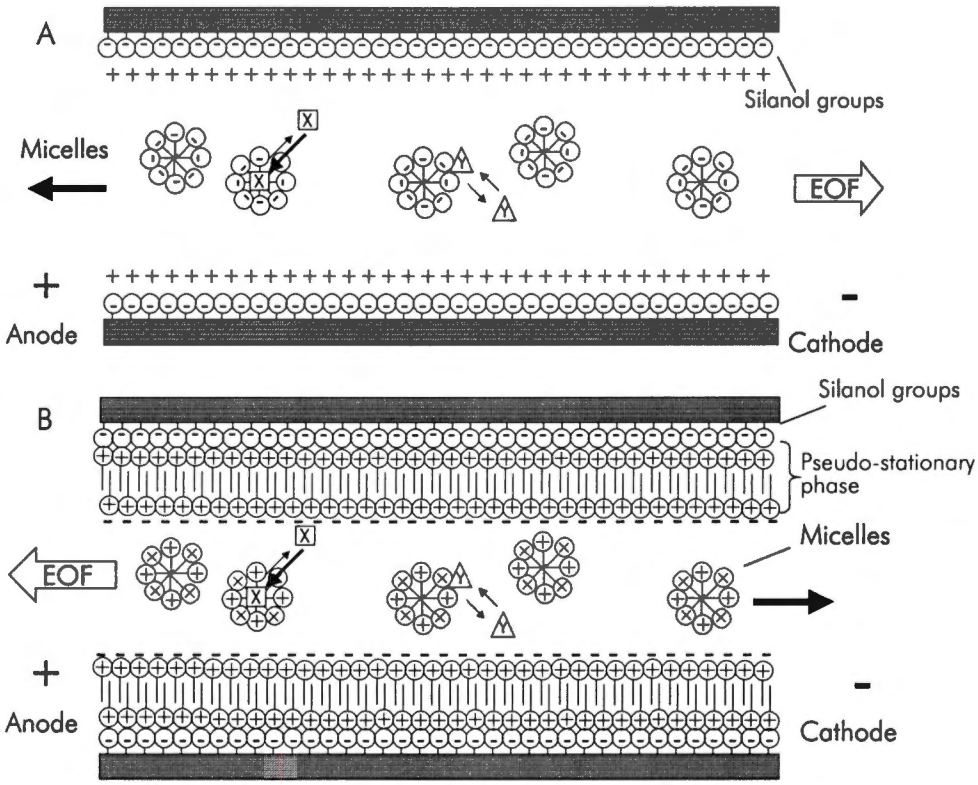

Fig. II-9. Surfactant interaction within capillary. Anionic surfactants migrate against the EOF (A). Cationic surfactants form a pseudo-stationary phase and reverse the flow of the EOF (B).

Part III

Indian Mustard and Allyl Isothiocyanates Inhibit *Sclerotium rolfsii*

Part III is a slightly revised version of a paper by the same name published in the Journal of the American Society of Horticultural Science in 2002 by Stephanie G. Harvey, Heather N. Hannahan and Carl E. Sams:

Harvey, Stephanie G., Heather N. Hannahan and Carl E. Sams. Indian mustard and allyl isothiocyanates inhibit Sclerotium rolfsii. J. Amer. Soc. Hort. Sci. 127:27-31.

My primary contributions to this paper include: (1) selection of the topic from several ongoing projects and development of the problem into a work relevant to my study of *Brassica* species as potential biofumigants, (2) identification, isolation and culturing of *Sclerotium rolfsii*, the pathogen used in this study, (3) implementation and analysis of data, (4) most of the gathering and interpretation of literature, (5) structuring and composition of this paper.

Abstract

Allyl isothiocyanate (AITC) is the predominant isothiocyanate produced by damaged tissues of Indian mustard *(Brassica juncea* (L.) Czerniak). This study investigated Indian mustard (IM) and AITC mediated suppression of mycelial growth and sclerotial germination of *Sclerotium rolfsii* Saccardo, a common soilborne pathogen. Indian mustard 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 $g \cdot L^{-1}$ (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 $\mu mol \cdot L^{-1}$ 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 $g \cdot L^{-1}$, respectively, with death resulting with > 2 $g \cdot L^{-1}$. 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 IC_{50} and IC_{90} of AITC were 249.0 and 528.8 $\mu mol \cdot L^{-1}$, 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.

Introduction

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 (GS), found in *Brassica* species, are of particular interest because their volatile degradation products have biocidal activity.

The genus *Brassica* L. contains over 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. (Pekinensis 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 GS.

Glucosinolates are organic anions containing ß-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, GS produce D-glucose, sulfate, isothiocyanates (volatile mustard oils), thiocyanates, and nitriles (Larsen, 1981; Poulton and Moller, 1993). Both GS 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 GS 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) have been reported since 1937 (Walker *et al.*, 1937). The chemical concentration necessary to demonstrate inhibition of a given fungus varies with the specific ITC. The type of ITC produced depends on the side chains of the glucosinolate from which it is derived. The concentration of ITC produced varies with environmental factors. Soil texture, temperature, microbial community, and pH can significantly affect the conversion of GS to ITC (Price, 1999).

Sclerotium rolfsii has an extensive host range of more than 270 plant species in the United States alone and includes dicotyledons and monocotyledons (Farr *et al.*, 1989). Many commercial crops including peanut (*Arachis hypogaea* L.), bean (*Phaseolus* L. sp.), sugarbeet [*Beta vulgaris* L. (Crassa Group)], sweet pepper (*Capsicum frutescens* L.) and tomato (*Lycopersicon esculentum* Mill.) are

affected (Singleton *et al.*, 1992). Some members of the Brassicaceae are reported to host *S. rolfsii* (Jyoti *et al.*, 1994). The fungus causes southern blight (seedling damping-off, blight, and stem rot) throughout the tropics, subtropics, and in areas of the southern and southeastern United States. *Sclerotium rolfsii* produces survival structures, called sclerotia that are typically brown to tan in color and measure 0.5-mm to 2.0-mm in diameter. A sclerotium is comprised of a melanized outer layer, a middle cortex, and an innermost medulla of loosely arranged filamentous hyphae (Chet *et al.*, 1969). Germinating sclerotia initiate new infections under conditions suitable for fungal growth.

In high value crops, southern blight incidence can be reduced with preplant applications of methyl bromide, chloropicrin, or metham-sodium (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-chlorophenyl)ethyl]-α-(1-1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol], flutolanil [N-[3-(1-methylethoxy)phenyl]-2-(trifluoromethyl) benzamide], and fluazinam [3-chloro-*N*-(3-chloro-5-trifluoromethyl-2-pyridyl)-trifluoro-2,6-dinitro-*p*-toluidine], 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 utilizing *Brassica* spp.

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 sclerotial 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, TN. 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 Experiment Station, Knoxville, TN. 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 at 30 °C (Kelvinator Commercial Products, Manitowoc, WI). 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 a 490-mL glass jar and 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 reconstituted FDM per liter of enclosed atmosphere were established. Deionized water was added to reconstitute FDM to its fresh weight (9.160 $\pm$ 0.001 g water per 1.000 $\pm$ 0.001 g FDM). Plugs (4.6 mm diameter) were cut from margins of an actively growing *S. rolfsii* culture and placed in the center of fresh PDA plates (100 x 15 mm). Plates with hyphal plugs were inverted, agar side down, over the mouth of the jars and sealed with wax film. This process was repeated for each treatment. Jars were incubated at 30 °C for 42 h. Jars were removed and radial growth of the colony on agar was measured. When mycelial growth from the plug was nonsymmetrical, four radii were taken and averaged. Percentage inhibition of growth was

calculated from the difference between growth of treated plugs and control. Plugs exhibiting zero (0) growth following treatment were transferred to fresh PDA and incubated at 30 °C for an additional 42 h to determine if suppression or death had occurred.

To determine the amount of inhibition that was attributable to the AITC released by the remoistened FDM, hyphal plugs were fumigated with AITC standard (diluted in ethanol) at concentrations of 0.3, 0.8, 1.7, 2.9 and 6.0 $\mu mol \cdot L^{-1}$ enclosed atmosphere. The dilutions were applied to filter paper (Whatman No. 2) in the bottom of the 490-mL jars. Plates with hyphal plugs were inverted over the mouth of each jar. The rest of the procedure was the same as that used with the FDM.

A completely randomized design was utilized 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 $Y=a/(1+e^{-(X-B/C)})$, the concentrations of FDM and AITC to produce 50% and 90% inhibition (IC_{50} and IC_{90}) 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, 0.05, 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, MO). 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 x 0.25 x 0.25 (Alltech Associates, Inc., Deerfield, IL). 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 utilized a completely randomized design with four replications. Linear regression was utilized 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 ($\geq$1 and <2-mm diameter). Five sclerotia were selected and placed on a 2-cm square of 0.2-mm polyester mesh (Fig. III-1). The mesh was then tied up around the five sclerotia. For each treatment, a culture tube was packed with 15.60 $\pm$ 0.05 g of oven-dried clay loam soil (fine, kaolinitic, thermic, Typic Paleudult). The soil was

brought to approximately 60% field capacity with the addition of 2.4 g deionized water. Additional soil (approximately 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 microorganisms. Following autoclaving, sterile deionized water was added to the tubes and beaker to return them to their pre-autoclave weight ($\approx$ 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 included 0.0, 4.0, 16.0, 64.0, 256.0, 1024.0, and 4096.0 μmole$\cdot$L^{-1}. The AITC treatment was injected through the septa and into the tube. Tubes were incubated at 30 °C for 24 h and then checked for AITC concentration using GC-MS (Price, 1999). If no AITC was detected, bags were removed from tubes and surface-sterilized with 0.5% sodium hypochlorite followed by a deionized water rinse. Sclerotia were removed and placed on PDA, an equal distance from each other and incubated at 30 °C for 42 h. Radial growth of mycelia from sclerotia was measured. Sclerotia exhibiting zero growth were monitored for 7 d to determine if suppression or death had occurred.

A completely randomized design with four replications and five samples per experimental unit was used. To examine the dosage effect, a nonlinear regression of percentage inhibition was performed. From the sigmoid equation $Y=a/(1+e^{-(x-}$

$^{B)/C}$), the concentrations to produce 50% and 90% inhibition (IC_{50} and IC_{90}) were calculated (SigmaPlot, 2000).

Results

Inhibition of mycelial growth by Indian mustard. Mycelia of *S. rolfsii* were sensitive to volatiles produced by FDM. Treatments in excess of 2.0 $g \cdot L^{-1}$ Indian mustard resulted in death of *S. rolfsii* (Table 1). The IC_{50} and IC_{90} values 0.74 and 0.98 $g \cdot L^{-1}$ of reconstituted FDM, respectively were calculated from the equation $Y=99.45/(1+e^{-(X-0.7435)/0.1034})$ ($R^2=0.91$; SE±13.82)(Fig. III-2).

AITC inhibition of mycelial growth was also investigated. *Sclerotium rolfsii* was sensitive to AITC (Table 2). The nonlinear regression of the inhibition of radial growth ($R^2=0.94$; SE±9.63) produced the equation $Y=98.59/(1+e^{-(X-1.6890)/0.8593})$, from which an IC_{50} and IC_{90} of AITC at 1.6 and 4.5 $\mu mol \cdot L^{-1}$, respectively, were calculated (Fig. III-3).

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 ($R^2=0.99$; SE±0.11). The equation describing this relationship was: AITC ($\mu mol \cdot L^{-1}$) = 0.41 × Indian mustard [g fresh weight (FW)/L] + 0.59 (Fig. III-4).

Indian mustard treatments were more effective than equivalent AITC treatments (Fig. III-5). 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 the Indian mustard. The IC_{50} and IC_{90} for the inhibition attributable to only the AITC were calculated at 2.7 and 7.6 g FW/L, respectively.

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, $Y=95.54/(1+e^{-(X-239.29)/103.81})$, from the nonlinear regression of inhibition ($R^2=0.96$; SE$\pm$9.54) was used to calculate the IC_{50} and IC_{90} of 249.0 and 528.8 μmol·L^{-1} AITC, respectively (Fig. III-6). Suppressed sclerotia germinated 6 to 8 d after transfer to PDA.

Discussion

Volatiles released from 2.0 g Indian mustard into a headspace volume of 1 L (equaling AITC at1.45 μmol·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 $\mu mol \cdot L^{-1}$. 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 $\mu mol \cdot L^{-1}$ soil airspace (based on 23 $\mu g \cdot g^{-1}$ soil (Hoynes *et al.*, 1999) assuming an average soil bulk density of 1.5 $g \cdot cm^{-3}$). 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 prior to treatment, lower concentrations of AITC/mustard could provide adequate inhibition of both the saprophytic and newly emerged mycelia 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* (Chellemi *et al*, 1997; Lewis and Fravel, 1996; Ristaino *et al.*, 1996). The 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.

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Appendix

Figure III-1. Sclerotial bags. Mesh bags were used to aid in retrieval of sclerotia following treatment. Each bag contained five sclerotia of *Sclerotium rolfsii*.

Table III-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[Z] (mm)	Inhibition[Z] (%)
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[Y]	**	**

[Z] Mean of four replications.

[Y] Analysis of dosage effect was performed using SigmaPlot nonlinear regression model ($Y=a/(1+e^{-(X-B/C)})$.

Dosages in excess of that needed to induce 100% inhibition were eliminated from the analysis.

** Significant at $P \leq 0.01$.

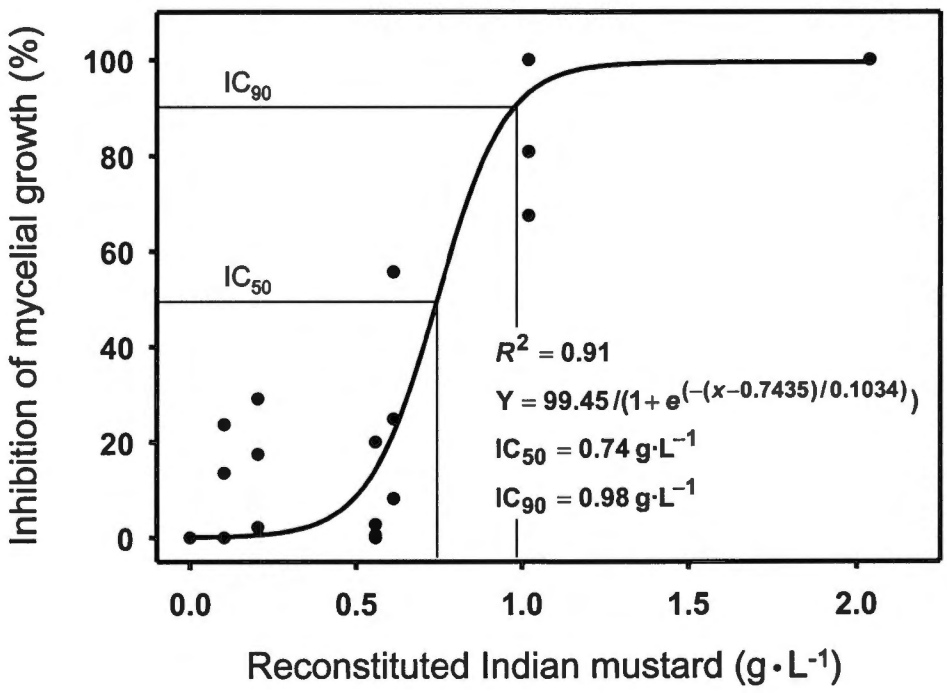

Fig. III-2. Inhibition of mycelial growth of *Sclerotium rolfsii* with treatments of reconstituted Indian mustard (*Brassica juncea*) residues. The IC_{50} and IC_{90} (inhibiting concentration at 50% and 90%) were calculated from the equation produced by the nonlinear regression.

Table III-2. Radial growth ±SD and inhibition of *Sclerotium rolfsii* mycelia at 42 h after treatment with known concentrations of allyl isothiocyanates (AITC).

AITC (μmol·L^{-1})	Radial growth of mycelia[z] (mm)	Inhibition[z] (%)
0.0	27.2 ± 1.3	0.0
0.3	21.2 ± 1.4	5.7
0.8	16.3 ± 1.4	39.7
1.9	11.8 ± 0.3	56.4
2.9	6.7 ± 2.9	70.0
6.0[w]	0.0 ± 0.0	100.0
Nonlinear regression[y]	**	**

[z] Mean of three replications.
[y] Analysis of dosage effect was performed using SigmaPlot nonlinear regression model ($Y=a/(1+e^{-(X-B/C)})$).
[w] Dosages in excess of that needed to induce 100% inhibition were eliminated from the analysis.
**Significant at $P \leq 0.01$.

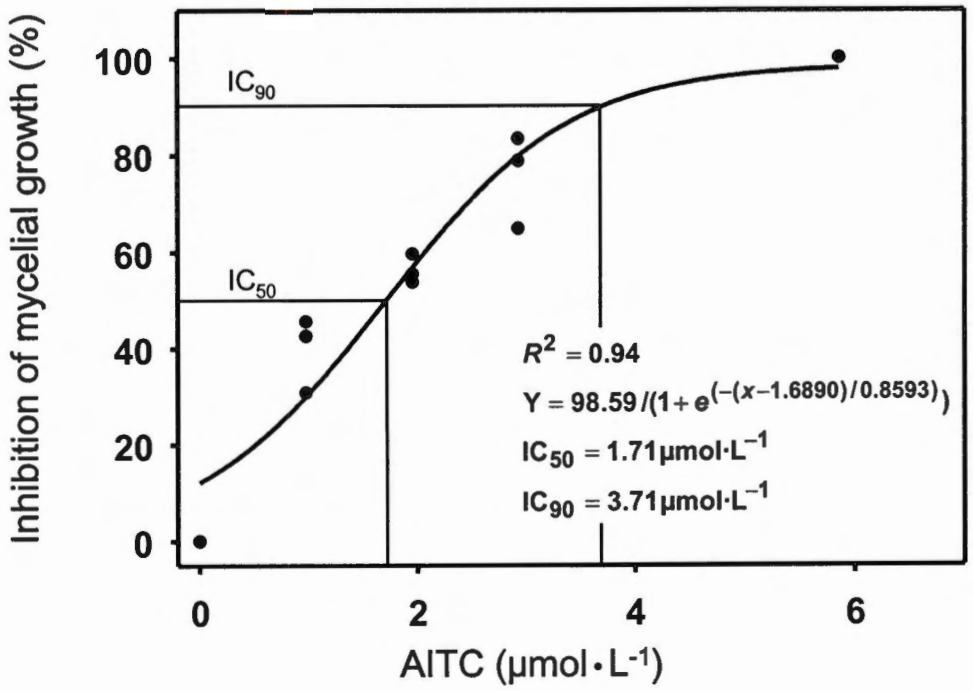

Fig. III-3. Inhibition of mycelial growth of *Sclerotium rolfsii* by known concentrations of allyl isothiocyanate (in $\mu mol \cdot L^{-1}$). The IC_{50} and IC_{90} (inhibiting concentration at 50% and 90%) were calculated from the equation produced by the nonlinear regression.

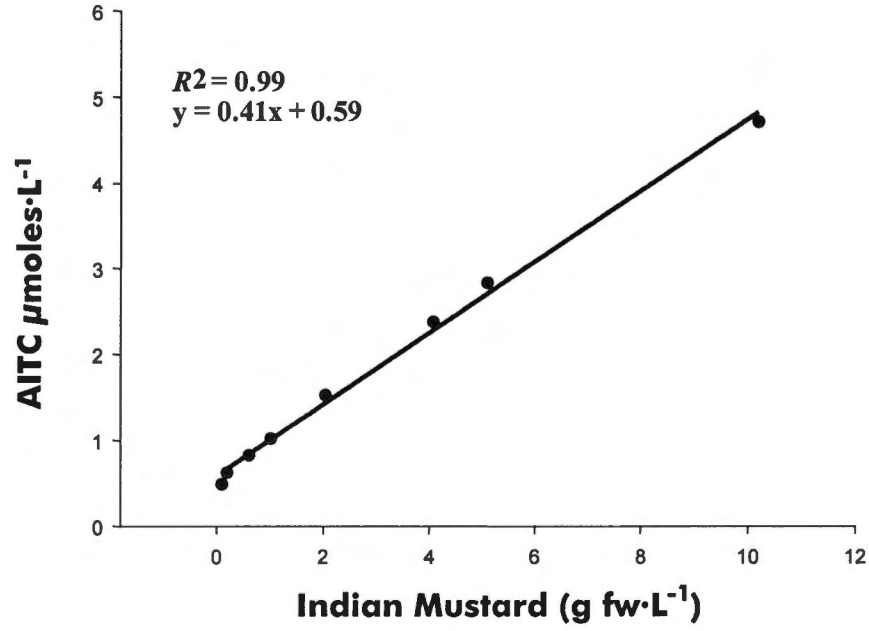

Fig III-4. Allyl isothiocyanate released from Indian mustard. Allyl isothiocyanates quantified from the head-volume above a given amount of Indian mustard.

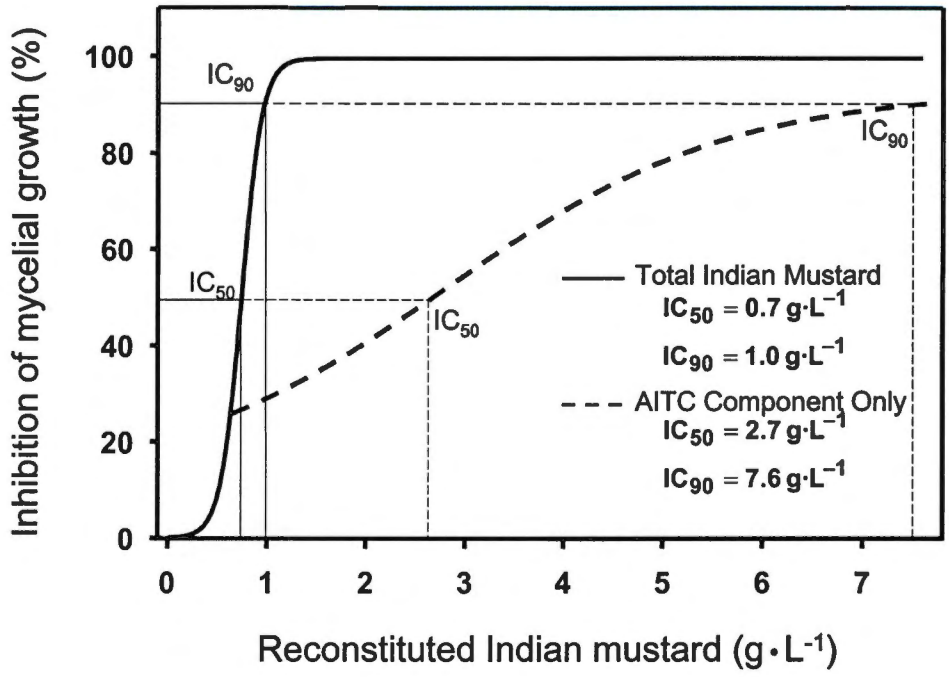

Fig III-5. Allyl isothiocyanate contribution to Indian mustard inhibition of *Sclerotium rolfisii* mycelial growth. Relationship between Indian mustard and inhibition of *Sclerotium rolfsii* mycelial growth (solid curve) and the projected amount of Indian mustard required to achieve the same inhibition, if AITC was the only factor involved in the inhibition (dashed curve). The IC_{50} and IC_{90} (concentration resulting in 50% and 90% inhibition, respectively) were calculated for each equation for comparison.

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

AITC ($\mu mol \cdot L^{-1}$)	Radial growth of mycelia [z] (mm)	Inhibition[z] (%)
0.0	7.2 ± 2.0	-
4.0	7.0 ± 2.7	6.4
16.0	6.4 ± 2.6	26.9
64.0	7.5 ± 1.7	13.7
256.0	4.2 ± 3.8	50.0
1024.0	0.7 ± 0.7	91.5
4096.0	0.0 ± 0.0	100.0
Nonlinear regression[y]	**	**

[z] Mean of four replications with five samples per replication.
[y] Analysis of dosage effect was performed using SigmaPlot nonlinear regression model ($Y=a/(1+e^{-(X-B/C)})$.
[w] Dosages in excess of that needed to induce 100% inhibition were eliminated from the analysis.

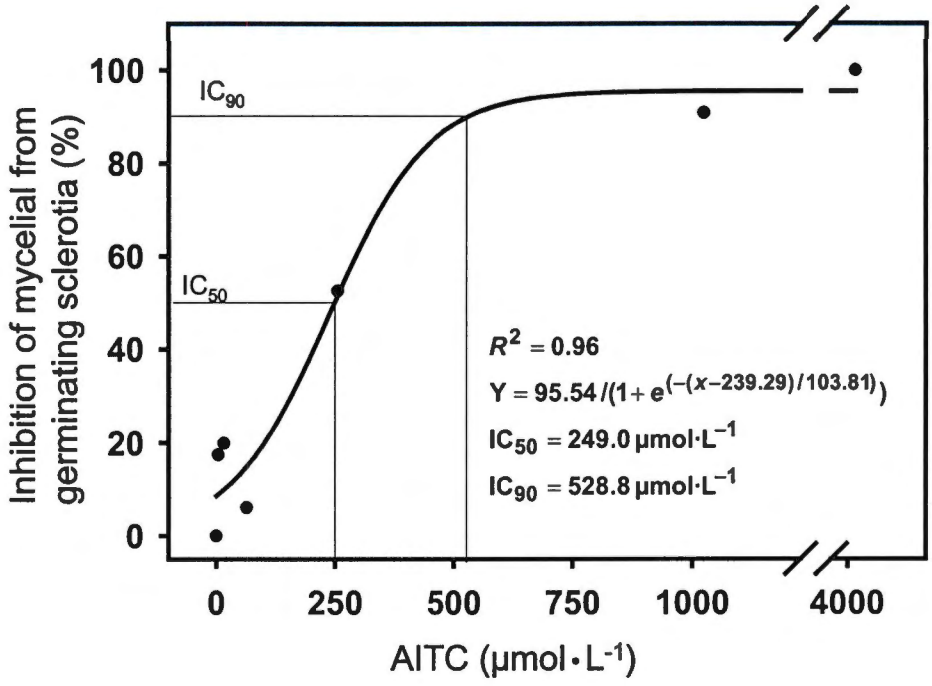

Fig. III-6. Inhibition of mycelial growth from germinating sclerotia of *Sclerotium rolfsii* by known concentrations of allyl isothiocyanate (in μmol·L^{-1}). The IC_{50} and IC_{90} (concentration resulting in 50% and 90% inhibition, respectively) were calculated from the equation produced by the nonlinear regression.

Part IV

Brassica Biofumigation Impacts Tomato Fruit Production

Part IV is a slightly revised version of a paper submitted for publication to American Journal of Alternative Agriculture in July 2002 by Stephanie G. Harvey and Carl E. Sams:

My primary contributions to this paper include: (1) selecting the topic and developing the problem into a work relevant to my study of *Brassica* species as potential biofumigants, (2) selecting the treatments used in this study, (3) implementing and analyzing data, (4) gathering and interpreting literature, (5) structuring and composing this paper.

Abstract

In previous experiments, allyl isothiocyanate (AITC) inhibited mycelial growth and sclerotial germination of *Sclerotium rolfsii*, a common soilborne pathogen of tomatoes. Volatiles from macerated *Brassica juncea*, (which included AITC) were more suppressive than AITC alone. The objective of this study was to determine the effect of biofumigation utilizing *Brassica* sp. on tomato yield in the field. The experiment used a split-plot array of treatments with three soil pH ranges (< 6.0, 6.5-7.0 and > 7.0) as main treatments. The subplot treatments were fall-planted, winter cover crops of Indian mustard (*B. juncea*) (two seeding densities (0.9 and 1.8 $g \cdot m^{-2}$), 'Fall Raab' (*B. campestris*) (1.2 $g \cdot m^{-2}$) and a control cover of winter rye. Cover crops were over wintered and then incorporated into the soil three weeks prior to tomato transplanting. Incorporation consisted of mowing the cover, tilling in the plant material, and forming plastic-mulch covered beds. Number and total weight of tomato fruit were measured. Neither pH nor the interaction between main and subplot treatments was significant. The number and total weight of marketable fruit (greater than 6.4 cm diam) differed significantly among the *Brassica* treatments and the control ($P < 0.01$). The 'Fall Raab' cover crop produced 18.5 % more marketable fruit with 19.2% greater marketable weight than did the Indian mustard cover crop and 45.4% and 40.0%, respectively, greater, than the rye control. Indian mustard treatments produced 22.7-23.5% more marketable fruit with 18.2% greater marketable weight, than the rye control

($P < 0.01$). No significant differences in number of fruit or weight were detected between the two Indian mustard densities. Variations in soil carbon and nitrogen content were not correlated to yield differences.

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is an economically important crop in Tennessee, Florida, and North Carolina. Commercial producers depend heavily on methyl bromide to control soilborne pests. Often grown without rotations, tomato fields can develop high pathogen inoculum densities. Without chemical control of the pathogens, losses can be devastating. The approaching ban of methyl bromide, widely used for control of soilborne pathogens, has been projected to produce an annual economic loss in excess of $350 million to tomato growers and consumers in the United States (USDA, 2000; Ferguson and Padula, 1994).

A possible alternative to methyl bromide and other synthetic chemicals for the control of soilborne pests is the utilization of glucosinolate degradation products, such as isothiocyanates, in a biofumigation system. Glucosinolates (GS), found in *Brassica* and other species, are naturally occurring secondary metabolites that exist in intact plant tissues. When tissue is damaged, glucosinolates are hydrolyzed by an enzyme, myrosinase, to produce D-glucose, sulfate, isothiocyanates, thiocyanates and nitriles (Fig. II-6) (Larsen, 1981; Poulton and

Moller, 1993). Both GS and myrosinase occur in cells throughout the plant, but are isolated from each other. The compounds mix and react (in the presence of water) when pests damage cells (Chew, 1988). Isothiocyantates have been demonstrated to control fungi (Harvey *et al.*, 2002; Charron and Sams, 1999; Sarwar *et al.*, 1998; Mayton *et al.*, 1996), bacteria (Delaquis and Mazza, 1995), nematodes (Mojtahedi *et al.*, 1993 and 1991) and some weed seeds in laboratory experiments (Al-Khatib, *et al.*, 1997).

Glucosinolates (Fig. II-3) are organic anions containing ß-D-thioglucose and sulfonated oxime moieties (Brown *et al.*, 1994). They have been found in only 15 dicotyledonous families, most predominately the Brassicaceae and Euphorbiaceae. Glucosinolates are produced from amino acids; methionine is the progenitor of the majority of GS, while tryptophan and phenylalanine are integrated into others (Underhill *et al.*, 1973). Found in cell vacuoles throughout the plant, GS account for less than 0.1% of the fresh weight in intact plants.

Sclerotium rolfsii Saccardo is a soilborne pathogen with an extensive host range of more than 500 plant species, including both dicotyledons and monocotyledons (Singleton *et al.*, 1992). The fungus causes "Southern Blight" of tomato (seedling damping-off, blight and stem rot) throughout the tropics, subtropics and in areas of the southern and southeastern United States where high temperatures permit its growth and survival (Punja, 1985).

Sclerotium rolfsii is one of a number of plant pathogenic fungi that form resistant, over-wintering survival structures called sclerotia. Sclerotia are typically brown to tan in color and measure 0.5-mm to 2.0-mm in diameter. A sclerotium is comprised of a two to four cell-layer thick, melanized outer layer, a middle cortex and innermost medulla of loosely arranged filamentous hyphae (Chet *et al.*, 1969). Infection of the host plants by *S. rolfsii* is preceded by an accumulation of mycelium at the host surface. Oxalic acid and polygalacturonases cause tissue death prior to hyphal penetration. The cell wall is weakened as oxalic acid sequesters calcium to produce calcium oxalates (Punja and Jenkins, 1984). In addition, this lowers the tissue pH to the optimum for endopolygalacturonase and cellulase activity (cell wall degrading enzymes). The necrotic and macerated appearance of infected tissues occurs as pectic substances in the cell walls are depleted in advance of the growing hyphae (Punja, 1985).

Sclerotium rolfsii can be controlled by soil fumigation with methyl bromide, chloropicrin and metham-sodium in preplant application (Jenkins and Averre, 1986). However, the future availability of all these chemicals is questionable. The use of ammonium fertilizers such as urea and ammonium bicarbonate lowers disease incidence and could be used to moderate the disease (Jenkins and Averre, 1986). The exact mechanism of action is not known but it is reported to directly inhibit sclerotial germination and retard mycelial growth. However, it is known that ammonium fertilizers can increase the incidence of fruit disorders such as blossom

end rot (Wilcox *et al.*, 1973). Alternatively, increased calcium levels in tissue following the application of calcium fertilizers such as calcium nitrate may provide some level of control by increasing the calcium content of host tissue cell walls which may offset the oxalic acid effect.

Pythium ultimum Trow. is an oomycete that is pathogenic on many plants, including tomato. It causes significant economic losses of crops, has a broad host range and is worldwide in distribution (Plaats-Niterink, 1981). *Pythium* spp. can produce both pre- and post-emergence damping-off. The taproots, root tips and feeder roots of mature plants may be attacked, stunting plants and reducing yields (Schlub and Lockwood, 1981). Chemicals are used in most agricultural systems to manage *Pythium*. Captan®, Arasan®, Chloroneb® and Apron® are a few of the possible treatments. The effectiveness of these pesticides can be lost when environmental or cultural conditions become too adverse for proper plant development.

The objective of this experiment was to determine the effectiveness of biofumigation, with *Brassica* sp. as a green manure, in controlling soilborne tomato pathogens, such as *Pythium ultimum* Trow and *Sclerotium rolfsii* Sacc., under field conditions. The effects of these treatments on tomato production were evaluated by disease ratings, fruit yield and fruit quality.

Materials and Methods

Field studies were conducted at the Knoxville Experiment Station, Knoxville, Tenn. The experimental design was a randomized complete block with split-plot treatment assignment with pH whole plot treatments and biofumigation as the subplot treatments. The field, 45.7 m × 30.5 m, was established with 15 rows prepared in 0.9-m wide beds (Fig. IV-1). Rows were created on 2.4-m centers with 5.5 m between sets of five rows. Four blocks were designated running north to south. Within each block, three whole plots were established, each 15.2 m long × 7.6 m wide and containing five rows. Within each whole plot, five subplots (rows) were available for individual treatment assignment.

Experiment -1999. The field was limed to maintain whole plot treatments of soil pH in the range of 5.5-6.0, 6.5-7.0 or >7.0. Within the three established pH plots, five subplot treatments were applied. Two control treatments were utilized: (1) no Indian mustard cover and no tomatoes and (2) no Indian mustard cover but with tomatoes. This allowed the establishment of a base line for seasonal changes in microbial density and for changes due to tomato cultivation. Three treatments consisted of Indian mustard seeding densities (PI 458928; U.S. Dept. Agr. / Agr. Res. Serv., Ames, Iowa) at 1.5 x 10^5 (low), 5.5 x10^5 (medium) and 8.5 x 10^5 (high) plants/ha. These densities were selected for biofumigation based on commercial application rates (Brandt, 1992; Bracy *et al.*, 1991; Singh and Singh, 1984; Kumar and Shastry, 1981).

On March 16, 1999, seeds were hand-scattered and gently tapped into soil to a depth of approximately 5 mm. Seeds were watered immediately following seeding. Plots were surveyed for germination after 15 days. Due to poor plant stand, a second seeding was conducted on April 2. Plant counts were conducted for each plot prior to incorporation to determine actual densities (Table IV-1).

The Indian mustard incorporation into soil was completed at anthesis on May 17, 1999. Plant material was roto-tilled into soil. Immediately behind the tiller, bedding equipment prepared the bed, laid T-tape for drip irrigation, and covered the bed with black plastic mulch. Time from mustard incorporation until bed formation was less than 1 min. Within a row, the plastic between blocks was cut and the end buried to prevent volatiles from potentially moving across treatments.

A Teflon® tube, 14.5 cm x 6 mm diameter perforated with holes (1.6-mm diameter), was inserted through plastic mulch and into soil at the approximate center of each subplot with 2.5 cm remaining above the bed surface. The end of tube was plugged to prevent soil from clogging the tube and the above surface end was capped with a septum to prevent volatiles from escaping. The plastic mulch was taped to the tube to minimize loss of volatiles.

Two hours after incorporation and covering, soil temperatures were recorded at a depth of 5 cm for each subplot using a Fluke digital thermometer (Everrett, WA) with a 15.2-cm probe, while 50 ml of air was extracted from the

Teflon tube using a 60-ml syringe. The syringe needle was then inserted into a 50-ml Vacutainer® (BD, Franklin Lakes, NJ) which was allowed to collect 40 ml from the 50-ml sample. The Vacutainers were appropriately labeled and placed in a cooler with ice for transport to the laboratory. Upon arrival in the laboratory, vials were stored at -30 °C.

The air samples were analyzed using a Solid-Phase-Microextraction (SPME) device (Supelco, St. Louis, MO). The SPME probe was then inserted into the injection port of a Hewlett Packard 5890 gas chromatograph (GC) equipped with a flame ionization detector (FID). The volatiles desorb from the fiber and are delivered to the GC Alltech EC Wax 30 X 0.25 X 0.25 column (Alltech Associates, Inc., Deerfield, IL). 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 (Sigma-Aldrich Corp, St. Louis, MO) standard curve.

Health of tomato plants was determined by way of visual weekly ratings of disease incidence using the Horsfall-Barratt rating scale (Horsfall and Barratt. 1945) for assessing foliar diseases (Early blight). Plants were inspected for the presence of S. *rolfsii* mycelial growth. In addition, tomato fruit yield was monitored on a subplot basis.

Soil Microbial Population. Soil samples were collected from the field. Four cores were taken, to a deep of 15 cm using a 2.5 cm soil auger, at random along a "W" pattern across the row within the tomato root zone for each treatment (within 15 cm of the center of the bed; total of 240 cores). Each set of four cores was taken to the lab where they were air-dried and homogenized. One-gram samples were taken from the bulked cores and added to 98-ml sterile water blanks and mixed on a vortex mixer for 2 minutes to produce a 0.01 $g \cdot ml^{-1}$ soil suspension.

Using a Spiral Biotech Autoplate™ 3000 (Bethesda, MD), a 50.0 μl aliquot was withdrawn from the dilution and placed on a prepared agar plate in a logarithmic pattern. This provides the equivalent of a 10^{-8} serial dilution. A calibrated counting grid was used to count colonies in high-density plates and full counts were taken on plates with lower population densities. Six plates were made from the soil suspensions: three PDA plates with chloramphenicol (50 mg L^{-1}) for general fungal count and three tryptic soy agar plates with cyclohexamide (75 mg L^{-1}) for general aerobic bacteria counts. Plates were read after 24 h for fast growing species such as *P. ultimum* and as late as 72 h for slower growing species. Additionally, *Pythium* propagules were investigated also using the soil-drop technique (Stanghellini and Hancock, 1970). A sample of 1 ml of the soil suspension was removed and spotted on two water agar plates (9-12 drops per plate in a circular pattern). Plates were incubated overnight (21-26°C) and

observed under a stereomicroscope. Colonies that consisted of single hyphae with rapid growth rate and short side branches were identified as *Pythium*.

The quantitative determination of *Sclerotium rolfsii* sclerotia was attempted using the flotation sieving method (Rodriquez-Kabana *et al.*, 1974). From the bulked soils, 75 g were mixed with a molasses solution with a specific gravity of 1.07. This was achieved by adding 250 ml of molasses to 750 ml of water. The soil-molasses mixture was stirred for 30 s and then decanted through a 0.6-mm sieve to extract sclerotia. Sclerotia were then counted under a stereomicroscope. Due to low recovery rates, results are not reported here.

Field Experiment – 2000. Using the same field and experimental design as in 1999, the 2000 experiment was implemented using *Brassica* plants that overwintered in the field as a cover crop. Prior to planting *Brassica*, the site was prepared by incorporation of fertilizer (12-24-24 at a rate of 336 $kg \cdot ha^{-1}$) and medium and high pH plots were limed to maintain pH levels. Subplot treatments were modified as follows. Control plots were maintained as in the 1999 experiment with winter rye as the covercrop. Two treatment densities of Indian mustard (PI 458934) were implemented: commercial density (0.9 $g \cdot m^{-2}$) and twice commercial density (1.8 $g \cdot m^{-2}$). The fifth treatment implemented was 'Fall Raab' (*B. rapa* L. [Ruvo Group]) at commercial density (1.2 $g \cdot m^{-2}$). Winter rye was planted on control blocks to provide cover for winter and to simulate non-GS plant cover. Seeds were planted using a grain drill on September 15, 1999.

Plant stand was assessed April 2000 and shoot biomass was determined (dry and fresh weight). Cover crops were incorporated on April 20 at *Brassica* anthesis. Plant material was mowed using a flail-mower and immediately tilled into the soil. The mowing step was added to the incorporation procedure to increase homogeneity of the incorporation. The bed laying equipment followed the tiller as described in Experiment 1. Soil was bedded with trickle irrigation tape laid and the bed was covered with black plastic mulch.

Small petri dishes (35 x 10 mm) containing 4 ml of PDA or water agar were inoculated with mycelia (4.6 mm diameter plug) of *S. rolfsii* and *P. ultimum*, respectively. Plates were used as indicators of fumigation and were inserted under the plastic mulch. Additionally, five (5) sclerotia of *S. rolfsii* were placed in a mesh bag (6.25 cm^2 with 0.2 mm mesh) with 15 ml of sand (sieved to < 0.50 mm). Three plates of each pathogen and three bags with sclerotia were placed in each subplot. One week after incorporation, plates and bags were removed and growth was assessed. Sclerotia were placed on fresh PDA for germination.

Temperatures were monitored at 5 cm beneath the soil surface using Hobo XT temperature loggers (Onset, Bourne, MA) with external thermocouples placed in beds. Soil atmosphere was sampled as in the above experiment except samples were sealed in syringes and not transferred to Vacutainers. Analysis was preformed on the GC-FID under parameters described above. Soil samples were taken to

determine microbial populations as in the 1999 experiment. Treatments were assessed as in the 1999 experiment.

Field Experiment – 2001. The field was prepared for fall planting of *Brassica* treatments and rye cover by strip tilling the raised bed area of the plot. Fertilizer (10-10-10) was applied at a rate of 101 kg/ha of nitrogen. Lime was applied to plots receiving the 6.5-7 or >7 pH treatment to raise the pH 0.3 pH units. The 2001 field experiment was conducted as described for the 2000 experiment with some modifications. Biofumigation material was changed in an effort to increase cover crops survival over winter. Based on a fall 1999 over-wintering trial (data not shown), *B. juncea* L. Czerniak cultivars, 'Southern Giant Curled Mustard' and 'Florida Broadleaf,' and *B. rapa* L. (Ruvo Group) 'Fall Raab' were selected to be planted October 1, 2000 at 1.5 times commercial density. The controls were maintained as in the previous experiments. Due to unusually cold winter conditions, the 'Southern Giant' and 'Florida Broadleaf' mustards did not survive. Plots were reseeded in April 2001. Plots containing 'Fall Raab' were incorporated at anthesis on April 6, 2001 while the controls and mustards were incorporated June 20, 2001 (also at anthesis). Indicator pathogens were again placed under plastic mulch during fumigation. *Pythium* mycelia were placed in mesh bags to facilitate soil water contact with pathogens and were placed under the plastic mulch. Bags were recovered three weeks after *Brassica* incorporation. Tomato transplants were set on July 16, 2001. Soil samples were taken from the

subplots with a pH range of 6.0-7.0 to determine microbial populations mentioned above. Effects on tomato production were assessed as in previous years. Tomato fruits were harvested twice a week from September 10 to October 11. All fruit was removed on the last harvest date regardless of maturity. Number and weight of fruit were recorded by grade and statistical analyses of treatment effects on total fruit, marketable fruit (diameter >6.4 cm), and non-diseased fruit were performed.

Results and Discussion

Field Experiment 1999. Prior to treatment, the average general bacterial count for the site was $5.0 \times 10^5 \pm 1.0 \times 10^6$ CFU (colony forming units) g^{-1} of soil. The average general fungal count for the site was $1.3 \times 10^4 \pm 1.2 \times 10^4$ CFU g^{-1} of soil. Statistical differences were observed among plots before biofumigation when bacteria counts were analyzed using a logarithmic transformation ($P<0.01$). Soils in plots designated for 2X density of Indian mustard treatments, had the highest bacterial population at 8.61×10^5 CFU g^{-1}, while soils intended for ½-commerical density Indian mustard treatments exhibited 1.58×10^5 CFU g^{-1} soil (Table IV-2). Neither fungal CFU nor *Pythium* CFU was significant among the soils sampled before biofumigation treatment ($P \geq 0.10$).

For post-biofumigation microbial analysis, only soil from the 6.5-7.0 pH range and the 2X Ind. Mustard plots were selected to compare with samples from the control plots. No significant differences were detected ($P>0.10$). The mean

bacterial count in soil that received the 2X density of Indian mustard treatment was 5.4 ± 2.0 X 10^5 CFU g^{-1} soil, while the mean fungal count was 3.2 ±1.0 X 10^4 CFU g^{-1} soil. Control plots exhibited a mean bacterial population of 6.8 ± 4.7 X 10^5 CFU g^{-1} soil, and a mean fungal population of 3.6 ±1.2 X 10^4 CFU g^{-1} soil. *Pythium* CFU levels were not analyzed due to the high incidence of seedling damping off observed following tomato transplanting. Twenty-five percent of the transplants died and had to be replaced. No significant differences were detected among treatments ($P>0.50$).

Samples from the soil atmosphere, taken from all plots at 2 h after incorporation, had no detectable ITC. Additional samples were taken from high concentration plots at 24 h, 48 h and 5 d after treatment. No ITC were detected in any of the samples.

Treatments were evaluated based on tomato plant health and yield. Tomato fruit was harvested from August 5 to 27, 1999 by subplots consisting of 10 plants. All fruit was removed on the last harvest date regardless of maturity. Number of fruit, total weight of fruit and average weight per fruit were analyzed for whole-plot, subplot and interaction using the mixed procedure of SAS (SAS institute, 1999) using data for marketable fruit (diameter >6.4 cm; tray sizes 4X5, 5X6 and 6X6), non-diseased fruit, and total fruit (Table IV-3). No statistical differences in fruit yield or size were observed among the soil pH treatments, biofumigation treatments, or the interaction ($P>0.10$). A trend for soil pH to affect number of

marketable fruit was observed and was significant for non-diseased fruit number ($P< 0.05$), when analyzed independently from the *Brassica* covers (Table IV-4). Tomato plants in plots with soil pH 5.0-6.0 produced significantly less fruit than those in soil with pH >7.0. Foliar disease incidence was recorded August 27, 1999, following final harvest. No significant differences were observed for applied treatments (Table IV-5). This was expected, as foliar disease is not typically affected by soil fumigation.

The lack of response to the biofumigation treatments may be attributed to the low biomass achieved relative to the volume of soil when incorporation took place. To facilitate early planting of mustard in spring 1999, the field had been tilled, beds formed and covered in plastic mulch in fall 1998. Plastic was removed and Indian mustard seed was sown on crown of the bed. When soil was tilled during incorporation, the area worked was at least twice that covered with the Indian mustard, thus diluting the intended concentration.

Field Experiment – 2000. Indian mustard densities were low due to abnormally warm fall/winter. Plants did not acclimate to cold temperatures and were actively growing when freezing temperature occurred. The biomass present was calculated based on visual assessment of living material as a percentage of the original stand. Living plant material was collected to determine the amount of plant tissue incorporated. Subplots planted with 1X Indian mustard at the commercial rate were calculated to have produced 4.1 ± 2.6 mT of shoot (dry

wt)·ha^{-1} (18% stand), while the subplots planted at 2X Indian mustard produced 5.1 ± 4.1 mT·ha^{-1} (19% stand). The subplots planted with 'Fall Raab' at the commercial rate were less affected by climatic conditions and produced 11.1 ± 5.2 mT·ha^{-1} (91% stand). Control cover crop of rye was unaffected and yielded 9.9 ± 0.8 mT·ha^{-1} (100% stand).

Temperatures under the black plastic mulch were monitored from bed forming until tomato planting. Temperatures for the first week following incorporation were low, dropping below 20 °C as a cold front moved into the region. During the second and third week, temperatures increased to >35 °C (Fig. IV-2). Sensors were calibrated at room temperature prior to use. The temperature difference record by the two sensors may have been due to angle of solar incidence. Sensor A was place on a south-facing slope, while sensor B was in a more north-facing plot.

Bacterial counts from soil dilutions following biofumigation did not significantly differ in plots with the control cover crop of rye compared with the 'Fall Raab' or 2X Indian mustard treatments (Table IV-2). Soil treated with 2X Indian mustard had less CFU g^{-1} than soils from other treatments (P= 0.06). Fungal count for the 'Fall Raab' treated soil was significantly less (8.50 X 10^3 CFU g^{-1}) than in soil treated with 2X Indian mustard or control biofumigation treatments, 1.35 X 10^4 and 1.33 X 10^4 CFU g^{-1} soil, respectively (Table IV-2). Pythium species counts for the control were significantly higher (1160 ± 940 CFU g^{-1}) than those of the 'Fall

Raab' (500 ± 370) or 2X Indian mustard treatments (580 ± 510) ($P<0.01$) and there was no damping-off of transplants. Sampling soil atmosphere to detect ITC again proved futile. Samples taken from all plots at 2 h after incorporation showed no indication of ITC. Samples were taken from high concentration plots at 24 h, 48 h and 5 d after treatment. Additionally, samples also were taken from a depth of 10 cm at 72 h. No ITC were detected in any of the samples. Indicator plates of *Pythium ultimum* and *Sclerotium rolfsii* mycelia exhibited equal growth in all treatments. *S. rolfsii* sclerotia also exhibited no response to fumigation with 100% germination following transfer to PDA.

The inability to detect ITC and the lack of response on indicator plates may indicate that the ITC was released and trapped in the soil water phase (Tsao *et al.*, 2000). Although ITC are not extremely soluble in water, there is evidence to suggest that the released volatiles can be trapped in the soil water under field conditions and very little is released to the soil atmosphere. This principal is used by "water-capping" fumigants such as Basamid™. Another possibility is that the breakdown compounds adsorb to soil particles and organic matter (Ashley, 1963). However, Bending and Lincoln (1999) failed to detect significant levels of AITC in either soil atmosphere or in direct extraction of soil.

Tomato fruits were harvested twice a week from July 13 to August 11. All fruit were removed on the last harvest date. Yield analysis of treatment effects on total fruit, marketable fruit (diameter >6.4 cm), and non-diseased fruit was

performed (Table IV-3). Due to its high variability, the cover biomass was analyzed as a covariant and was not significant ($P > 0.50$). The interaction between pH and biofumigation treatments was also not significant ($P > 0.50$). Soil pH did not significantly affect number of fruit or total weight of fruit produced ($P > 0.05$), although a trend for increased total fruit numbers with increased pH ($P = 0.06$) was observed. The biofumigation treatments significantly affected number of fruit and total fruit weight, marketable fruit weight, and non-diseased fruit weight ($P \leq 0.01$). Tomato plants transplanted into beds fumigated with 'Fall Raab' produced significantly more marketable fruit (17.3 ± 2.2 fruit and 3.14 ± 0.49 kg fruit) than did the Indian mustard treatment (1X: 14.6 ± 3.4 fruit and 2.63 ± 0.69 kg fruit; 2X: 14.7 ± 3.4 fruit and 2.62 ± 0.50 kg fruit) or the rye control (11.9 ± 2.8 fruit and 2.18 ± 0.49 kg fruit) ($P \leq 0.05$). Tomato production, following the mustard treatments, was not significantly different from one another, but differed significantly from production on the rye control ($P \leq 0.05$). Neither pH nor biofumigation treatment affected the average fruit weight ($P > 0.50$). Physical defects of fruit and other tomato fruit diseases was not affected by treatments utilized ($P > 0.10$).

Tomato plants were assessed for disease using the Horsfall-Barratt visual rating scale and for presence of *S. rolfsii* mycelial mats. Soil pH significantly affected *S. rolfsii* disease incidence and visual rating ($P \leq 0.05$). Plots with soil pH > 7.0 exhibited less disease than plots with a pH of 5.0 to 6.0, while plots with a soil pH of 6.5 to 7.0 did not separate from either the higher or the lower pH plots.

Biofumigation treatments did not have an effect on foliar disease incidence of tomato plants (Table IV-5).

Field Experiment – 2001. The late planting of the tomato transplants, due to the failure of the mustard covercrops, appeared to have a dramatic impact on the effectiveness of the biofumigation treatments and on fruit yield and quality.

Soil microbial community analysis by soil dilution plating revealed no significant difference in fungal CFU among biofumigation treatments ($P>0.95$). Significant differences were observed for bacterial CFU among biofumigation treatments ($P<0.01$) when analyzed using logarithmic transformation (Table IV-2). Soil from plots treated with 'Fall Raab' and 'Fl. Broadleaf' had significantly lower bacterial counts ($9.92 \pm 6.18 \times 10^5$ and $6.13 \pm 2.26 \times 10^5$, respectively) than the soil treated with Southern giant curled ($1.65 \pm 0.96 \times 10^6$) and soil from the control without tomato plants ($2.42 \pm 2.65 \times 10^6$). The control plots in which tomato was grown was not different ($1.18 \pm 0.71 \times 10^6$) from either group. Plated *Pythium* CFU was also significantly different among treatments when analyzed using a square root transformation ($P<0.05$). Soil biofumigated with 'Fall Raab' had significantly less (1001 ± 635 CFU g^{-1}) than the non-*Brassica* treated soils with 2368 ± 400 and 3094 ± 80 CFU g^{-1} soil for the control with tomatoes and without tomatoes, respectively and from the 'Southern giant curled' with 2810 ± 390 (Table IV-2). Growth of indicator pathogens (*Pythium* spp and *S. rolfsii*) from mesh bags exposed to biofumigation treatments did not significantly differ among pH or

biofumigation treatments ($P<0.10$). Sclerotia placed in the treatments as indicators of effective biofumigation, also exhibited no significant differences among treatments ($P>0.05$).

Soil pH did not significantly affect number or weight of the total, marketable, or non-diseased fruit produced (Table IV-3)($P>0.10$). The biofumigation treatments did not significantly affect either number of total fruit or non-diseased fruit and did not significantly affect weight of total fruit, marketable fruit or non-diseased fruit ($P>0.10$). Number of marketable fruit was significantly affected by biofumigation ($P<0.05$). Control plots yielded approximately 7.4 fruit per plant while biofumigated plots produced 5.4-5.6 fruit per plant. The interaction between pH and biofumigation treatments was not significant ($P>0.05$). Physical defects of fruit and other tomato fruit diseases were not affected by treatments utialized ($P>0.50$).

Tomato plants were assessed for disease using the Horsfall-Barratt visual (foliar) rate scale and for presence of *S. rolfsii* mycelial mats. Neither soil pH nor biofumigation treatment significantly affected *S. rolfsii* disease incidence or visual rating ($P> 0.10$). The interaction between soil pH and biofumigation treatment was significant for foliar disease ($P<0.04$). Necrotic spotting of tomato foliage was less on plots treated with 'Fall Raab' and 'Fl. broadleaf' with soil pH of 6.5-7.0 (10.5% and 11.8%, respectively) than in plots treated with 'Southern giant curled' at 6.5-7.0 (29.5%) and 'Fall Raab' at pH >7.0 (38.8%). Disease ratings for

tomato in the 'Fall Raab' with soil pH >7.0 plots also differed significantly from the control at pH >7.0, 'Fall Raab' at pH 5.0-6.0 and from the control at pH 6.5-7.0 (Table IV-5).

The failed Indian mustard crops probably did not contribute to the biofumigation procedure. Under controlled laboratory conditions, air-dried mustard tissue evolved only 75 percent of the ITC concentration produced by freeze-dried tissue (unpublished). In nature, soil microorganisms can quickly break down glucosinolates in the dead tissues without the release of ITC, further reducing the ITC concentration available for biofumigation (Ohtsuru *et al.*, 1973; Reese *et al.*, 1958). In addition, the dead/dried biomass may have increased disease incidence by acting as host material for soilborne pathogens. This may account for the lower yields of marketable tomato fruit.

The late summer planting of the tomato transplants affected marketable fruit yield and disease pressure. The heat during early bloom and fruitset, reduced the number of fruit produced as tomato pollen loses viability at >89°F (Sato *et al.*, 2000). The irrigation was inadequate during the hottest months and the resulting water fluctuations produced growth cracks in >60% of the fruit harvested. Disease pressure was high early in the season; however, as the cooler temperatures of fall approached both, the foliar diseases and southern blight incidence decreased and plants began to recover. Any benefit from the biofumigation may have been negated with the loss of disease pressure.

Conclusions

Biofumigation with *Brassica* spp. is beneficial in tomato production under disease pressure. Although disease was not fully controlled during first two years with these treatments, yields improved substantially. This may be due to the beneficial microorganisms that are favored by the biofumigation process. However, there was no noticeable effect under the lower disease pressure conditions of fall. It appears important that biofumigation material (Indian mustard or other *Brassica*) should be incorporated as green material. Dead or dried material produce lower concentrations of the ITC needed in biofumigation. The debris may increase microbial populations and act as host for soilborne pathogens. Dynamics of the microbial community following biofumigation need further investigation. When integrated with an IPM system, biofumigation could increase control of soilborne pathogens in tomato production systems and may provide an alternative for use in organic systems.

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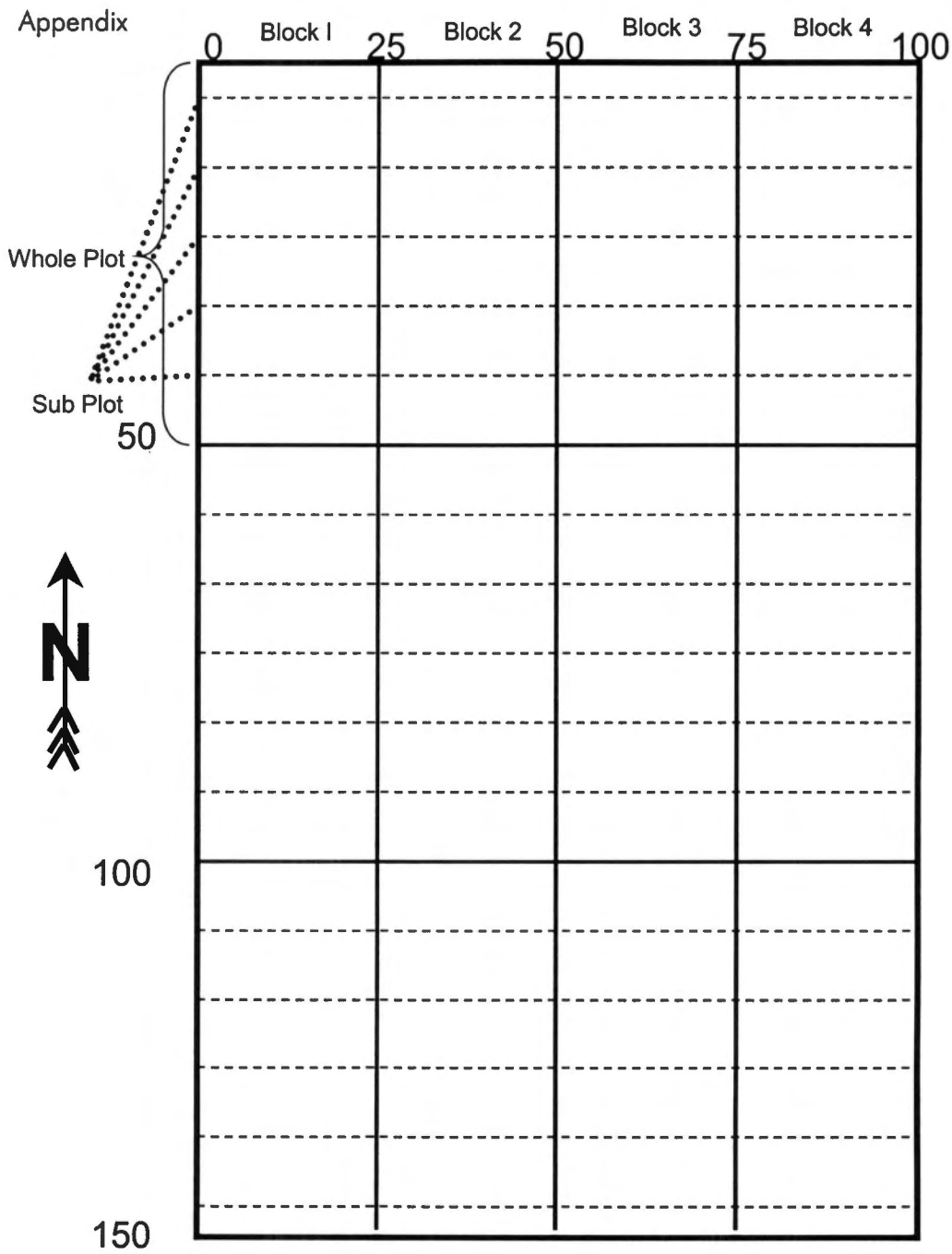

Fig. IV-1. Field design. The field is a split-plot design with pH as the whole plot treatment and the biofumigation treatments as the sub-plots.

Table IV-1. *Brassica juncea* densities. Plant counts conducted prior to incorporation Spring 1999.

Soil pH	Brassica plants (thousands per ha)		
	Low density	Medium density	High density
5.5-6.0	62	302	425
6.0-7.0	77	271	483
>7.0	78	332	486

Table IV-2. Bacterial, fungal and *Pythium* soil populations in the 6.5-7 soil pH plots

Year	Biofumigation	Colony forming units per gram soil (thousands)		
		Bacterial	Fungal	*Pythium* sp.
1999	Prior to Biofumigation:			
	½X Ind. Mustard[y]	158.43 ± 48.8 c[x]	8.22 ± 7.59	28.89 ± 23.2
	1X Ind. mustard	364.90 ± 69.8 bc	12.56 ± 11.61	33.89 ± 22.5
	2X Ind. mustard	861.04 ± 129.8 a	14.88 ± 15.48	30.00 ± 19.4
	Control with tomato	165.00 ± 73.9 c	12.28 ± 10.90	36.11 ± 20.3
	Control WITHOUT TOMATO	766.05 ± 145.5 b	16.53 ± 15.18	28.99 ± 21.7
		(P <0.01; Log[w])	(P >0.05)	(P >0.50)
1999	After Biofumigation:			
	2X Ind. mustard	536.38 ± 195.2	32.17 ± 10.0	.
	Control with tomato	676.30 ± 474.0	36.33 ± 12.2	.
		(P >0.10)	(P >0.10)	
2000	After Biofumigation:			
	2X Indian mustard	338.75 ± 69.4 b	13.50 ± 11.93 a	0.58 ± 0.51 B
	'Fall raab'	418.25 ± 127.1 a	8.50 ± 6.20 b	0.50 ± 0.37 B
	Control (rye)	413.17 ± 78.8 a	13.25 ± 10.35 a	1.16 ± 0.94 A
		(P = 0.06)	(P <0.05)	(P <0.01)
2001	After Biofumigation:			
	'Fl. Broadleaf'	613.42 ± 226.3 b	6.17 ± 7.26	1.62 ± 0.69 bc
	'Southern Giant Curled'	1650.42 ± 959.7 a	5.00 ± 4.86	2.81 ± 0.39 ab
	'Fall raab'	992.92 ± 618.1 b	5.33 ± 5.48	1.00 ± 0.64 c
	Control with tomato	1175.75 ± 712.7 ab	6.50 ± 4.10	2.37 ± 0.42 ab
	Control without tomato	2419.75 ± 2653.4 a	3.83 ± 2.89	3.09 ± 0.08 a
		(P <0.01; Log[x])	(P >0.50)	(P < 0.05; SqRoot[w])

[z] Samples were taken before biofumigation in 1999 and following biofumigation in 1999, 2000, and 2001; population estimates by soil dilution plating.

[y] Indian mustard (PI 458928) at: ½X = commercial seeding density (0.45 $g \cdot m^{-2}$); 1X=commercial density (0.9 $g \cdot m^{-2}$); 2X=twice commercial density (1.8 $g \cdot m^{-2}$).

[x] Mean separation by protected LSD (P<0.05).

[w] Log = a logarithmic transformation was necessary to normalize data; SqRoot = a square root transformation was necessary to normalize data.

Table IV-3. Quantity and weight tomato fruit. Marketable (fruit diameter > 6.43 cm) and total fruit were analyzed for 1999, 2000, and 2001. No significant interaction was observed. Letters denote mean separation within treatment type and

Treatment	Marketable Tomato Fruit			Total Tomato Fruit (per 10 plants)	
	Quantity (no. / plant)	Weight (kg/plant)	Mean Weight (g)	(No.)	Total Weight (kg)
1999					
Soil pH					
5.0-6.0	8.41 ± 1.36 [z] a	1.27 ± 0.25 a	150.5 ± 9.8	14.44 ± 2.00 a	1.72 ± 0.26 a
6.5-7.0	9.61 ± 2.16 a	1.45 ± 0.34 a	151.2 ± 8.7	15.43 ± 3.44 a	1.91 ± 0.44 a
> 7.0	10.52 ± 3.24 a	1.58 ± 0.57 a	148.2 ± 10.5	16.22 ± 2.82 a	2.00 ± 0.55 a
	(P ≥0.10)	(P ≥0.10)		(P ≥0.10)	(P ≥0.50)
Biofumigation Cover					
High density	9.61 ± 3.09 a	1.44 ± 0.52 a	148.7 ± 10.6	15.96 ± 3.43 a	1.92 ± 0.53 a
Medium density	9.58 ± 2.42 a	1.44 ± 0.42 a	149.1 ± 9.2	14.96 ± 3.19 a	1.83 ± 0.48 a
Low density	9.52 ± 2.56 a	1.44 ± 0.43 a	150.3 ± 8.9	15.73 ± 2.28 a	1.92 ± 0.40 a
No cover	9.35 ± 2.12 a	1.42 ± 0.35 a	151.8 ± 10.7	14.79 ± 2.56 a	1.83 ± 0.38 a
	(P ≥0.50)	(P ≥0.50)		(P ≥0.50)	(P ≥0.50)
2000					
Soil pH					
5.0-6.0	13.61 ± 3.77 a	2.44 ± 0.67 a	172.6 ± 46.0	28.49 ± 5.68 b	3.69 ± 0.9 b
6.5-7.0	14.75 ± 2.97 a	2.64 ± 0.55 a	190.4 ± 52.0	31.37 ± 5.58 ab	4.46 ± 0.9 ab
> 7.0	15.51 ± 3.29 a	2.85 ± 0.64 a	156.9 ± 32.7	32.92 ± 6.08 a	4.77 ± 0.9 a
	(P ≥0.10)	(P ≥0.10)		(P ≤0.01)	(P ≤0.05)
Biofumigation Cover					
1X Ind. Mustard	14.57 ± 3.41 b	2.63 ± 0.69 b	170.7 ± 42.7	31.54 ± 5.17 a	4.40 ± 1.0 b
2X Ind. Mustard	14.66 ± 2.69 b	2.62 ± 0.50 b	170.2 ± 42.7	31.68 ± 6.36 a	4.37 ± 0.8 b
Fall Raab	17.33 ± 2.23 a	3.14 ± 0.49 a	178.4 ± 45.4	34.77 ± 4.25 a	5.09 ± 0.8 a
Rye (Control)	11.93 ± 2.83 c	2.18 ± 0.49 c	173.9 ± 55.6	25.73 ± 4.43 b	3.72 ± 0.8 b
	(P ≤0.01)	(P ≤0.01)		(P ≤0.01)	(P ≤0.01)
2001					
Soil pH					
5.0-6.0	5.66 ± 1.91 a	1.02 ± 0.34 a	180.5 ±12.9	23.77 ± 4.36 b	4.08 ±0.6 a
6.5-7.0	6.23 ± 2.85 a	1.20 ± 0.69 a	191.3 ± 55.1	23.20 ± 4.92 ab	4.11 ±0.9 a
> 7.0	5.84 ± 2.40 a	1.04 ± 0.42 a	177.6 ±11.2	24.38 ± 3.82 a	4.38 ±1.1 a
	(P ≥0.50)	(P ≥0.50)		(P ≥0510)	(P ≥0.10)
Biofumigation Cover					
Fl. Broadleaf	5.68 ± 2.09 b	1.02 ± 0.35 a	181.5 ±12.6	22.45 ± 4.01 a	3.90 ±0.6 a
S.Giant Curled	5.31 ± 1.68 b	1.11 ± 0.69 a	199.8 ±62.1	24.07 ± 2.65 a	4.38 ±0.8 a
Fall Raab	5.31 ± 2.18 b	0.93 ± 0.38 a	175.6 ±11.1	22.77 ± 3.77 a	4.01 ±0.8 a
Rye control	7.34 ± 3.02 a	1.28 ± 0.51 a	175.6 ±11.3	25.85 ± 5.89 b	4.46 ±1.2 a
	(P ≤0.05)	(P ≥0.10)		(P ≥0.10)	(P ≥0.10)

[z] Mean separation by LSD (P ≤0.05).

Table IV-4. Effect of soil pH on tomato fruit size and yield, 1999.

Whole plot treatment	Fruit Yield		Average Fruit
Soil pH	(no. / plant)	(kg / plant)	(g/fruit)
5.0 - 6.0	12.7 ± 2.2 b[y]	1.05 ± 0.14	84.4 ± 13.4
6.0 - 7.0	14.3 ± 3.4 ab	1.06 ± 0.25	75.3 ± 17.3
> 7.0	15.4 ± 2.8 a	1.11 ± 0.21	72.8 ± 11.3
	($P < 0.05$)	(NS)	(NS)

[y] LSD mean separation ($P < 0.05$) was used to determine groupings.
[NS] Not Significant.

Table IV-5. Foliar disease (%) incidence in 1999, 2000 and 2001 was analyzed for soil pH and *Brassica* cover density. Interactions between soil pH and biofumigation treatments were significant in 2001 ($P < 0.05$). Letters represent mean separation.

Year	Biofumigation	Disease(%) Soil pH 5.0-6.0	Soil pH 6.5-7.0	Soil pH > 7.0	Across pH
1999					
	½X Ind. mustard[z]	22.5 ± 14.1	16.1 ± 14.7	13.5 ± 12.7	17.4 ± 13.1
	1X Ind. mustard	18.1 ± 13.2	18.3 ± 22.5	9.9 ± 3.5	15.4 ± 14.3
	2X Ind. mustard	20.5 ± 17.2	12.2 ± 7.5	12.3 ± 13.4	15.0 ± 12.7
	None	19.8 ± 3.6	10.5 ± 8.5	10.3 ± 5.3	13.5 ± 7.2
	Across Biofumigation	20.2 ± 11.8	14.3 ± 13.4	11.5 ± 8.9	
2000					
	1X Indian mustard	76.1 ± 11.4	70.5 ± 24.2	53.1 ± 33.2	66.6 ± 25.6
	2X Indian mustard	77.8 ± 21.1	64.4 ± 22.6	66.3 ± 19.3	69.5 ± 21.0
	'Fall raab'	74.3 ± 18.4	61.9 ± 27.4	54.9 ± 22.4	63.7 ± 23.5
	Control (Rye)	70.1 ± 35.4	71.3 ± 20.2	66.9 ± 22.1	69.4 ± 25.6
	Across Biofumigation	74.6 ± 22.3	67.0 ± 22.9	60.3 ± 24.4	
2001	'Fl. Broadleaf'	21.8 ± 12.0 abc[y]	11.8 ± 5.5 c	21.8 ± 12.0 abc	18.4 ± 10.5
	'Southern Giant Curled'	21.8 ± 12.0 abc	29.5 ± 17.0 ab	21.8 ± 12.0 abc	24.3 ± 13.1
	'Fall raab'	17.3 ± 5.5 bc	10.5 ± 6.8 c	38.8 ± 28.4 a	22.2 ± 20.0
	Control (Rye)	21.8 ± 12.0 abc	19.0 ± 13.7 bc	12.0 ± 9.2 bc	17.6 ± 11.5
	Across Biofumigation	20.6 ± 9.8	17.7 ± 13.1	23.6 ± 18.3	

[z] Indian mustard (PI 458928) at: ½X = commercial density (0.45 $g \cdot m^{-2}$); 1X=commercial density (0.9 $g \cdot m^{-2}$); 2X=twice commercial density (1.8 $g \cdot m^{-2}$).

[y] Letters represent mean separation across rows and columns; mean separation by protected LSD ($P<0.05$).

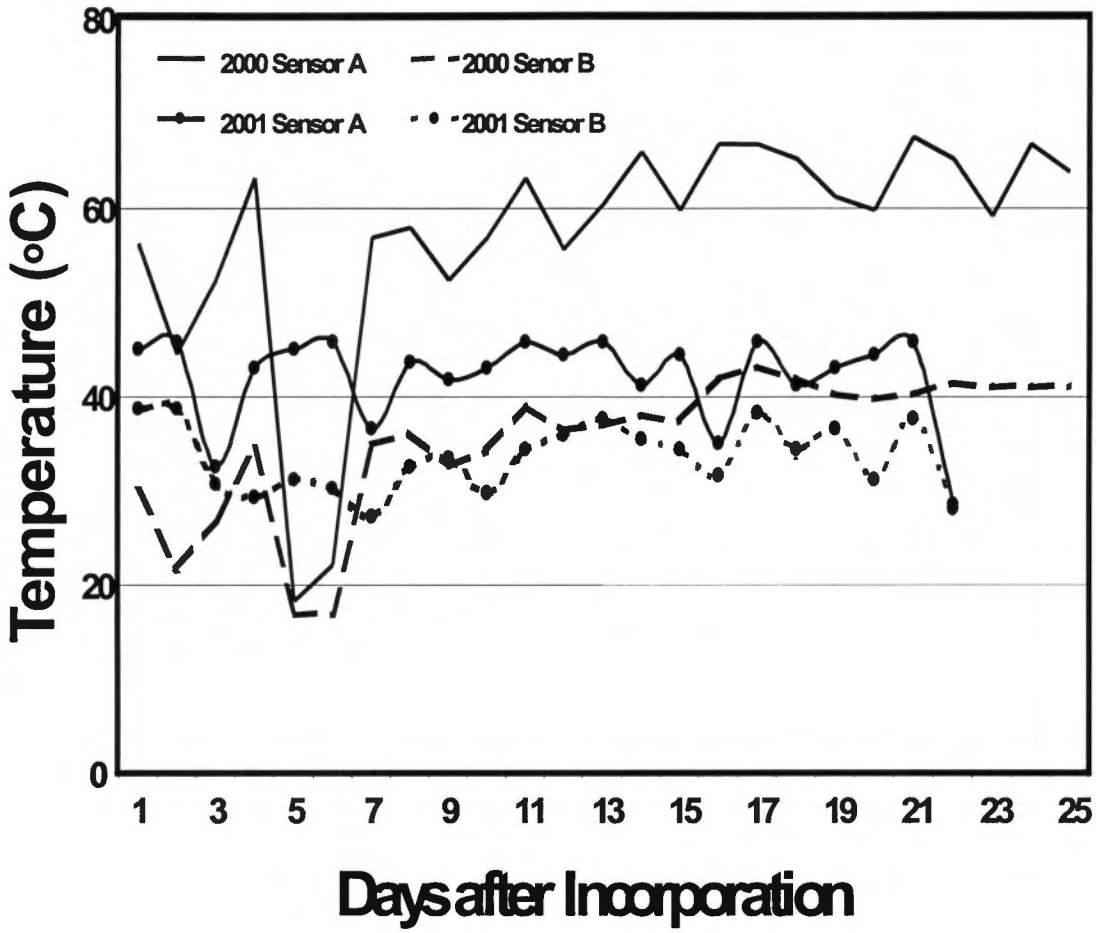

Fig. IV-2. Soil temperatures for 2000 and 2001. Soil temperatures recorded 5 cm beneath black plastic mulch during biofumigation.

Fig. IV-3. *Sclerotium rolfsii* Sacc. mycelia growing at the base of an infected tomato plant (photo by Martin Lyons).

Part V

Capillary Electrophoretic Separation of Glucosinolates from Seed of Selected Plant Species and the Relative Toxicity of the Corresponding Isothiocyanates to Four Important Plant Pathogens

Abstract

Glucosinolates (GS) are secondary plant metabolites involved in plant defense. A highly variable side chain results in over 120 identified GS in a diverse group of plants including members of the Brassicaceae, Capparaceae, Phytolaccaceae and Resedaceae. When hydrolyzed by the enzyme myrosinase, GS produce D-glucose, sulfate, isothiocyanates, thiocyanates, and nitriles. Isothiocyanates (ITC), the predominante breakdown product at physiological pH, have been shown to have biocidal activity. The objectives of this study were: 1) identification and quantitation of intact GS from seeds using capillary electrophoresis (CE), 2) comparing the relative toxicity of extracts from GS contain seed in a bioassay and 3) comparison of the relative toxicity of ITC in a bioassay. Seeds were extracted using 95 °C water and then processed using solid phase extraction. Aliquots of each extract were analyzed on an HP3D capillary electrophoresis system using MECC separation techniques and on an HPLC using the ISO desulfo-GS method. Crude seed extracts (100 μL) and myrosinase (50 μL) were applied to potato dextrose agar along with a *Sclerotium rolfsii* mycelial plug. Plates were incubated and growth was determined at 24 h. Plates treated with crude seed extracts from *Brassica oleracea* L. (Germmifera Group), *B. napus* L. (Neobrassica Group) and *Hesperis matronalis* L. did not exhibit growth significantly different from the control. All other extracts demonstrated significant inhibition of

mycelial growth with > 20 percent suppression ($P< 0.05$). *Lepidium sativum* L. exhibited the most activity with 93.7% inhibition of mycelial growth.

Six ITC were applied in a bioassay against *Botrytis cinera, Pythium myriotylum, Penicillium expansum, Sclerotium rolfsii* and *Agrobacterium tumefaciens*. Methyl ITC exhibited the best control of *B. cinerea* with an IC_{90} (concentration producing 90% inhibition) of 153 μMoles mL^{-1}. Phenethyl ITC inhibited growth of *P. myriotylum, S. rolfsii* and *P. expansum* at low concentrations with IC_{90}s of 11-, 27-, and 22 μMoles mL^{-1}, respectively. *Agrobacterium tumefaciens* was less sucseptable to the tested ITC. Phenethyl ITC exhibited inhibitory activiety with an IC_{90} at 213 μMoles mL^{-1}.

Introduction

Isothiocyanates (ITC) exhibit a variety of biological activities that have drawn the interest of scientists for many decades. In recent years more emphasize has been placed on the investigation of natural and synthesized ITC for both their toxicity and anti-carcinogenic properties. Isothiocyanates are one of the breakdown products resulting for the enzymatic degradation of glucosinolates (GS). Glucosinolates are secondary plant metabolites that are thought to be involved in plant defense. All members of the plant family Brassicaceae contain GS. Although not as pervasive, GS can be found in members of other families, such as Euphorbaceae and Phytolaccaeae. In excess of 200 naturally occurring GS have

been identified. The concentrations and types of GS vary among species and cultivars, presenting a vast array of distinct GS and therefore distinct ITC profiles.

The use of plants containing GS for control of soilborne pests has potential. Isothiocyanates have been demonstrated to control fungi (Harvey *et al.*, 2002; Charron and Sams, 1999; Sarwar *et al.*, 1998; Mayton *et al.*, 1996), bacteria (Delaquis and Mazza, 1995), nematodes (Mojtahedi *et al.*, 1993 and 1991) and some weed seeds in laboratory experiments (Al-Khatib, *et al.*, 1997). To improve the results achieved from "biofumigation" the relative lethality of various glucosinolates needs to be investigated.

Plant Pathogens. The pathogens selected for this study represent several types of plant pathogens of economic importance in agriculture: *Pythium myriotylum* Drechs., *Sclerotium rolfsii* Sacc., *Botrytis cinerea* Pers.: Fr., *Penicillium expansum* Link, and *Agrobacterium tumefaciens* (Smith and Townsend) Conn.

Pythium myriotylum is one of several species of *Pythium* that affects agricultural crops by producing both pre- and post-emergence damping-off. The taproots, root tips and feeder roots of mature plants may be attacked, stunting plants and reducing yields (Schlub and Lockwood, 1981). *Pythium* exists as thread-like strands of cells called hyphae that can grow into a thick mat referred to as a mycelium. A soilborne pathogen, *Pythium* often persists in the soil or in crop residue. However, it is frequently associated with streams and lakes, which can serve as a source of inoculum.

Sclerotium rolfsii, also a soilborne pathogen, has an extensive host range of more than 500 plant species, including both dicotyledons and monocotyledons (Singleton *et al.*, 1992). The fungus causes "Southern Blight" (seedling damping-off, blight and stem rot) throughout the tropics, subtropics and in areas of the southern and southeastern United States where high temperatures permit its growth and survival. It is also a soilborne plant pathogen. A dense white mycelial mat is often visible at the base of infected plants. Infected plants appear to wilt suddenly and die. Sclerotia, the overwintering structure of *S. rolfsii*, may also be visible at the soil/stem interface.

Gray mold, caused by *Botrytis cinerea,* is of significant economic importantance. Unlike many of the host specific *Botrytis* species, *B. cinerea* has an extensive host range of over 200 species and impacts yields under field, greenhouse, and storage conditions (Chardonnet *et al.*, 2000; Rosslenbroich and Stuebler, 2000). *Botrytis* overwinters as sclerotia on dead tissue. In the spring during cool humid weather, spores form and spread, by wind or water, to wounded or soft plant tissues. In addition to spore-induced infections, growth of fungal strands from previously infected plant parts can play an important role in gray mold occurance. The fungus can survive on decaying vegetation, so it can infect healthy plants throughout the growing season. Thriving in cool, damp environments, *B. cinerea* is one of the leading causes of post-harvest loss.

Blue mold, like gray mold, is primarily a post-harvestdisease (Walker, 1969). *Penicillium expansum*, the causative agent, is not typically a problem in orchards. A wound parasite, spores often infects the fruit following mechanical damage during handling. Aged fruit also is softer and more susceptible to penetration. Storage temperatures facilitate rapid colonization and fruit rot. .

Agrobacterium tumefaciens is a pathogen that causes the production of galls in many dicotyledons. The bacterium survives in the soil on plant debris and infects its host through wounds. The bacteria induce galls or tumors on the roots, crowns, trunks, and canes of infected plants (Stafford, 2000). These galls interfere with water and nutrient flow in the plants. Severely infected plants may become weakened, stunted, and unproductive.

The objectives of this study were: 1) identification and quantitation of intact GS using capillary electrophoresis (CE) and high performance liquid chromatography (HPLC), and 2) comparison of the relative toxicity of the GS breakdown products against five plant pathogens.

Materials and Methods

Capillary Electrophoresis. The extraction and separation of intact GS using capillary electrophoresis (CD) require three steps: (1) crude extraction, (2) clean-up using solid phase extractions (SPE), and (3) separation using CE. The extraction procedure for intact GS is a modification of the method outlined by Declercq and

Daun (1989). Seed material was selected based on GS content as reported in the literature (Table V-1)(Daxenbichler *et al.*, 1991; Fahey *et al.*, 2001). All seeds were obtained from commercial sources. Seeds were stored at 5 °C until used.

Crude extraction began with 200 mg of seed being weighed into a test tube. Four milliliters of boiling water were added to the sample, which was then ground using a Polytron tissue homogenizer (Brinkmann Instruments, Inc., Westbury, NY). The tube was placed in a heating block at 95°C for 15 min and 1 mL of an internal standard (1 mM Benzyl GS or 1 mM Allyl GS) was added to the mixture. Both types of GS are available commercially and the one not found in a specific seed was used. The tube was capped and quickly vortexed. Then samples were returned to a heating block for an additional 3 min. Each sample was allowed to cool and then centrifuged at 900 g. Resulting supernatant was transferred to a graduated centrifuge tube containing 150 μL of 0.5 M barium/lead acetate solution. The pellet was extracted with an additional 4 mL of boiling water for 3 min at 95°C, allowed to cool, and then centrifuged at 900 g. Supernatants from the two extraction steps were combined and the volume adjusted to 10 mL. The mixture was centrifuged (900 g) and supernatant was retained for GS purification.

Purification of crude extracts was carried out using ion exchange chromatography on a prepared DEAE-Sephadex A-25 column. Dry DEAE (100 mg) was weighed into a syringe (0.8 X 4 cm) and hydrated with deionized water,

and stirred to remove air bubbles. Columns were constructed with bed volumes of 15 mm X 8 mm and reservoir at the top of column. Five milliliters of 0.05 N sodium hydroxide were passed through the columns, and then was followed by 10.0 mL water, prior to sample application. Elution was checked to make sure the pH was neutral. Columns were changed to acetate form by the addition of 5.0 mL of 0.5 M pyridine acetate solution. Ten mL deionized water was washed through the column, leaving a meniscus of water on the top of the column for sample application.

From the retained supernatant, 3.0 mL of the extract was added to the prepared columns. Columns were washed with a total of 4.0 mL water, 4.0 mL 30% formic acid and then 4.0 mL water. The eluate was discarded each time. The GS were eluted with 9.5 mL of 0.3 M potassium sulfate then adjusted to 10.0 mL.

Separation of Glucosinolate. Separation of intact GS was preformed by modifing the MECC (micellar electorkinetic capillary chromatography) method described by Michaelsen *et al*. (1992). Run buffers were prepared from the following stock solutions: (1) Sodium tetraborate - 100 mM, (2) Sodium phosphate - 150 mM, and (3) CTAB (hexadecyl-trimethylammonium bromide)- 100mM. From the above stocks, a run buffer of 9 mM tetraborate, 15 mM phosphate and 25 mM CTAB was created and adjusted to pH 7.0. The buffer was filtered through a 0.45-μm-membrane filter prior to use. Separation was performed utilizing a Hewlett-Packard (Palo Alto, CA) 3D CE in MECC mode. GS elutioned from the DEAE

column were injected by pressure (50 mbars) for 10 seconds. Separation was preformed at 30°C and 20 kV, with negative polarity at the injection end. Ultraviolet (UV) detection at 235 nm using a deuterium lamp was utilized, however, a complete spectrum was recorded. The capillary column was 720 mm in total length and the length from injection end to detector was 500 mm. The capillary was washed with buffer between each analysis for 4-7 min and was washed for 2-4 min. with 1.0 and 0.1 M NaOH after 5 runs, daily. Quantitation of the response from each GS detected was calculated based on the internal standards. Tentative identification was based on comparison of relative retention times.

Seed profiles were verified using the desulfo-GS method for high performance liquid chromatograph (HPLC) published by Spinks *et al.* (1984). Analysis was preformed on Hewlett Packard 1050 HPLC (Palo Alto, CA) on a Hypersil C18 column 250 x 4.6 mm (Phenomenex, Torrance, CA).

Bioassay for Lethality. A series of experiments were conducted to determine the biocidal potential of identified glucosinolates. In the first experiment, crude seed extracts were applied in a growth inhibition assay on *S. rolfsii*. Mycelial plugs (4.5-cm dia) of S. rolfsii were placed on potato dextrose agar (PDA) in microplates (35 x 15 mm). Filter sterilized (0.02 μm) seed extracts (100 μL) were applied to the PDA, 1 cm from the plug. To illicit production of the potentially toxic ITC, 30 μg myrosinase (aqueous) (Sigma-Aldrich; St. Louis, MO) was pipetted into the seed extracts on PDA. The controls for this assay consisted of non-treated mycelial plugs

and plugs treated with Indian mustard seed extract but without the addition of the myrosinase and plugs treated with myrosinase only. Plates were incubated at 30 °C for 42 h. Results were recorded as net radial growth. Inhibition (%) was calculated as difference from the control. Statistical analysis was performed using a mixed procedure of SAS (2002).

In the second experiment, individual isothiocyanates of interest were purchased from LKT Laboratories, Inc (St. Paul, MN) and Aldrich (St. Louis, MO). The original goal of extracting the intact GS to obtain the ITC was an exhaustive process and was deemed unnecessary when the ITC became commercially available. Brassinin, a non-isothiocyanate phytoalexin from cabbage, also was included in this study (Table V-2).

Botrytis cinerea strain ATCC 90870 was provided by Dr William S. Conway (Horticultural Crops Quality Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture. Beltsville, MD). *Sclerotium rolfsii* was cultured from an infected tomato plant, grown at the Knoxville Experiment Station, and was stored as sclerotia until needed. *Penicillium expansum* was isolated from apples and maintained in the laboratory. *Pythium myriotylum* and *Agrobacterium tumefaciens* strain C58 were obtained from Dr. Bonnie H. Ownley (Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN).

Test compounds were dissolved in dimethyl sulfoxide (DMSO) and multiple dilutions (Table V-3) were made to achieve a range of concentrations. Dilutions were placed in an airtight vials with Teflon lined lids and stored at 0 °C prior to use. Small petri plates containing 4.5 mL of potato dextrose agar (PDA) or clarified V-8 agar were used in this assay. Hyphal plugs (4.5-cm dia) were cut from the growing margins of pathogen cultures. *Sclerotium rolfsii, P. expansum* and *B. cinerea* plugs were placed on PDA, while *P. myriotylum* plugs were placed on clarified V-8 agar. After placing a hyphal plug in the center of the plate, 10 μL of the diluted compound was pipetted onto the agar 1 cm from the plug. The process was repeated for each dilution and pathogen. Three replications were conducted.

The first assay (assay 1) was conducted with only allyl ITC (AITC) against *P. expansum* and *S. rolfsii* to determine any potential problems. Five-, 10-, 20-, 30-, and 40 μL of AITC were diluted to 1 mL with DMSO. After placing the hyphal plugs, 10 μL was pipetted onto the plate as described above. This affected a series of treatments of AITC at 0.11-, 0.22-, 0.44-, 0.64- and 0.87 mMoles mL^{-1}. Control plates receiving no treatment compounds and plates receiving DMSO also were included. Plates were incubated at 21 °C. Mycelial growth was measured after 24 h for *S. rolfsii* and after 48 h for *P. expansum*. Radial growth was determined by measuring the diameter of the mycelium less the initial plug diameter and then dividing by two. Inhibition was calculated as the difference in radial growth as a percentage of the control. The experimental design consisted of

a completely randomized design with dosage nested within tested compound and three replications. Data were analyzed using the mixed procedure of SAS (2002).

The second assay was conducted in a similar manner. Benzyl, 3-phenylproply and phenyl ITC were diluted (w/v) to produce dilution of 502-, 50.2-, 5.0-, 0.05 mMoles mL^{-1} and resulted in dosages of 1115.6-, 111.6-, 11.2-, 0.1 μMoles mL^{-1} agar. As in assay 1, the resulting dilutions were tested against both *S. rolfsii* and *P. expansum*. The experimental design consisted of a completely randomized design with dosage nested within tested compound and three replications. The data were analyzed using the mixed procedure of SAS (2002).

The third assay consisted of the pathogens *P. myriotylum and B. cinerea*. The compounds assayed for inhibitory activity included: methyl ITC, allyl ITC, 3-indomethanol, erucin, cheirolin, berteroin, brassinin and alyssin. Each compound was weighed and diluted to the desired concentrations of 1-, 10-, 100-, and 1000 μMoles mL^{-1}. The application of 10 μL resulted in treatments of 0.002-, 0.02-, 0.22-, and 2.22 μMoles mL^{-1}, respectively. For *B. cinerea*, allyl ITC dosages did not include a 0.002 μMoles mL^{-1} concentration and 3-indomethanol dilutions consisted of only 2.2- and 0.22 μMoles mL^{-1} concentration. The same dosages were used for the *P. myriotylum* plates except that the allyl ITC and methyl ITC included only the 0.22 μMoles mL^{-1} concentration. Growth was determined after 24 h for *P. myriotylum* and after 48 h for *B. cinerea*. Statistical analysis was performed as stated above for assay 2.

Based on the results of assay 2 and assay 3, compounds and concentrations were selected for a fourth assay including *B. cinerea, P. myriotylum, P. expansum* and *S. rolfsii.* Allyl-, methyl-, and phenethyl ITC were weighed and diluted to the desired concentrations of 1-, 10-, 50-, 100-, and 1000 mMoles mL^{-1} with the application of 10 μL per 4.5 mL of agar. This resulted in treatment dosages of 2.2-, 22.2-, 111.1-, 222.2-, and 2222.2 μMoles mL^{-1}, respectively. Due to limited availability, cheirolin treatment dosages were limited to 2.2-, 22.2-, 111.1-, and 177.6 μMoles mL^{-1}. Brassinin dosages were limited to 2.2-, 22.2-, and 111.1 μMoles mL^{-1}, while erucin was tested at the limited dosages of 2.2-, 22.2-, and 35.6 μMoles mL^{-1}. Brassinin, cheirolin and erucin dilutions were made at the highest concentration possible with the limited quantity of the standards available. Lower concentrations were necessary due to the limited amount of the standards available. Growth was determined after 24 h for *P. myriotylum* and after 48 h for *B. cinerea, P. expansum* and *S. rolfsii.* Concentrations responsible for 50 and 90 % inhibition (IC_{50} and IC_{90}, respectively) were calculated for each compound and pathogen using regression statistics (SigmaPlot, 2000).

Based on assay 4, three combinations were investigated for potential synergistic activity for the pathogens *B. cinerea,* and *S. rolfsii.* The two compounds with greatest inhibition were added to the plates at 5 μL each. The same was done for the two compounds with the least inhibitory activity. The third combination consisted of one high and one low inhibition compound. To look for synergistic

activity, the pathogens also were treated with the individual compounds for comparision. The combinations were selected specifically for each pathogen and concentrations were chosen to produce less than optimal inhibition such that synergistic activity could be more readily assessed (Table V-3).

With the same concentration used in assay 4, a fifth assay was developed to investigate the toxicity of these compounds on *A. tumefaciens*. This was accomplished using liquid culture and measuring the absorbance. A single *A. tumefaciens* colony from a streak-plate was transferred to an Erlenmeyer flask containing 50 mL of nutrient broth (NB). The flask was gently shaken for 48 h at room temperature (~20 °C). Culture tubes containing 9 mL of NB were sterilized and 980 μL of inoculum (2.3×10^7 colony forming units (CFU) mL^{-1}) were added to each tube. After vortexing to disperse the bacteria, 20 μL of the diluted test compounds were injected. Mixtures were again vortexed, and then gently shaken for 24 h.

Inoculum concentration was determined via serial dilution and plating on NB agar plates. Bacterial growth was measured using a Shimadzu UV-260 spectrophotometer (Columbia, MD.) as a function of absorbance at a wavelength of 500 nm. As with the fungi, synergistic interactions were investigated with *A. tumefaciens* in a subsequent assay (Table V-3).

Results

Seed Glucosinolate Content. Capillary electrophoretic separation of intact GS produced results similar to the desulfo-GS method on HPLC (Table V-4). The extract from collard seed produced the highest concentration of GS at 241.5 μMole g $^{-1}$ seed weight with the majority being epiprogoitrin and sinigrin (allyl GS). Watercress seed also yielded a high GS concentration of 224.5 μMole g $^{-1}$. Gluconasturtiin comprised 88% of the watercress GS profile. Some seed extracts, such as radish and Brussels sprouts, had diverse profiles consisting of over six identified GS. In contrast, profiles of extracts from peppergrass and caper bush seeds showed little diversity with only one GS detected.

Bioassay for Lethality. The extracts from seed of GS containing plants inhibited mycelial growth of *S. rolfsii* (Fig. V-1). Peppergrass was the most effective ($P<0.01$), inhibiting growth 99.1 % compared to the control. Uplandcress, watercress and 'Fall Raab' were effective at inhibiting over 50% of the mycelial growth. The extracts from arugula, woad, radish, caper, English wallflower, alyssum, turnip, weld, watercress and 'Fall Raab' exhibited more than 20% inhibition of mycelial growth. Extracts from seed of rutabaga, dame's rocket and Brussels sprouts had the least activity with less than 10% inhibition and were not significantly different than the control ($P>0.05$).

Assay 1 was used primarily to determine if DMSO exhibited any inhibitory activity on growth of the pathogens. Mycelial growth of *P. expansum* among

control plugs and DMSO treated plugs were not significantly different ($P< 0.001$). *Sclerotium rolfsii* plugs had 10% more growth when treated with DMSO ($P<0.01$). The AITC treatments significantly impacted mycelial growth of both pathogens across dosage ($P<0.001$). Dosages in excess of 652 μMoles mL^{-1} caused growth inhibition of 95% for *S. rolfsii*. *Penicillium expansum* experienced only 63 % inhibition with 652 μMole mL^{-1}.

The four compounds selected for use in assay 2 have a similar chemical structure, differing only in a methyl addition (Fig V-2). However, significant differences among the treatments were observed. Benzyl ITC provided the best control with 100% inhibition of mycelial growth from *Sclerotium rolfsii* plugs across all dosages (Table V-5). Phenethyl and 3-phenylpropyl ITC had inhibition levels significantly different from benzyl and phenyl ITC, inhibiting growth by 78.2 ± 28.3 and 71.4 ± 36.7%, respectively, across dosages ($P< 0.05$). Phenyl ITC exhibited the least amount of activity, inhibiting growth by 41.7 ± 37.5% across dosage levels (Table V-5). A dosage response was observed for these ITC with the exception of Benzyl ITC (due to the 100% inhibition).

Similar results were obtained when these compounds were tested on *P. expansum* (Table V-6). *Penicillium expansum* appeared more resistant to phenethyl, phenyl, and 3-phenylproply ITC than did *S. rolfsii*. Benzyl ITC provided 100% control of mycelial growth across all dosages and was significantly different from the other compounds ($P< 0.05$). Although phenyl ITC exhibited the least

amount of activity, inhibiting growth by only 33.8 ± 38.4% across dosages, its level of inhibition was not significantly different from phenethyl and 3-phenylpropyl. These ITC had inhibition across dosages at 64.2 ± 39.5 and 59.2 ± 42.87%, respectively. A dosage response within treatment was observed except with benzyl ITC.

The dilutions selected for the compounds in the third assay produced less inhibition of *P. myriotylum* and *B. cinerea* than expected (Table V-7). Of the eight compounds assayed against *P. myriotylum,* only cheirolin and erucin caused inhibition (16.3 ± 13.7 and 17.2 ± 8.0 %, respectively), across dosages, significantly different from the control ($P<0.05$). Against *B. cinerea,* only methyl and allyl ITC elicited levels of inhibition significantly different from the control (16.6 ± 14.4 and 22.5 ± 11.8 %, respectively) when examined across all dosages ($P<0.05$). Even at the highest dosage level, few treatments separated from the control. The 2.22 μMole mL^{-1} dose of erucin provided the best control of *P. myriotylum* with 22.2 ± 1.4 % inhibition. All erucin treatments produced significantly more inhibition than the control. In addition, cheirolin at 0.22 - and 2.22 μMoles mL^{-1}, and brassinin at 2.22 μMoles mL^{-1} suppressed growth significantly more than the control. Results from *Botrytis cinerea* appear incongruous at best. For example the 0.02- and 0.22 μMoles mL^{-1} allyl ITC treatments inhibited mycelial growth by 25.3 ± 6.5 and 31.9 ± 8.3 %, respectively, while the 2.22 μMoles mL^{-1} treatment yielded only 10.2 ± 8.4 % control and did not differ significantly from the control ($P>0.05$).

Berteroin had a similar response; 0.02 μMoles mL^{-1} inhibited growth by 26.5 ± 5.5 %, while 2.22 μMoles mL^{-1} only induced 4.3 ± 7.0 % inhibition. These erratic results may be the result of the near-threshold levels used in this assay. Difference in response may represent genetic and environmental difference in the specific plugs used in the assay. The extremely low dosages may have magnified these differences.

The fourth assay conducted utilized compounds that had demonstrated activity in one of the previous assays. Selected compounds were applied to all four fungal pathogens. *Botrytis cinerea* was sensitive to the allyl, methyl, and phenethyl treatments (Fig. V-3; Table V-8). They suppressed radial growth significantly across dosage levels ($P<0.05$). Plugs treated with 222.2- and 2222.2 μMoles mL^{-1} of allyl ITC exhibited 80.2 ± 24.5 and 100.0 ±0.0 %, respectively. Brassinin at111.1 μMoles mL^{-1} significantly affected mycelial growth by reducing it 37.7 ± 9.4 %. Both methyl and phenethyl ITC treatments at dosages of 111.1-, 222.2-, and 2222.2 μMoles mL^{-1} significantly reduced growth of *B. cinerea* relatived to the control ($P<0.05$).

All compounds, at all dosages reduced *P. expansum* growth significantly (Fig. V-3; Table V-8; P. <0.05). At the lowest application of 2.2 μMoles mL^{-1}, these compounds suppressed mycelial growth by 71-78 %. Complete suppression (100%) was achieved at 111.1 μMoles mL^{-1} of phenethyl ITC and at 222.2 μMoles mL^{-1} of allyl and methyl ITC.

Pythium myriotylulm mycelial growth was suppressed by all of the compounds across dosage levels ($P<0.05$). Only allyl ITC, brassinin and methyl ITC at the 2.2 μMoles mL $^{-1}$ dose did not significantly reduce pathogen growth (Fig. V-4; Table V-8). Treatments applied in excess of 222.2 μMoles mL $^{-1}$ induced 100% inhibition. Cheirolin, erucin and phenethyl ITC provided the greatest level of suppression at the low dosage of 22.2 μMoles mL $^{-1}$ with inhibition of 84.8 ± 26.3, 93.4 ± 3.7 and 100.0 ± 0.0 %, respectively.

Mycelial growth of *S. rolfsii* was suppressed by all of the compounds across dosage levels ($P<0.05$). A dosage effect however, was observed (Fig V-4; Table V-8). Erucin produced the greatest level of suppression (81.8 ± 4.7 %) at the lower dosage of22.2 μMoles mL $^{-1}$, while phenethyl ITC affected 75.7 ± 20.1% inhibition. The other compounds demonstrated less than 20 % inhibition at the same dosage.

Agrobacterium tumefaciens was utilized also in this series of assays. Allyl, methyl and phenethyl ITC, as well as cheirolin treated cultures, exhibited suppressed growth, and differed significantly from the control cultures across dosage (Fig. V-5; $P<0.05$). Phenethyl ITC exhibited the most control of bacterial growth with 48.0 ± 4.4 % inhibition at the 111.1 μMoles mL^{-1} dosage and 92.5 ± 0.3 % at 222.2 μMoles mL^{-1} dosage. Allyl ITC also demonstrated control of bacterial growth at the 222.2- and 2222.2 μMoles mL^{-1} dosages, inhibiting bacterial growth 41.0 ± 3.1 and 93.6 ± 0.5 %, respectively (Table V-9).

The assay to investigate potential synergistic interactions was completed using the treatments listed in Table V-3. When 55.6μMoles mL $^{-1}$ each of methyl and allyl ITC were added to a *B. cinerea* plate, inhibition increased 86.5 % compared to either compound separately at 111.1 μMoles mL $^{-1}$. The combination of 88.8 μMoles mL $^{-1}$ cheirolin and 17.6 μMoles mL $^{-1}$ erucin increased inhibition by 6.6 % over the individual compounds at 177.6 and 35.2 μMoles mL $^{-1}$, respectively. Only a 2 % increase was observed with the 55.6 μMoles mL $^{-1}$ methyl ITC –and 88.80 μMoles mL $^{-1}$ cheirolin combination. Two of the combinations tested against *S. rolfsii* caused a marked increase in inhibition. The combination of 55.6 μMoles mL $^{-1}$ brassinin and 11.2 μMoles mL $^{-1}$ phenethyl caused a 12 % increase in inhibition. The combination of 55.6 μMoles mL $^{-1}$methyl ITC and 55.6 μMoles mL $^{-1}$brassinin demonstrated a 144 % increase in inhibition over the individual compounds. Increased inhibition was also caused by the combinations tested against *A. tumefaciens*. While the combination of 55.6 μMoles mL $^{-1}$ phenethyl ITC and 55.6 μMoles mL^{-1} brassinin and the combination 111.1 μMoles mL $^{-1}$ allyl and 55.6 μMoles mL $^{-1}$ phenethyl ITC produced a modest 8 % increase in inhibition, the combination of 55.6 μMoles mL $^{-1}$brassinin and 55.6 μMoles mL $^{-1}$ cheirolin affected a 90% increase in inhibition.

Regression statistics were used to calculate the IC_{50} and IC_{90} for each compound against each pathogen from assay 4 (Table V-10). As expected, the pathogens had different sensitivities to the different compounds. Methyl ITC was

the most effective inhibitor of *B. cinerea* mycelial growth with an IC_{90} of 153 μMoles mL^{-1}. In contrast, it would require 253 μMoles mL^{-1} allyl ITC and 483 μMoles mL^{-1} of phenethyl ITC to obtain similar levels of suppression. *Botrytis cinerea* was however the exception to the rule; the other pathogens are highly sensitive to phenethyl. Less than 30 μMoles mL^{-1} of phenethyl ITC were need to produce 90% inhibition of the fungi, *P. myriotylum, P. expansum* and *S .rolfsii*. They in turn were more tolerant of methyl ITC. For both *P. expansum* and *S. rolfsii*, the IC_{90} for methyl ITC was in excess of 250 μMoles mL^{-1}. *Agrobacterium tumefaciens* was also more sensitive to phenethyl ITC (IC_{90} = 213 μMoles mL^{-1}) than to methyl ITC (IC_{90} = 2095 μMoles mL^{-1}).

During data collection, it was noted that brassinin induced asymmetric growth in *B. cinerea* (Fig. V-6a). To determine if this was a diffusion phenomenon, plates were treated with 2.5 μL of 50 mMoles mL^{-1} brassinin at each cardinal point. The resulting mycelial growth was symmetric and was not significantly different from the single 10 μL treatment application. It also was noted that although the radial growth was not significantly different, erucin and cheirolin did affect hyphal morphlogy of *B. cincerea* and *S.rolfsii*. Botrytis treated with erucin and cheirolin exhibited a reduced mycelial mat (Fig. V-6b).

Conclusions

Glucosinolates show extensive genetic variation within and among plant species due the heterogenous selection pressure from the generalist insect herbivores and pathogens (Daxenbichler *et al.*, 1991; Halkier and Du, 1997; Kliebenstein *et al.*, 2001). The resulting breakdown products including isothiocyanates exhibit an equally extensive diversity. The toxicity of the ITC can vary greatly from benign to highly toxic. There appears to be some specificity to the ITC. Phenethyl ITC, for example, was highly effective in controlling Penicillium expansum, Pythium myriotylum and Sclerotium rolfsii, concentration 40 times stronger were needed to control B. cinerea mycelial growth.

The differences in inhibitory activity among the ITC tested against these five pathogens suggest that there might be benefits to using mixtures of plant material. A diverse selection of ITC may provide better control soilborne pathogens and other pests than a single species biofumigation cover. In field settings, with multiple plant pathogens and pest present, a mixture of ITC would increase the spectrum of effectiveness. In addition, the potential additive and synergistic effects of mixtures may provide further protection. The composition of plant glucosinolates profiles may be more important than total GS concentration (Rask et al., 2000). Further investigation is needed to understand these reactions.

The ban of methyl bromide evinced agriculture dependence on synthesized chemicals for control of agronomic pests. The effort to find alternatives has increased the interest in non-synthetic alternatives. Biofumigation is one potential alternative. Identifying naturally occurring compounds with biocidal activty can help improve the viablity of this system. Breeding to modify GS content in an effort to increase efficacy of biofumigation is ongoing (Brown et al., 2001). Knowledge concerning toxicity, specificity, and synergistic reactions will help direct these efforts. The vast array of naturally occurring GS, and their corresponding ITC, are a resource that is just beginning to be investigated.

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Table V-1. Species selected for extraction of seed glucosinolates.

Common Name	Cultivar	Genus	Species	Authority
Alyssum	Saxatile	*Lobularia*	*maritima*	(L.) Desv.
Arugula		*Eruca*	*vesicaria ssp.* sativa	(L.) Cav.
Broccoli	Decicco	*Brassica*	*oleracea* (Botrytis Group)	L.
Brussels sprouts	Long Island	*Brassica*	*oleracea* (Gemmifer Group)	L.
Cabbage	Late Flat Dutch	*Brassica*	*oleracea* (Capitata Group)	L.
Caper Bush		*Capparis*	*spinosa ssp.* inermis	L.
Collards	Vates	*Brassica*	*oleracea* (Acephala Group)	L.
Dames Rocket		*Hesperis*	*matronalis*	L.
English Wallflower		*Cheiranthus*	*cheiri*	L.
Fall Raab	Salade	*Brassica*	*rapa* (Ruvo Group)	L.
Kohlrabi	Early Vienna Purple	*Brassica*	*oleracea* (Gongylodes Group)	L.
Mustard	Florida Broadleaf	*Brassica*	*juncea*	(L.) Czern.
Mustard	Southern Giant Curled	*Brassica*	*juncea*	(L.) Czern.
Peppergrass		*Lepidium*	*sativum*	L.
Pokeweed		*Phytolacca*	*americana*	L.
Radish	Scarlet Globe	*Raphanus*	*sativus* (Radicula Group)	L.
Radish	White Icicle	*Raphanus*	*sativus*	L.
Rutabaga		*Brassica*	*Napus* (Neobrassica Group)	L.
Turnip	Seven Top	*Brassica*	*rapa* (Rapifera Group)	L.
Upland Cress		*Barbarea*	*verna*	(Miller) Asch.
Watercress		*Nasturtium*	*officinalis*	L.
Weld		*Reseda*	*luteola*	L.
Woad		*Isatis*	*tinctoria*	L.

Table V-2. Compounds selected for use in toxicity assay and their glucosinolate progenitor.

Compounds	Glucosinolate Progenitor	Formula Weight (amu)
Allyl ITC[z]	2-Propenyl GS (Sinigrin)	99.1
Alyssin	5-(Methylsulfinyl)pentyl GS	191.3
Benzyl ITC	Benzyl GS (Glucotropaeolin)	196.1
Berteroin	5-(Methylthio)pentyl GS	175.4
Cheirolin	3-(Methylsulfonyl)propyl GS	179.3
Erucin	4-(methylthio)butyl GS	193.3
3-Indomethanol	Indole-3-ylmethyl GS (Glucobrassicin)	147.2
Methyl ITC	Chemically synthesized	73.0
Phenyl ITC	Phenyl GS	135.9
Phenethyl ITC	2-Phenylethyl GS (Gluconasturtiin)	163.2
3-Phenylpropyl	Chemically synthesized	177.3
Brassinin	Non-Isothiocyanate phytoalexin	236.4

[z] ITC= Isothiocyantate.

Table V-3. Combinations of chemicals and their concentrations evaluated for synergistic interactions.

Inhibitory activity	Assay Pathogens					
	Botrytis cinerea		*Sclerotium rolfsii*		*Agrobacterium tumefaciens*	
	Chemical	Conc. (μMoles mL^{-1})	Chemical	Conc. (μMoles mL^{-1})	Chemical	Conc. (μMoles mL^{-1})
High	Methyl ITC[z]	55.6	Phenethyl	11.2	Phenethyl	55.6
	Allyl ITC	55.6	Erucin	17.6	Allyl ITC	111.1
Low	Cheirolin	88.8	Methyl ITC	55.6	Brassinin	55.6
	Erucin	17.6	Brassinin	55.6	Cheirolin	55.6
Mixed	Methyl ITC	111.1	Phenethyl	11.2	Phenethy	55.6
	Cheirolin	88.8	Brassinin	55.6	Brassinin	55.6

[z] ITC = Isothiocyanate

Table V-4. Glucosinolate content of seeds.

	Glucosinolates[z] (μMoles g^{-1})								
	Benzyl	Brassicanapin	Gluco-barbarin	Gluco-capparin	Glucoerucin	Gluco-brassicin	Gluco-hesperin	Glucoibarin	Gluconapin
Alyssum	1.0	25.2	-	-	-	-	36.8	-	48.2
Arugula	3.4	-	-	-	114.3	-	-	-	-
Broccoli	4.7	-	-	-	-	3.9	-	-	-
Brussels sprouts	1.4	4.8	-	-	-	8.4	-	-	4.5
Cabbage	-	11.2	-	-	-	16.1	-	-	-
Caper	-	-	-	106.1	-	-	-	-	-
Collards	3.9	-	-	-	-	6.2	-	-	10.6
English Wallflower	2.7	25.8	-	-	-	-	-	-	-
Fall Raab	4.3	22.9	-	-	-	-	-	-	82.3
Fl. Broadleaf	3.7	-	-	-	-	-	-	-	-
Kohlrabi	4.5	11.2	-	-	-	6.2	-	-	-
Peppergrass	56.2	-	-	-	-	-	-	-	-
Pokeweed	3.4	-	-	-	-	-	-	-	-
Radish	1.6	-	-	-	-	-	-	-	2.9
Rutabaga	2.2	4.5	-	-	-	-	-	-	6.2
Dame's Rocket	50.3	-	-	-	-	-	-	-	10.3
Turnip	3.7	20.0	-	-	-	8.9	-	-	101.9
Uplandcress	1.2	-	-	-	-	-	-	-	-
Watercress	3.7	-	-	-	-	-	-	23.1	-
Weld	0.7	-	35.3	-	-	-	-	-	-
Woad	3.9	-	-	-	-	-	-	-	6.5

Table V-4. (continued).

continued	Glucosinolates (μMoles g^{-1})								
	Gluco-nasturtiin	Goitrins	Neogluco-brassicin	Raphanin	Raphanen	Sinalbin	Singrin	Unknown peak 1	Total Glucosinolates
Alyssum	56.1	-	-	-	-	-	-	-	167.3
Arugula	-	-	-	-	-	-	-	-	117.7
Broccoli	-	-	-	171.3	-	-	-	5.6	185.5
Brussels sprouts	-	97.1[y]	-	-	-	-	34.8	-	151.0
Cabbage	-	125.4	-	-	-	-	72.6	-	225.3
Caper	-	-	-	-	-	-	-	-	106.1
Collards	-	134.9	-	-	-	-	85.8	-	241.4
English Wallflower	-	-	-	-	-	-	-	-	28.5
Fall Raab	-	-	-	-	-	-	-	-	109.5
Fl. Broadleaf	-	-	-	-	-	-	86.4	17.7	107.8
Kohlrabi	-	178.6[y]	-	-	-	-	3.8	-	204.3
Peppergrass	-	-	-	-	-	-	-	-	56.2
Pokeweed	-	-	-	-	-	-	-	-	3.4
Radish	5.1	119.5	10.1	-	45.8	-	-	35.6	220.6
Rutabaga	-	79.6	3.4	-	-	-	-	-	95.9
Dame's Rocket	83.5	-	-	-	-	-	-	-	144.1
Turnip	-	-	-	-	-	-	-	-	134.5
Uplandcress	134.8	-	-	-	-	-	-	-	136.0
Watercress	197.7	-	-	-	-	-	-	-	224.5
Weld	-	-	-	-	-	-	-	-	36.0
Woad	-	10.2	4.2	-	-	11.5	-	-	36.3

[z] Concentrations of glucosinolates from seed extractions were determined using the HPLC desulfoglucosinolate method.

[y] Concentration may represent a combination of glucoiberin and goitrins.

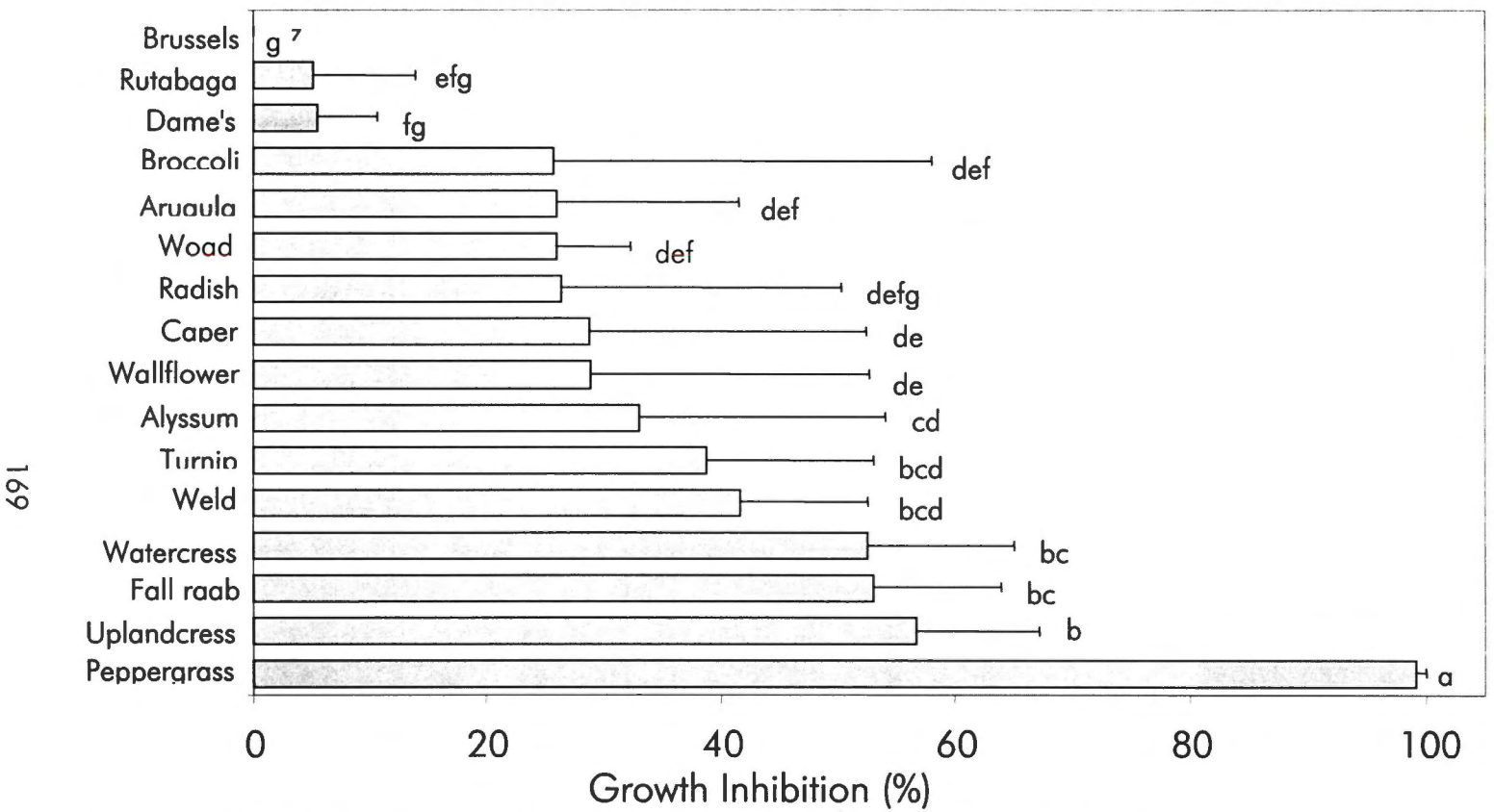


Figure V-1. Inhibition of *Sclerotium rolfsii* mycelial growth by seed extracts from 16 species

[z] Letters represent mean separation by LSD (P <0.05); bar represent standard error.

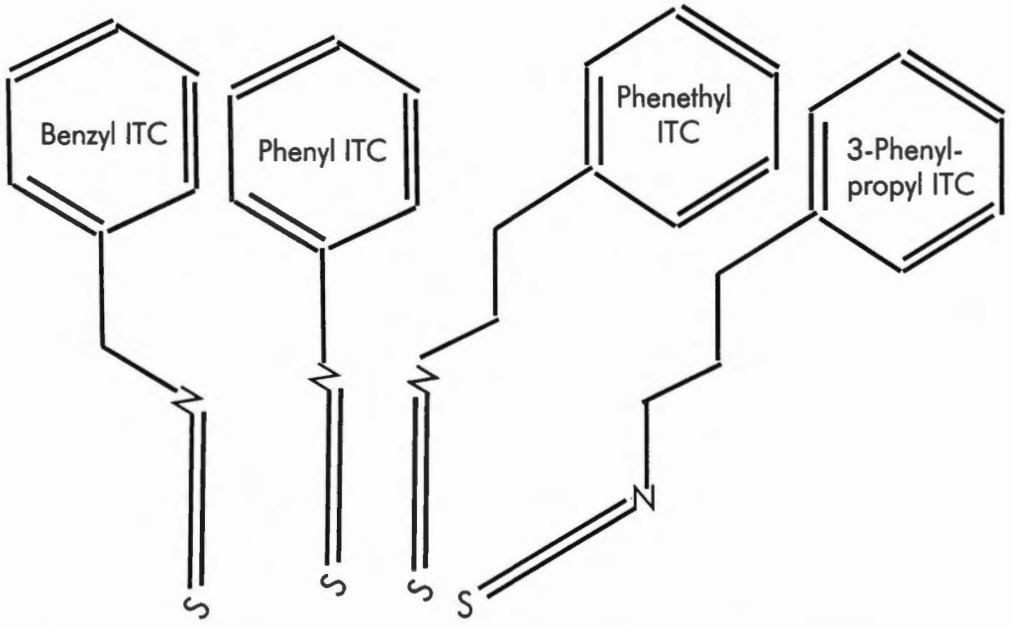


Fig. V-2. Molecular structure of 4 isothiocyanates (ITC) used in assay 2.

Table V-5. Lethality of 3-phenyl, phenethyl, phenyl and benzyl isothiocyantes (ITC) to *Sclerotium rolfsii*.

Concentration (μMoles)	Mycelium inhibition (%)			
	Benzyl ITC	Phenethyl ITC	Phenyl ITC	3-Phenylproply ITC
0.1	-	39.1 ± 20.8 c	8.3 ± 4.9 e	17.4 ± 5.2 d
11.2	100 ± 0.0 a[z]	73.6 ± 11.5 ab	19.2 ± 12.6 d	68.3 ± 23.1 b
111.6	100 ± 0.0 a	100.0 ± 0.0 a	39.5 ± 4.5 c	100.0 ± 0.0 a
1115.6	100 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Mean	100 ± 0.0 A[y]	78.2 ± 21.0 B	41.7 ± 37.5 C	71.4 ± 36.7 B

[z] Lower case letters represent mean separation among dose and ITC by LSD ($P<0.05$) using the mixed procedure of SAS (2002)

[y] Upper case letters represent mean separation for each ITC across dose by LSD ($P<0.05$) using the mixed procedure of SAS (2002)

Table V-6. Lethality of 3-phenyl, phenethyl, phenyl and benzyl isothiocyantes (ITC)to *Penicillium expansum*.

Concentration	Mycelial inhibition (%)			
(μMoles)	Benzyl ITC	Phenethyl ITC	Phenyl ITC	3-Phenylproply ITC
0.1	-	11.5 ± 2.3 bc	10.8 ± 4.9 bc	13.5 ± 2.5 c
11.2	100 ± 0.0 a[z]	45.3 ± 5.9 ab	8.8 ± 6.8 bc	23.2 ± 3.0 bc
111.6	100 ± 0.0 a	100.0 ± 0.0 a	19.0 ± 2.0 bc	100.0 ± 0.0 a
1115.6	100 ± 0.0 a	100.0 ± 0.0 a	96.7 ± 5.7 ab	100.0 ± 0.0 a
Mean	100 ± 0.0 A[y]	64.2 ± 39.5 B	33.8 ± 38.38 B	59.2 ± 42.8 B

[z] Lower case letters represent mean separation among dose and ITC by LSD ($P<0.05$) using the mixed procedure of SAS (2002)

[y] Upper case letters represent mean separation for each ITC across dose by LSD ($P<0.05$) using the mixed procedure of SAS (2002)

Table V-7. Lethality of eight compounds to *Pythium myriotylum* and *Botrytis cinerea*

Pathogen	Compound	Mycelial inhibition (%)			
		0.002 (μMoles mL^{-1})	0.02 (μMoles mL^{-1})	0.22 (μMoles mL^{-1})	2.2 (μMoles mL^{-1})
Pythium myriotylum	Allyl ITC	-	-	10.7 ± 4.1	-
	Alyssin	0.5 ± 0.8	9.2 ± 7.4	5.4 ± 2.5	7.4 ± 6.9
	Berteroin	2.3 ± 3.9	1.2 ± 1.1	8.0 ± 8.1	8.1 ± 13.3
	Brassinin	4.2 ± 3.8	0.8 ± 1.3	1.8 ± 3.1	17.0 ± 4.0 *
	Cheirolin	6.3 ± 7.3	10.9 ± 3.3	13.4 ± 6.6 *	34.7 ± 14.6 *
	Erucin	13.9 ± 5.0 *	13.6 ± 12.6 *	19.3 ± 9.4 *	22.7 ± 1.4 *
	3-Indomethanol	-	-	11.6 ± 9.3	4.4 ± 3.8
	Methyl ITC	-	-	3.3 ± 3.4	-
Botrytis cinerea					
	Allyl ITC	-	25.3 ± 6.5 *	31.9 ± 8.3 *	10.2 ± 8.4
	Alyssin	13.1 ± 6.2	12.5 ± 13.9	2.3 ± 4.0	13.6 ± 14.9
	Berteroin	8.4 ± 5.9	26.5 ± 5.5 *	1.1 ± 1.4	4.3 ± 7.0
	Brassinin	3.6 ± 6.2	14.0 ± 8.7	16.5 ± 14.4	18.3 ± 3.5 *
	Cheirolin	1.3 ± 1.7	0.2 ± 0.3	5.7 ± 5.6	23.5 ± 8.6 *
	Erucin	5.4 ± 4.9	2.0 ± 3.0	2.0 ± 3.4	11.8 ± 5.8
	3-Indomethanol	-	-	0.9 ± 1.6	3.9 ± 5.9
	Methyl ITC	26.2 ± 15.1 *	21.3 ± 2.8 *	11.6 ± 20.2	7.3 ± 12.7

* Identifies treatments with net growth significantly different from the control ($P<0.05$).

Table V-8. Lethality of selected compounds to *Botrytis cinerea*, *Pythium myriotylum*, *Sclerotium rolfsii* and *Penicillium expansum*.

Pathogen	Compound		Inhibition (%) 2.2 (μMoles mL^{-1})	22.2 (μMoles mL^{-1})	111.1 (μMoles mL^{-1})	222.2 (μMoles mL^{-1})	2222 (μMoles mL^{-1})
Botrytis cinerea	Allyl ITC	A[z]	1.1 ± 2.0 ef	2.8 ± 4.2 def	18.7 ± 16.5 c	80.2 ± 24.5 a	100.0 ± 0.0 a
	Brassinin	C	0.0 ± 0.0 f	4.0 ± 4.2 de	37.7 ± 9.4 b	-	-
	Cheirolin	D	0.0 ± 0.0 f	0.0 ± 0.0 f	0.0 ± 0.0 f	5.0 ± 0.8[y] d	-
	Erucin	D	0.0 ± 0.0 f	3.5 ± 6.0 def	3.6 ± 3.2[w] de	-	-
	Methyl ITC	A	0.8 ± 1.4 ef	0.5 ± 0.9 ef	50.9 ± 11.8 b	98.5 ± 2.6 a	100.0 ± 0.0 a
	Phenethyl ITC	B	0.0 ± 0.0 f	0.2 ± 0.3 ef	15.9 ± 5.7 c	46.4 ± 14.5 b	94.5 ± 9.5 a
Pythium myriotylum	Allyl ITC	C	4.5 ± 1.7 f	33.7 ± 13.1 d	88.8 ± 9.8 a	100.0 ± 0.0 a	100.0 ± 0.0 a
	Brassinin	E	0.2 ± 0.3 g	16.8 ± 1.4 e	57.7 ± 2.3 c	-	-
	Cheirolin	B	15.3 ± 6.4 ab	84.8 ± 26.3 a	100.0 ± 0.0 a	100.0 ± 0.0[y]a	-
	Erucin	B	17.3 ± 3.7 e	93.4 ± 3.7 a	97.1 ± 5.0[w] a	-	-
	Methyl ITC	D	4.1 ± 1.8 f	19.4 ± 3.8 e	70.8 ± 9.7 bc	100.0 ± 0.0 a	100.0 ± 0.0 a
	Phenethyl ITC	A	17.6 ± 7.9 e	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Penicillium expansum	Allyl ITC	C	25.3 ± 7.9 j	38.1 ± 9.7 h	60.9 ± 6.4 e	83.0 ± 6.9 cd	100.0 ± 0.0 a
	Brassinin	E	26.0 ± 5.9 j	33.5 ± 5.9 hij	48.1 ± 8.4 g	-	-
	Cheirolin	D	27.3 ± 0.6 ij	36.1 ± 3.4 hi	51.4 ± 4.9 fg	53.7 ± 5.0[y] efg	-
	Erucin	B	29.9 ± 3.7 hij	78.1 ± 9.2 d	94.5 ± 5.0[w] ab	-	-
	Methyl ITC	C	27.9 ± 5.6 ij	29.9 ± 5.7 hij	60.2 ± 10.8 ef	78.5 ± 3.9 d	100.0 ± 0.0 a
	Phenethyl ITC	A	34.4 ± 4.3 hij	90.5 ± 4.4 bc	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Sclerotium rolfsii	Allyl ITC	C	3.3 ± 4.7 hi	2.7 ± 2.6 i	41.4 ± 3.7 fg	89.4 ± 4.7 ab	100.0 ± 0.0 a
	Brassinin	E	0.0 ± 0.0 i	16.1 ± 9.5 h	48.4 ± 11.1 f	-	-
	Cheirolin	D	5.5 ± 5.3 hi	12.5 ± 6.9 hi	69.2 ± 9.1 de	63.2 ± 7.4[y] e	-
	Erucin	B	3.4 ± 5.3 hi	81.8 ± 4.7 bcd	84.5 ± 22.6[w] bc	-	-
	Methyl ITC	CD	0.0 ± 0.0 i	0.2 ± 0.3 i	30.4 ± 9.9 g	79.6 ± 6.7 bcd	100.0 ± 0.0 a
	Phenethyl ITC	A	3.4 ± 5.9 hi	75.7 ± 20.1 cde	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a

[z] *Botrytis* mean separation preformed using square root transformation; *Pythium* transformation by exponent 0.40; LSD ($P<0.05$);
[y] Cheirolin was applied at 177.6 μMoles mL^{-1} instead of 222.2 μMoles mL^{-1}.
[w] Erucin was applied at 35.2 μMoles mL^{-1} instead of 111.1 μMoles mL^{-1}.
* Identifies treatments with net growth significantly different from the control ($P<0.05$).

Table V-9. Lethality of selected compounds against *Agrobacterium tumefaciens*.

Compound	Mycelial inhibition (%)				
	2.2 (μMoles mL^{-1})	22.2 (μMoles mL^{-1})	111.1 (μMoles mL^{-1})	222.2 (μMoles mL^{-1})	2222.2 (μMoles mL^{-1})
Allyl ITC	0.2 ± 0.3	7.8 ± 4.1	9.1 ± 2.7	41.0 ± 3.1*	93.6 ± 0.5*
Brassinin	0.6 ± 1.0	14.6 ± 3.7*	27.4 ± 4.7*	-	-
Cheirolin	13.4 ± 2.7	5.1 ± 3.0	20.2 ± 5.6*	40.6 ± 10.2[z]*	-
Erucin	2.2 ± 2.6	16.1 ± 3.4	23.2 ± 5.7[y]*	-	-
Methyl ITC	2.0 ± 3.4	2.3 ± 2.7	10.8 ± 2.5	18.7 ± 2.0*	92.3 ± 1.2*
Phenethyl ITC	6.5 ± 4.0	22.4 ± 4.3*	48.0 ± 4.4*	92.5 ± 0.3*	93.1 ± 0.0 *

[z] Cheirolin was applied at 177.6 μMoles mL^{-1}instead of 222.2 μMoles mL^{-1}.
[y] Erucin was applied at 35.2 μMoles mL^{-1}instead of 111.1 μMoles mL^{-1}.
* Identifies treatments with net growth significantly different from the control ($P<0.05$).

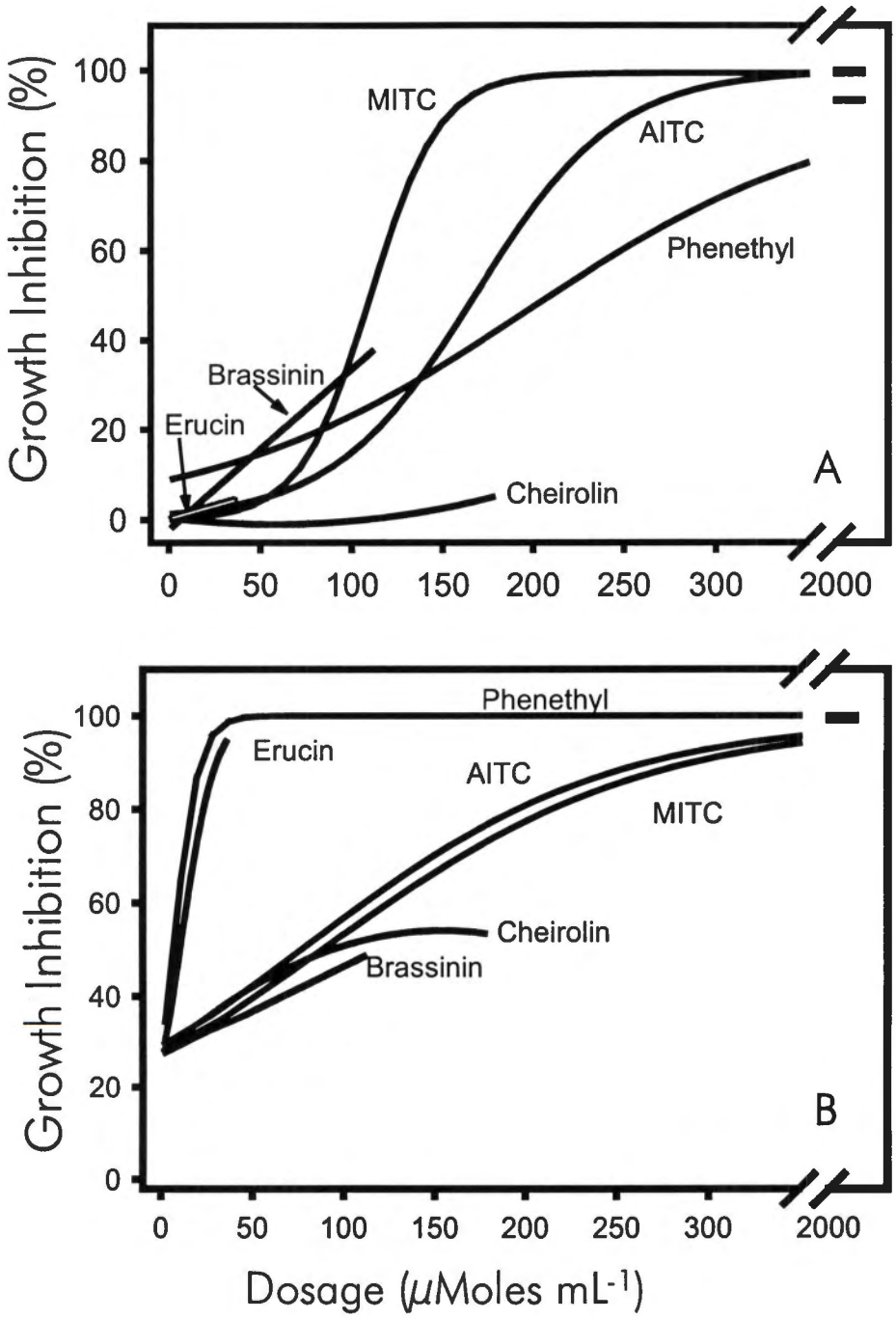


Fig. V-3. Dosage response of *Botrytis cinerea* (A) and *Penicillium expansum* (B) to selected isothiocyanates and brassinin.

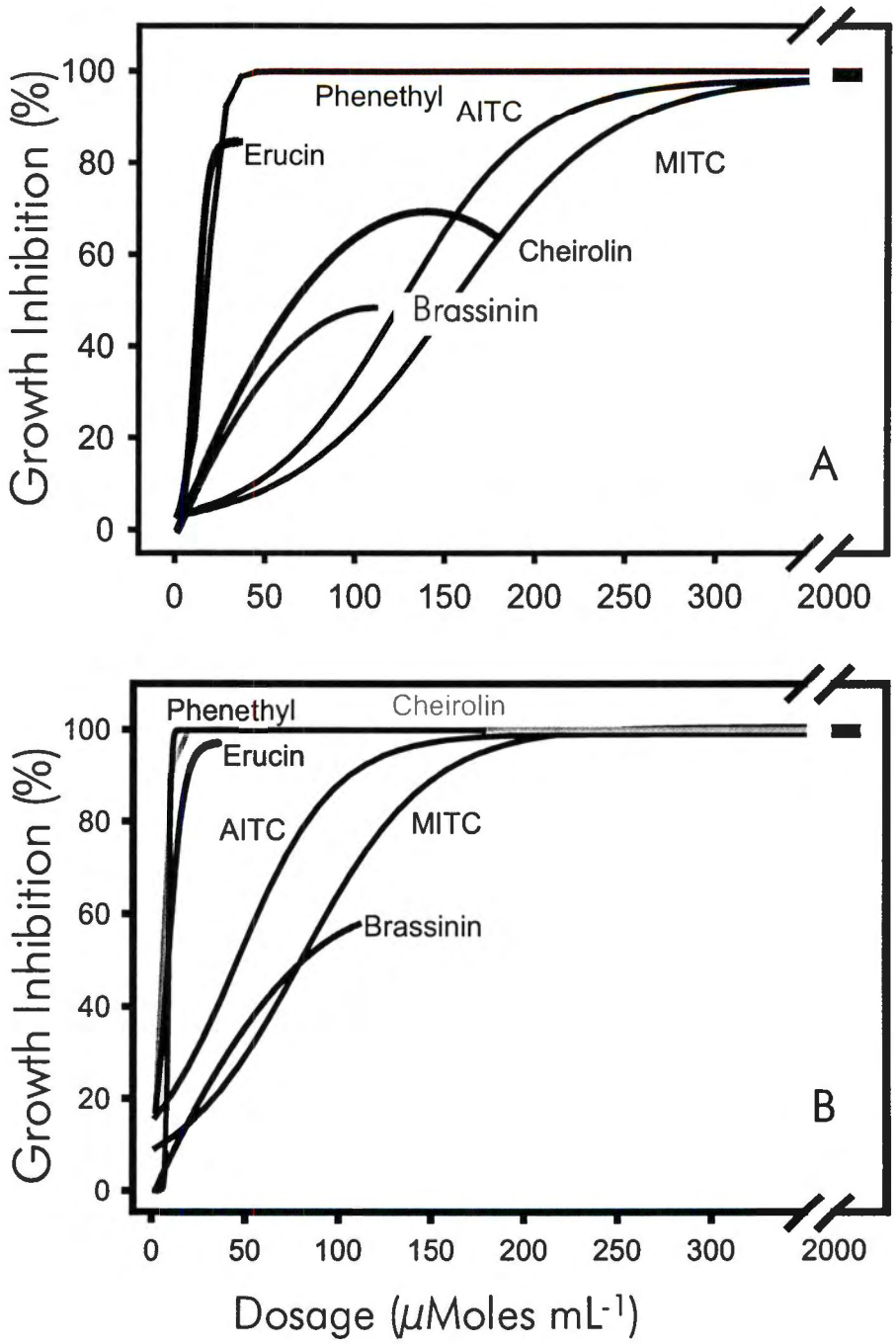


Fig. V-4. Dosage response of *Sclerotium rolfsii* (A) and *Pythium myriotylum* (B) to selected isothiocyanates and brassinin.

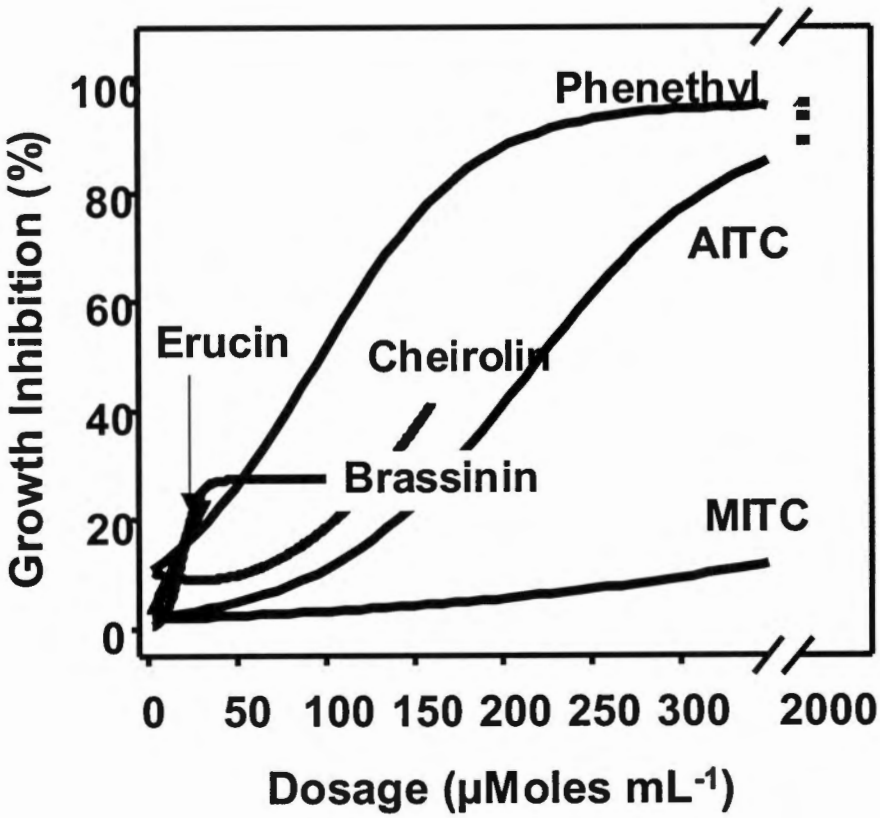


Fig. V-5. Dosage responses of *Agrobacterium tumefaciens* to selected isothiocyanates and brassinin.

Table V-10. Calculated[z] chemical concentrations needed to produce 50 and 90% growth inhibition (IC_{50} and IC_{90}).

Pathogen	Compound	IC_{50} (μMoles mL^{-1})	IC_{90} (μMoles mL^{-1})	Equation	R^2
Botrytis cinerea	Allyl ITC	168	253	Sigmoid[y]	0.94
	Brassinin	15	26	Linear[x]	0.92
	Cheirolin	408	526	Quadratic[w]	0.96
	Erucin	444	801	Linear	0.18
	Methyl ITC	110	153	Sigmoid	0.99
	Phenethyl ITC	210	483	Sigmoid	0.70
Pythium myriotylum	Allyl ITC	46	106	Sigmoid	0.96
	Brassinin	95	173	Quadratic	0.99
	Cheirolin	6	10	Sigmoid	0.99
	Erucin	9	19	Sigmoid	0.99
	Methyl ITC	80	153	Sigmoid	0.99
	Phenethyl ITC	9	11	Sigmoid	0.82
Penicillium expansum	Allyl ITC	77	267	Sigmoid	0.95
	Brassinin	119	327	Linear	0.71
	Cheirolin	132	404	Linear	0.91
	Erucin	10	30	Sigmoid	0.96
	Methyl ITC	88	291	Sigmoid	0.96
	Phenethyl ITC	7	22	Sigmoid	0.99
Sclerotium rolfsii	Allyl ITC			Sigmoid	0.99
	Brassinin	113	208	Linear	0.87
	Cheirolin	112	219	Linear	0.80
	Erucin	17	33	Linear	0.92
	Methyl ITC	155	256	Sigmoid	0.98
	Phenethyl ITC	17	27	Sigmoid	0.96
Agrobacterium tumefaciens	Allyl ITC	223	396	Sigmoid	0.99
	Brassinin	191	358	Linear	0.80
	Cheirolin	177	236	Quadratic	0.84
	Erucin	69	126	Linear	0.87
	Methyl ITC	1112	2095	Linear	0.96
	Phenethyl ITC	97	213	Sigmoid	0.98

[z] Concentrations were calculated from the regression equation (SigmaPlot, 2002) for each compound by pathogen.

[y] Sigmoid equation: $y = a/(1+e^{(-(x-c)/b)})$; y=inhibition; x= concentration of compound.

[x] Linear equation: $y= a(x) + b$; y=inhibition; x= concentration of compound.

[w] Quadratic equation: $y=c+ax+bx^2$; y=inhibition; x= concentration of compound.

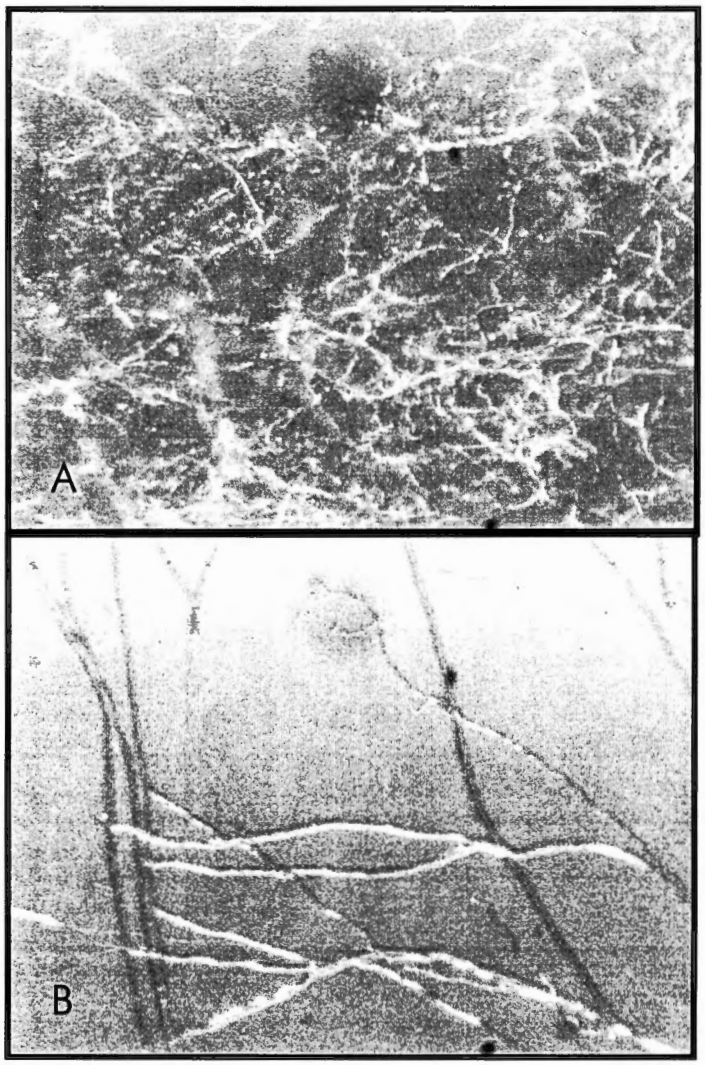


Figure V-6. Morphological differences in *Botrytis cinerea* treated with erucin: A) Normal growth, B) Growth following treatment. Radial growth of mycelia was not significantly different (Magnification:100 X).

Part VI

Conclusions

The objectives of this project, as addressed in Part III, IV and V, were to examine to potental of biofumigation as an alternative method of controlling soilborne pathogens. The volatiles released from 2.0 g Indian mustard into a headspace volume of 1 L (equaling AITC at1.45 $\mu mol \cdot L^{-1}$) proved effective for lethal inhibition of *S. rolfsii* mycelial growth. It was projected that, based on average soil porosity and Indian mustard production of leaf biomass at 12 $t \cdot ha^{-1}$ (Duke, 1984), the biomass needed for fumigation (approximately 1.68 $t \cdot ha^{-1}$) was achievable in field plantings. 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.

Biofumigation with *Brassica* spp. is beneficial in tomato production under disease pressure and would be a viable alternative for controlling soilborne pathogens in organic or "green" systems. When integrated with an IPM system, biofumigation could increase control of soilborne pathogens in tomato production systems and may provide an alternative for use in organic systems.

Glucosinolates show extensive genetic variation and therefore results in breakdown products including isothiocyanates exhibit an equally extensive diversity. The differences in inhibitory activity among the ITC tested against these five pathogens suggest that there might be benefits to using mixtures of plant material.

A diverse selection of ITC may provide better control soilborne pathogens and other pests than a single species biofumigation cover. In field settings, with multiple plant pathogens and pest present, a mixture of ITC would increase the spectrum of effectiveness. In addition, the potential additive and synergistic effects of mixtures may provide further protection.

The ban of methyl bromide evinced agriculture dependence on synthesized chemicals for control of agronomic pests. The effort to find alternatives has increased the interest in non-synthetic alternatives. Biofumigation is one potential alternative. Identifying naturally occurring compounds with biocidal activty can help improve the viablity of this system. Breeding to modify GS content in an effort to increase efficacy of biofumigation is ongoing (Brown *et al.*, 2001). Knowledge concerning toxicity, specificity, and synergistic reactions will help direct these efforts. The vast array of naturally occurring GS, and their corresponding ITC, are a resource that is just beginning to be investigated.

APPENDIX

Inhibition of mycelial growth attributable to the AITC released from reconstituted Indian mustard – Nonlinear Regression by SigmaPlot 2000.

$Y = 0.41X + 0.59$

$1 = 0.41(1) + 0.59$

$1.41 = 0.41(2) + 0.59$

$2.23 = 0.41(4) + 0.59$

$3.05 = 0.41(6) + 0.59$

$3.87 = 0.41(8) + 0.59$

$4.69 = 0.41(10) + 0.59$

$Y = 98.59 / (1 + e^{(-(x-1.6890)/0.8593)})$

$30.53 = 98.59 / (1 + e^{(-(1-1.6890)/0.8593)})$

$41.36 = 98.59 / (1 + e^{(-(1.41-1.6890)/0.8593)})$

$64.32 = 98.59 / (1 + e^{(-(2.23-1.6890)/0.8593)})$

$81.80 = 98.59 / (1 + e^{(-(3.05-1.6890)/0.8593)})$

$91.37 = 98.59 / (1 + e^{(-(3.87-1.6890)/0.8593)})$

$95.68 = 98.59 / (1 + e^{(-(4.69-1.6890)/0.8593)})$

1. Based on the equation relating AITC (μmoles L^{-1}) release to Indian mustard (IM) (g L^{-1}), AITC production was calculated at 5 treatment levels of 1, 2, 4, 6, 8, and 10 g IM.

2. The projected AITC produced from the above treatment levels was then plugged into the equation representing the relationship between AITC and inhibition of mycelial growth to predict the level of inhibition by the AITC released from a given amount of mustard.

Indian mustard (g L^{-1})	Inhibition of mycelial growth due to AITC (%)
1	30.53
2	41.36
4	64.32
6	81.80
8	91.37
10	95.68

3. The curve in Fig III-5, was constructed by relating the grams of IM to the inhibition by AITC based on the projected coordinates listed to the left.

Nonlinear Regression

[Variables]
x = col(1)
y = col(2)
[Parameters]
a = max(y) "Auto {{previous: 98.5929}}
b = xwtr(x,y,.5)/4 "Auto {{previous: 2.09621}}
x0 = x50(x,y,.5) "Auto {{previous: 2.68068}}
[Equation]
f=a/(1+exp(-(x-x0)/b))
fit f to y
[Constraints]

[Options]
tolerance=0.000100
stepsize=100
iterations=100

R = 1.00000000 Rsqr = 0.99999999 Adj Rsqr = 0.99999999

Standard Error of Estimate = 0.0026

	Coefficient	Std. Error	t	P
a	98.5929	0.0033	30070.1188	<0.0001
b	2.0962	0.0002	8785.0130	<0.0001
x0	2.6807	0.0002	12098.9166	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	2	3628.5716	1814.2858262428838.4888		<0.0001
Residual	3	0.0000	0.0000		
Total	5	3628.5716	725.7143		

PRESS = 0.0001

Durbin-Watson Statistic = 3.5539

Normality Test: Passed (P = 0.2900)

Constant Variance Test: Passed (P = 0.0600)

Power of performed test with alpha = 0.0500: 1.0000

Regression Diagnostics:

Row	Predicted	Residual	Std. Res.	Stud. Res.	Stud. Del. Res.
1	30.5290	0.0010	0.3796	0.6036	0.5258
2	41.3623	-0.0023	-0.8861	-1.1537	-1.2629
3	64.3171	0.0029	1.0941	1.5842	3.1995
4	81.8024	-0.0024	-0.9087	-1.2077	-1.3757
5	91.3696	0.0004	0.1534	0.1895	0.1557
6	95.6796	0.0004	0.1569	0.2790	0.2308

Influence Diagnostics:

Row	Cook'sDist	Leverage	DFFITS
1	0.1856	0.6045	0.6501
2	0.3084	0.4101	-1.0530
3	0.9175	0.5231	3.3506
4	0.3727	0.4339	-1.2045
5	0.0063	0.3447	0.1129
6	0.0561	0.6837	0.3393

95% Confidence:

Row	Predicted	Regr. 5%	Regr. 95%	Pop. 5%	Pop. 95%
1	30.5290	30.5225	30.5355	30.5184	30.5396
2	41.3623	41.3570	41.3677	41.3524	41.3723
3	64.3171	64.3111	64.3232	64.3068	64.3275
4	81.8024	81.7969	81.8079	81.7924	81.8124
5	91.3696	91.3647	91.3745	91.3599	91.3793
6	95.6796	95.6727	95.6865	95.6687	95.6904

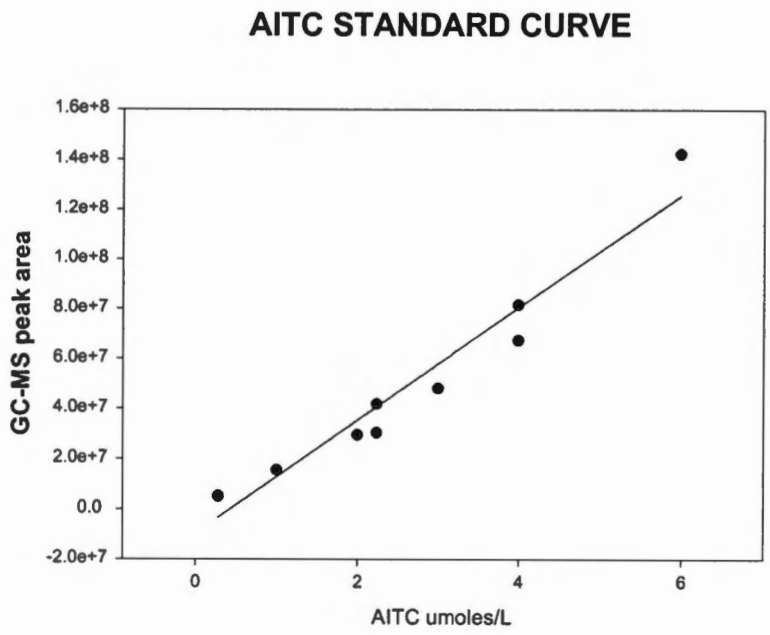
Calculation of LC_{50}:

$$50 = 98.59(1 + e^{(-(x-2.6807)/2.0962)})$$
$$1.97 = (1 + e^{(-(x-2.6807)/2.0962)})$$
$$0.97 = e^{(-(x-2.6807)/2.0962)}$$
$$-0.029 = -(x - 2.6807)/2.0962)$$
$$-0.060 = 2.6807 - x$$
$$x = 2.741$$

Calculation of LC_{90}:

$$90 = 98.59(1 + e^{(-(x-2.6807)/2.0962)})$$
$$1.095 = (1 + e^{(-(x-2.6807)/2.0962)})$$
$$0.095 = e^{(-(x-2.6807)/2.0962)}$$
$$-2.349 = -(x - 2.6807)/2.0962)$$
$$-4.924 = 2.6807 - x$$
$$x = 7.605$$

AITC Standard Curve on GC-FID - SigmaPlot 2000:



Nonlinear Regression

[Variables]
x = col(1)
y = col(2)
'Automatic Initial Parameter Estimate Functions
F(q)=ape(x,y,1,0,1)
[Parameters]
y0 = F(0)[1] "Auto {{previous: -9.68776e+006}}
a = F(0)[2] "Auto {{previous: 2.2589e+007}}
[Equation]
f=y0+a*x
fit f to y
[Constraints]
[Options]
tolerance=0.000100
stepsize=100
iterations=100

R = 0.97283110 Rsqr = 0.94640035 Adj Rsqr = 0.93970039

Standard Error of Estimate = 10293248.6778

	Coefficient	Std. Error	t	P	
y0	-9687764.2930		5754978.8729	-1.6834	0.1308
a	22588979.8097		1900619.4425	11.8851	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	1	14966071978761194.0000		14966071978761194.0000	
	141.2547	<0.0001			
Residual	8	847607746749885.5000		105950968343735.6900	
Total	9	15813679725511080.0000		1757075525056786.7000	

PRESS = 2043142747593276.5000

Durbin-Watson Statistic = 1.8555

Normality Test: Passed (P = 0.6746)

Constant Variance Test: Passed (P = 0.2583)

Power of performed test with alpha = 0.0500: 0.9999

Regression Diagnostics:

Row	Predicted	Residual	Std. Res.	Stud. Res.	Stud. Del. Res.	
1	-3380921.1301		8614643.1301	0.8369	0.9780	0.9750
2	-3380921.1301		8378812.1301	0.8140	0.9512	0.9448
3	40769239.9079		1143096.0921	0.1111	0.1172	0.1097
4	40769239.9079		-10346904.9079		-1.0052	-1.0610
	-1.0706					
5	80419676.1679		1238796.8321	0.1204	0.1326	0.1241
6	80419676.1679		-13092124.1679		-1.2719	-1.4011
	-1.5087					
7	12840225.2712		2712826.7288	0.2636	0.2905	0.2731
8	35365955.9374		-5795000.9374	-0.5630	-0.5963	-0.5706
9	57893945.5017		-9757763.5017	-0.9480	-1.0039	-1.0045
10	125473396.3983		16903618.6017		1.6422	2.3567
	3.9866					

Influence Diagnostics:

Row	Cook'sDist	Leverage	DFFITS
1	0.1748	0.2677	0.5895
2	0.1654	0.2677	0.5713
3	0.0008	0.1024	0.0371
4	0.0642	0.1024	-0.3616
5	0.0019	0.1759	0.0574
6	0.2095	0.1759	-0.6970
7	0.0091	0.1767	0.1265
8	0.0217	0.1086	-0.1992
9	0.0612	0.1083	-0.3501
10	2.9419	0.5144	4.1033

95% Confidence:

Row	Predicted	Regr. 5%	Regr. 95%	Pop. 5%	Pop. 95%
1	-3380921.1301		-15662292.5228		8900450.2625 -
30106242.2225		23344399.9622			
2	-3380921.1301		-15662292.5228		8900450.2625 -
30106242.2225		23344399.9622			
3	40769239.9079		33174919.7397		48363560.0761
	15847674.8884		65690804.9274		
4	40769239.9079		33174919.7397		48363560.0761
	15847674.8884		65690804.9274		
5	80419676.1679		70464931.5458		90374420.7900
	54680452.3121		106158900.0236		
6	80419676.1679		70464931.5458		90374420.7900
	54680452.3121		106158900.0236		
7	12840225.2712		2862761.1197	22817689.4227	-
12907793.9749		38588244.5173			
8	35365955.9374		27543343.5387		43188568.3362
	10373877.8136		60358034.0612		
9	57893945.5017		50080988.0260		65706902.9773
	32904887.7219		82883003.2814		
10	125473396.3983		108448984.4240		142497808.3727
	96263101.8555		154683690.9411		

SEEDS AND SEED SOURCES:

Early Spring 1999 covers:

Brassica juncea L.
Indian mustard
PI 458934
U.S. Dept. Agr./Agr. Res. Serv.
Ames, Iowa

Fall 1999 covers:

Brassica juncea L.
Indian mustard
PI 458928
U.S. Dept. Agr./Agr. Res. Serv.
Ames, Iowa

Brassica rapa L. (Ruvo Group)
Fall Raab – 'Salade'
Stokes Seeds Inc
Buffalo, New York 14240

Fall 2000 covers:
Secale cereale L.
Winter rye - 'Dacold'
Kermit's Farm Center, Watertown, S.D.
Lot# 027
Germination 85%; tested 8/2000

Fall Raab – As above

Southern Giant Curled Mustard
Lot #: k3623-l18-8-203
Producing company=s name: unknown
Place of harvest: Oregon
Date of harvest: fall 1999
Test date: 05/00

Mustard, Florida Broadleaf
Lot #: k3627-l111-9-jfb
Producing company=name: unknown
Place of harvest: Oregon
Date of harvest: fall 1999
Test date: 01/00

Lycopersicum esculentum
Tomato – 'Celebrity'-hybrid
Knoxville Seed and Greenhouse Supply
5001 Rutledge Pike
Knoxville, TN 37914
Lot k-5316

SEEDS FOR EXTRACTION PROCEDURE:

Brassica juncea (L.) Czern.

'Southern Giant Curled' Mustard
Purchased from: Knoxville Seed & Greenhouse Supply Corp, Knoxville, TN 37914
Lot: K3623-L18-80203
Purity: 99%
Germination 85%; tested 01/2001

'Florida Broadleaf Mustard'
Purchased from: Knoxville Seed & Greenhouse Supply Corp, Knoxville, TN 37914
Lot: K3627-K111-9-JFB
Purity: 99%
Germination 85%; tested 01/2001

Barbarea verna (Miller)Asch.
Cress - Upland
Purchased from: Knoxville Seed & Greenhouse Supply Corp, Knoxville, TN 37914
Lot: K3230-89532
Purity: 99%
Germination 86%; tested 01/2001

Raphanus sativus (Radicula Group) L.

Radish- "Scarlet Globe"
Purchased from: Knoxville Seed & Greenhouse Supply Corp, Knoxville, TN 37914
Lot K-3708
Tested 2001

Radish- "White Icicle"
Purchased from: Knoxville Seed & Greenhouse Supply Corp, Knoxville, TN 37914
Lot K-5091
Tested 2001

Lepidium sativum L.
Peppergrass – *Lepidium Sativum*
Purchased from: Knoxville Seed & Greenhouse Supply Corp, Knoxville, TN 37914
Packaged by: The Page Seed Co. Greene, NY 13778
Run#: 1
Packaged 2001

Eruca vesicaria spp. sativa (L.) Cav.
Arugula
Purchased: Companion plants, 7247 North Coolville Ridge, Athens, OH 45701
ERU10X A2'3' FS/CM

Isatis tinctoria L.
Dyers Woad
Purchased: Companion plants, 7247 North Coolville Ridge, Athens, OH 45701
ISA10X B 4' FS/D LO

Isatis tinctoria L.
Dyers Woad
Purchased: Herb Society of America Seed Exchange, 9019 Kirtland Chardon Road, Kirtland, Ohio 44094
Collected WRHS Herb Garden Cleaveland OH Summer 2000

Phytolacca americana L.
Pokeweed
Purchased: Companion plants, 7247 North Coolville Ridge, Athens, OH 45701
PHY30X HP 8' PS-FS/ M N
Phytolacca americana – Pokeweed
Purchased: Herb Society of America Seed Exchange, 9019 Kirtland Chardon Road, Kirtland, Ohio 44094
Collected at Wayland MA, 2000

Reseda luteola L.
Weld
Purchased: Companion plants, 7247 North Coolville Ridge, Athens, OH 45701
RES10X B 4'FS/D
Reseda luteola - Weld
Purchased: Herb Society of America Seed Exchange, 9019 Kirtland Chardon Road, Kirtland, Ohio 44094
Collected Frankenmuth, MI, 1999.

Cheiranthus cheiri L.
English Wallflower
Purchased: Earthlygoods
Lot 061; tested 4/01

Hesperis matronalis
Dames Rocket
Purchased: Earthlygoods
Lot 016; tested 3/01
Purchased: Herb Society of America Seed Exchange, 9019 Kirtland Chardon Road, Kirtland, Ohio 44094
Collected 1999.

Capparis spinosa inermis L.
Caper Bush
Purchased: Redwood city Seed Co., P.O. Box 361, Redwood City, CA, 94064
ECOSEEDS
Packaged for 2001.

Nastriuim officinalis L.
Watercress
Purchased: Comstock, Ferre & Co., 263 Main Str. P.O. Box 125, Wethersfield, CT 6109-1890
Seeds by Design
Lot HER 4768.
Germ 60%; 10/01

Brassica rapa L. (Ruvo Group)
Fall Raab – 'Salade'
Stokes Seeds Inc
Buffalo, New York 14240

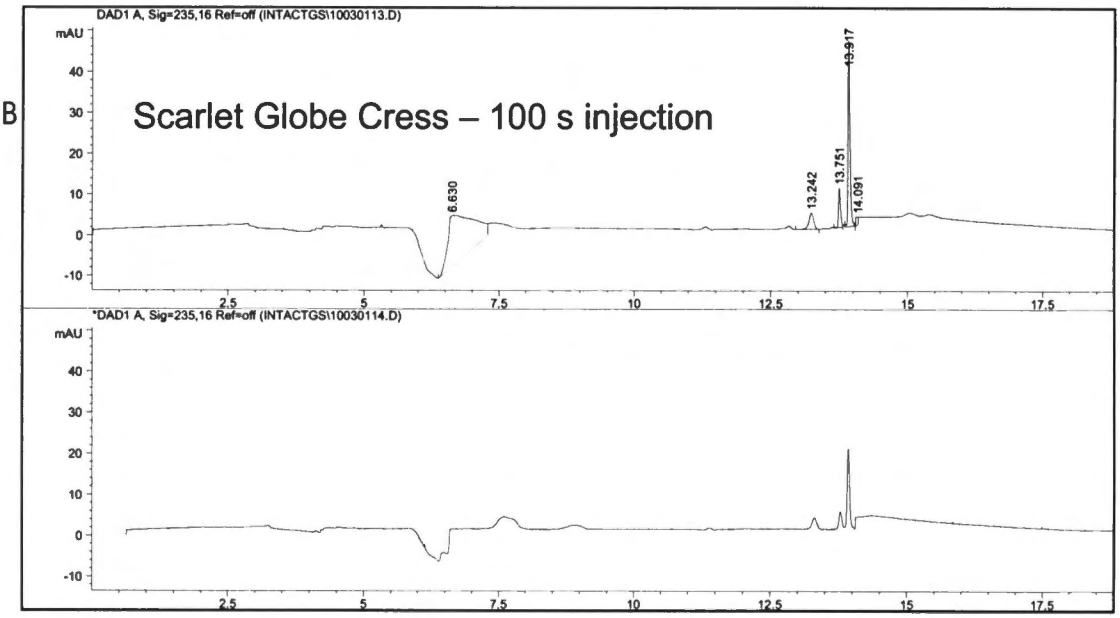
Fall Raab – 'Late Fall Supremo'
Purchased: Comstock, Ferre & Co., 263 Main Str. P.O. Box 125, Wethersfield, CT 6109-1890
Grower: Condor Seed Production
Lot 7260
Germ 96%; 06/00

Lobularia maritime (L.) Desv.
Alyssum 'Saxatile' (perennial)
Purchased: Mayo's Seeds, Knoxville, TN 37919
Lot A Packed for 2001

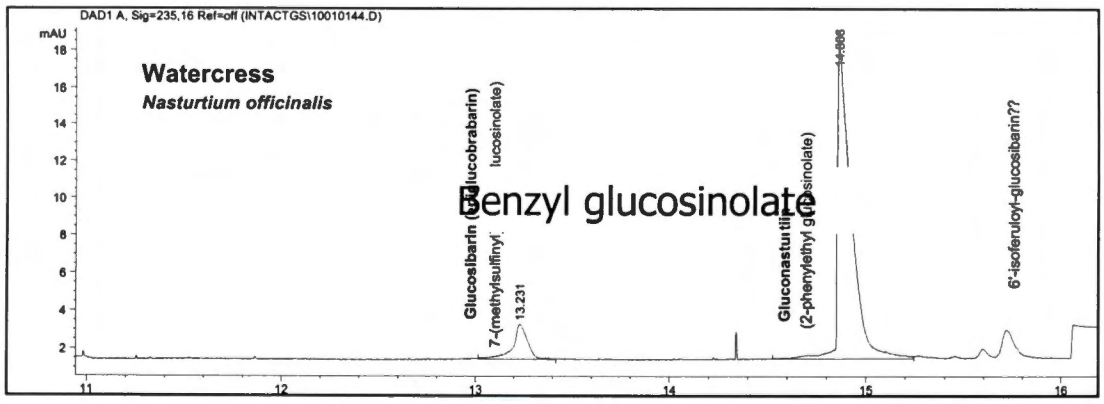
Brassica oleracea (Capitata Group) (L.)
Cabbage – 'Late Flat Dutch'
Knoxville Seed & Greenhouse Supply
5001 Rutledge Pike
Knoxville TN 37914
Lot K2853

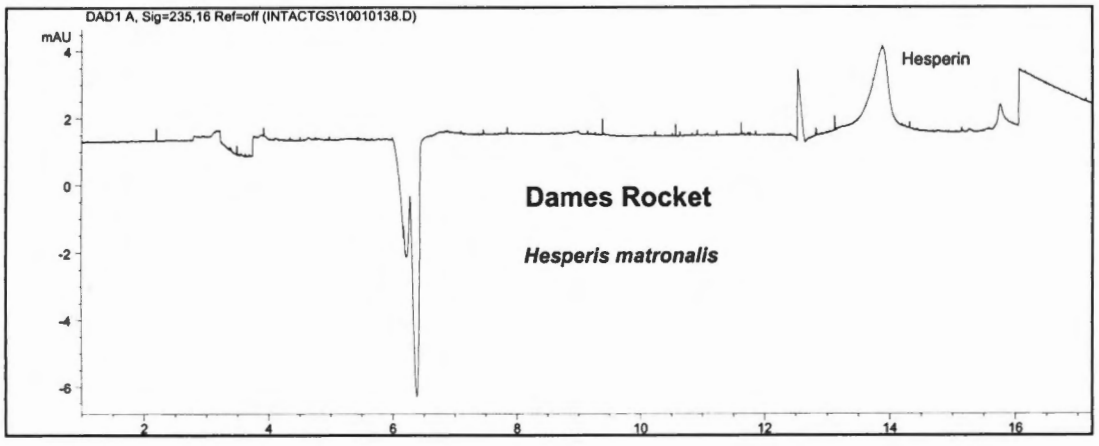
Electropherograms

A



B

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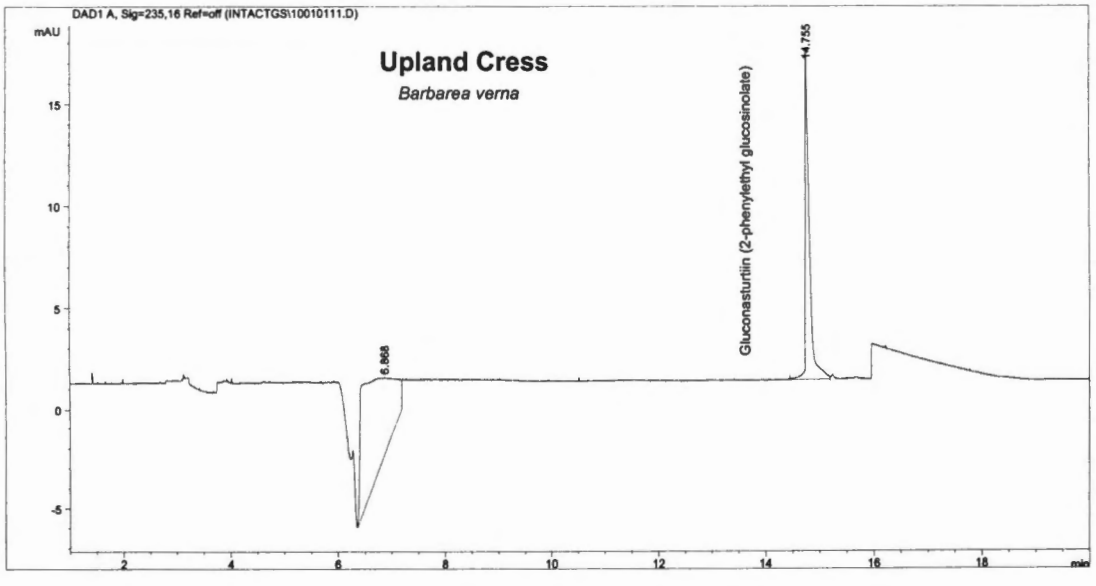
mAU
Hesperin
Dames Rocket
Hesperis matronalis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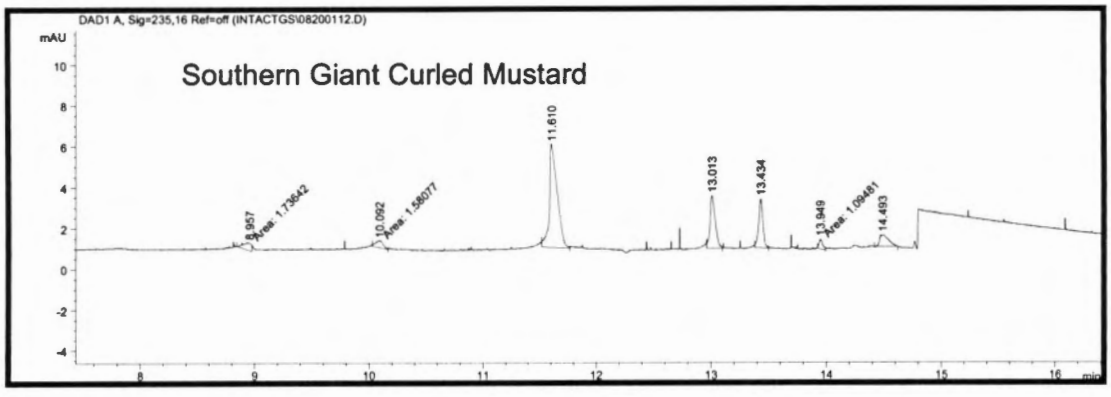
mAU
Upland Cress
Barbarea verna
Gluconasturtiin (2-phenylethyl glucosinolate)
14.755
6.668
min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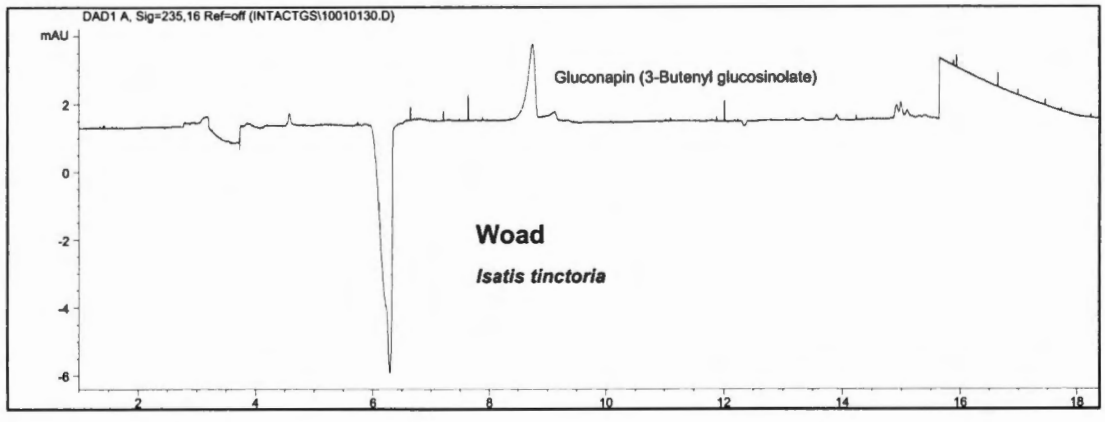
mAU
Southern Giant Curled Mustard
9.957
Area: 1.73642
10.092
Area: 1.58077
11.610
13.013
13.434
13.949
Area: 1.09481
14.493
min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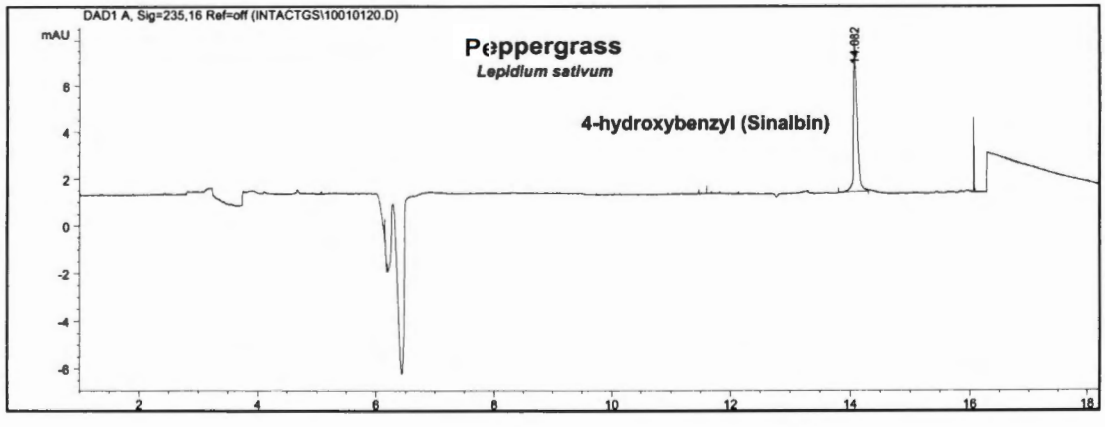
mAU
Gluconapin (3-Butenyl glucosinolate)
Woad
Isatis tinctoria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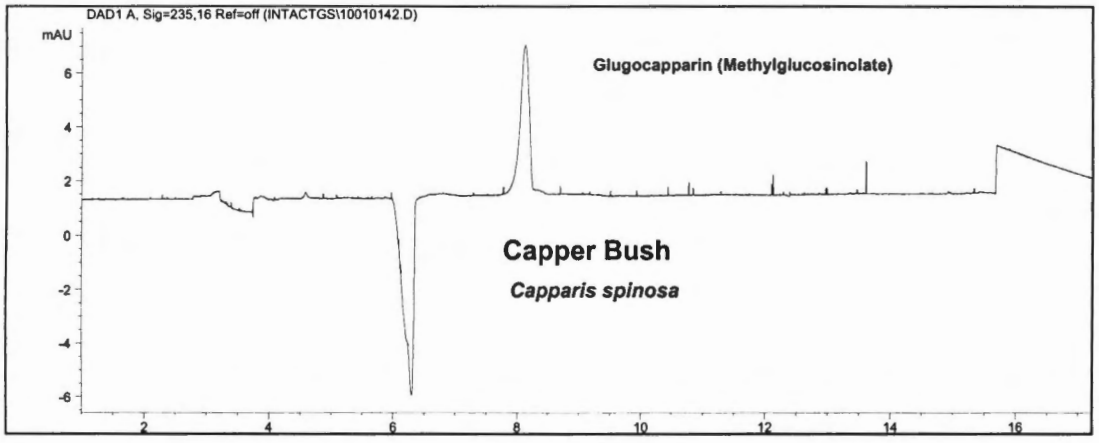
mAU
Peppergrass
Lepidium sativum
4-hydroxybenzyl (Sinalbin)
14.082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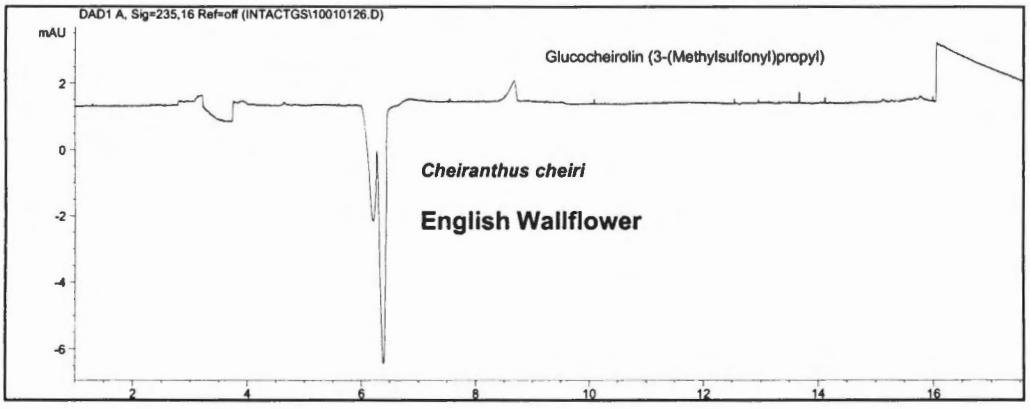
mAU
Glugocapparin (Methylglucosinolate)
Capper Bush
Capparis spinosa


DAD1 A, Sig=235,16 Ref=off (INTACTGS\10010126.D)
mAU
Glucocheirolin (3-(Methylsulfonyl)propyl)
Cheiranthus cheiri
English Wallflower

VITA

Stephanie Gail Harvey was born in Macon, Georgia in 1970. She lived in Fort Valley, Georgia for most of her life and graduated from Peach County High School. Ms. Harvey attended Wesleyan College, Macon where she obtained an *A.B.* in Biology in 1992. Awarded a graduate teaching assistantship at Georgia College and State University, Milledgeville, Georgia, Ms. Harvey worked on a M.S. in Biology and completed that degree in1997. In the Department of Plant and Soil Sciences at the University of Tennessee, Ms Harvey worked as a graduate research assistant for Dr. Carl E. Sams. Following completion of her Doctor of Philosophy in Plant and Soil Sciences, she accepted an assistant professorship at Georgia Southwestern State University in Americus, Georgia.