Correlation of Fungicidal Activity of *Brassica* Species with Allyl Isothiocyanate Production in Macerated Leaf Tissue

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ABSTRACT

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Brassica species were tested for production of volatile fungicidal compounds from macerated green leaf tissue. Tissue (10, 20, and 40 g) of one cultivar each of six *Brassica* species was assayed for inhibition of *Fusarium sambucinum*. Only cultivars of *B. nigra* and *B. juncea* suppressed radial growth (>50% inhibition of control). Plant introduction accessions (PIs) of *B. juncea*, *B. carinata*, *B. nigra*, and *B. napus* were screened for suppression of *F. sambucinum*. Only *B. nigra*, *B. juncea*, and *B. carinata* PIs had suppressive activity, but activity varied among PIs within these species. The concentration of allyl isothiocyanate (AITC), a breakdown product of allyl glucosinolate, in leaf tissue was measured using gas chromatography. AITC was detected in PIs of *B. nigra*, *B. juncea*, and *B. carinata*. Radial growth of *F. sambucinum* was negatively correlated ($P < 0.05$) with AITC concentrations emitted from *Brassica* leaf tissue. All *Brassica* PIs with AITC concentrations greater than 0.10 mg/g of leaf tissue were suppressive to *F. sambucinum* in radial growth assays. Growth of five other plant pathogenic fungi was also suppressed by a *B. juncea* cultivar that contained high concentrations of AITC.

Additional keywords: biological control, green manures, soilborne pathogens.

Green tissue of *Brassica* species can decrease population densities of some fungal and nematode pathogens when incorporated into soil. *Brassica* amendments reduced populations of *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *conglutinans* (Wollenweb.) W. C. Snyd. & H. N. Hans., *Verticillium dahliae* Kleb., and *Meloidogyne chitwoodi* Golden et al., and decreased root rot of pea caused by *Aphanomyces euteiches* Drechs. in field studies (11,12, 14,15,18). Fresh leaf tissue of *Brassica* species suppressed root rot of bean and sesame caused by *Thielaviopsis basicola* (Berk. & Broome) Ferraris (1,13). Control of plant pathogens by *Brassica* species is attributed to their production of glucosinolates (9).

Glucosinolates are sulfur compounds composed of a thioglucose group, a variable carbon side chain (R-group), and a sulphonated oxime. Glucosinolates are named according to the structure of their R-group. Approximately 100 different glucosinolates have been identified in plant tissue (5). Enzymatic hydrolysis of glucosinolates by membrane-bound thioglucosidase produces numerous compounds including isothiocyanates, nitriles, thiocyanates, epinitriles, and glucose. Some of the hydrolytic breakdown products have antimicrobial, fungicidal, and insecticidal properties (4,22). The end product of the hydrolytic reaction is determined by the R-group of the glucosinolate and the physical and chemical conditions under which hydrolysis takes place (5,21).

Allyl glucosinolate is one of the predominant glucosinolates in *B. nigra* (L.) W. Koch, *B. carinata* A. Braun, and *B. juncea* (L.) Czernj. & Coss., and is generally converted to allyl isothiocyanate (AITC) at a pH of 4.0 or greater (2,5,6,19,21). Borek et al. (2) reported that, in soil, AITC was the predominant hydrolytic byproduct formed from allyl glucosinolate. AITC, a volatile compound, is as toxic to fungi as methyl isothiocyanate, an active ingredient in commercial soil fumigants (9,22).

Soil fumigants are widely used for control of pathogenic fungi, nematodes, and weeds. Environmental and human health hazards associated with the manufacture, transport, and application of these pesticides may greatly restrict their availability in the future. *Brassica* green manures may have the potential to control soilborne pathogens. Because volatile compounds are dispersed effectively in soil, *Brassica* species with high concentrations of fungicidal volatile compounds may provide greater control of soilborne plant pathogens than species with little volatile activity. The objectives of this project were to i) select *Brassica* genotypes that produce volatile fungicidal compounds; and ii) determine if fungal inhibition was correlated to the concentration of AITC among *Brassica* accessions and cultivars.

MATERIALS AND METHODS

Plant material. Cultivars of *B. nigra*, *B. napus* L., *B. carinata*, *B. juncea*, *B. campestris* L., and *B. hirta* Moench (synamorph *Sinapis alba* L.) were obtained from the Agriculture Canada Research Station, Saskatoon, Saskatchewan. Plant introduction accessions (PIs) of *B. nigra*, *B. napus*, *B. carinata*, and *B. juncea* were obtained from the USDA-ARS Regional Plant Introduction Station in Ames, IA. Plants of 10 to 15 PIs of each species were grown from seed in the greenhouse under a 12-h photoperiod. After 1 to 2 weeks, the seedlings were transplanted into greenhouse soil mix containing a complete fertilizer (20-20-20). The plants were watered daily without additional fertilizer. All of the plants were 40- to 50-days-old and flowering when they were harvested for the assays.

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Fungal cultures. An isolate of *Fusarium sambucinum* Fuckel, a cause of potato tuber dry rot, was used in experiments 1 to 5, in which *Brassica* genotypes were assessed for their relative suppressiveness. This isolate was obtained from a potato tuber lesion and is highly virulent (7). Working stock cultures were maintained on V8 agar at 4°C for up to 6 months. Cultures were transferred to water agar supplemented with streptomycin sulfate (90 mg/liter) and penicillin G (100,000 units/liter) (WA + SP) and grown at 22°C to produce inoculum plugs for assays. In experiment 6, *Brassica* species were tested for suppressive activity against isolates of five fungal pathogens. Pathogenic isolates of *Verticillium dahliae*, *V. albo-atrum* Reinke & Berthier, *Pythium ultimum* Trow, *Colletotrichum coccodes* (Wallr.) S. J. Hughes, and *Rhizoctonia solani* Kühn were obtained from our own culture collection or from the Department of Plant Pathology Teaching Collection, Cornell University, Ithaca, NY. Cultures were transferred onto V8 agar plates amended with streptomycin sulfate (100 mg/liter) and penicillin G (100,000 units/liter) (V8 + SP) to produce inoculum plugs for assays.

Bioassay. We used a modification of an assay (16) designed for testing the efficacy of volatile fungicides. Freshly harvested leaf tissue was macerated in a food processor for about 2 min, and then placed into wide-mouth glass jars (500 ml). In experiments 1 to 5, agar plugs (3 mm) were transferred from 7-day-old cultures of *F. sambucinum* to petri plates (90 mm) containing WA + SP. In experiment 6, fungi were grown for 3 to 7 days, depending on the growth rate of the pathogen. Agar plugs (5 mm) were then transferred onto plates of V8 + SP. The plates were inverted onto jars containing the macerated plant tissue and sealed with Time Tape (TimeMed Labeling Systems, Inc., Burr Ridge, IL) before incubating for 7 days at 21°C in darkness. Empty jars were included as control treatments in all experiments and are subsequently referred to as the unamended control. Treatments were considered "suppressive" if the radial growth after 7 days was <50% of the unamended control.

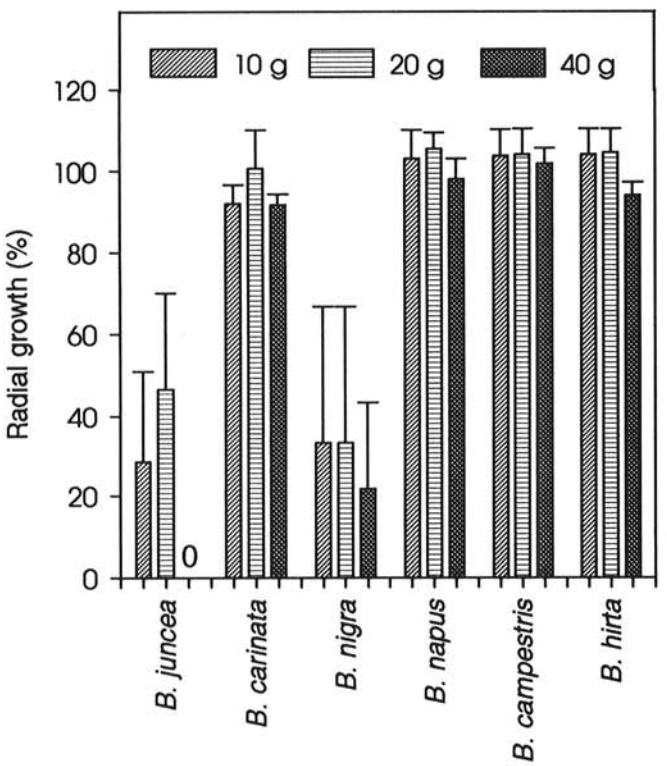

Fig. 1. Radial growth of *Fusarium sambucinum* (% of the unamended control) as affected by *Brassica nigra* cv. Type 1, *B. napus* cv. Midas, *B. carinata* cv. Dodolla, *B. juncea* cv. Cutlass, *B. campestris* cv. Torch, and *B. hirta* cv. Ochre. Radial growth was assessed in bioassays that measured fungicidal activity of volatile compounds released from macerated leaf tissue added at the rate of 10, 20, or 40 g per jar. Vertical bar indicates the standard error of the mean of three replicates. No error bar indicates no variation among replicates.

In experiments 1 to 5, *Brassica* cultivars and PIs were tested for production of volatile compounds fungicidal to *F. sambucinum*. In experiment 1, one cultivar each of *B. nigra*, *B. napus*, *B. carinata*, *B. juncea*, *B. campestris*, and *B. hirta* were compared using 10, 20, and 40 g of leaf tissue using a factorial design. In experiments 2 to 5, PIs within *B. juncea*, *B. napus*, *B. carinata*, and *B. nigra* were compared. In these studies, 25 g of leaf tissue for *B. nigra* and 40 g of tissue for the other species were used. Based on the results from experiment 1, *B. juncea* cv. Cutlass and *B. napus* cv. Midas were used as positive and negative controls, respectively, in experiments 2 to 5. At the conclusion of the bioassays, the agar plugs of *F. sambucinum* from selected treatments were transferred to fresh WA + SP to assess fungicidal activity.

In experiment 6, the sensitivity of five fungal pathogens to volatile compounds emitted from *B. juncea* cv. Cutlass and *B. napus* cv. Midas was tested. Twenty-five grams of macerated leaf tissue was used. Inoculum plugs were transferred to V8 + SP plates to test viability of the fungi at the termination of the experiment.

There were three replicates for each treatment in experiments 1 to 5 and four replicates in experiment 6, arranged in a completely randomized design. All experiments (1 to 6) were repeated at least once with similar results. Radial growth data from experiments 1 and 2 were analyzed using Minitab (Minitab Inc., State College, PA).

Glucosinolate analysis. Glucosinolates were extracted from 50 or 100 g of leaf tissue that was a composite of three to four plants. Leaf tissue was collected from plants of selected cultivars and PIs and stored in plastic bags at −10°C until processed. The plant tissue was microwaved for 2 to 3 min to thaw it and destroy any endogenous thioglucosidase activity. The tissue was then blended with 30% aqueous methanol for approximately 2 min and allowed to stand in the blender for 1 h. The slurry was filtered through four layers of cheesecloth, after which the methanol and most of the water were removed by rotoevaporation. The aqueous residue was placed into a centrifuge tube and 1 ml of a 1:1 solution of barium and lead acetate (0.5 M each) was added before centrifuging for 5 min at 3,200 × *g*. The supernatant was removed, frozen, and lyophilized.

The freeze-dried plant residue was suspended in 15 ml of a potassium-phosphate buffer (0.05 M; pH 7.5). Dichloromethane (15 ml) that contained 1 mg/ml of the internal standard benzyl isothiocyanate (Aldrich Chemical Co., Milwaukee, WI; 98%) was added, followed by 25 mg of thioglucosidase (Sigma Chemical Co., St. Louis) that enzymatically cleaves the thioglucoside bond of the glucosinolates present. The suspension was shaken at 21°C for 2 h, and then transferred to a separatory funnel.

The quantity of AITC in 1 µl of the CH_2Cl_2 suspension was determined using a Hewlett-Packard 5890A gas chromatograph and a Hewlett-Packard 3393A integrator (Hewlett-Packard Co., Palo Alto, CA). The parameters were: oven temperature, 50°C; injection temperature, 180°C; and flame ionization detector, 290°C. A DB-1 fused silica capillary column (0.25-mm inside diameter × 15-m length; 0.25 µm of film; J & W Scientific, Folsom, CA) was used and each sample was run twice. Concentrations of AITC were calculated based on peak areas in relation to the peak area of the internal standard benzyl isothiocyanate (Aldrich Chemical Co.). Analyses were repeated once using another group of plants.

Radial growth of *F. sambucinum,* expressed as percent unamended control, was regressed against the AITC concentration using the linear regression package of Minitab Inc. All treatments for which both AITC concentration and radial growth were available in experiments 1 to 5 were included in this analysis.

RESULTS

Brassica genotypes differed greatly in their ability to suppress the growth of *F. sambucinum* (Fig. 1). In experiment 1, both *B.*

nigra cv. Type 1 and *B. juncea* cv. Cutlass were suppressive (>50% inhibition of radial growth compared with an unamended control). Inhibition of radial growth was not affected by the quantity of tissue of the two cultivars. In contrast, the cultivars of *B. napus* (Midas), *B. carinata* (Dodolla), *B. campestris* (Torch), and *B. hirta* (Ochre) were not suppressive at any dose (Fig. 1). Results from the glucosinolate analysis (Table 1) revealed that *B. juncea* cv. Cutlass (0.18 mg of AITC/g of tissue) and *B. nigra* cv. Type 1 (0.17 mg of AITC/g of tissue) had the highest concentrations of AITC. *B. carinata* cv. Dodolla contained only 0.06 mg of AITC/g of tissue, and none of the other *Brassica* species produced AITC at detectable levels. When the experiment was repeated, glucosinolate concentrations were similar to those reported, except that *B. napus* cv. Midas contained a trace amount of AITC.

The suppressiveness of the *Brassica* genotypes also varied within species (Fig. 2). Four of the ten *B. juncea* PIs suppressed radial growth of *F. sambucinum* (>50% inhibition of the control), but growth varied from 0 to 100% of the unamended control. The concentration of AITC in *B. juncea* PIs also varied greatly (Table 1). PIs with the largest suppressive effect generally had the highest concentrations of AITC. Little or no AITC was detected in the nonsuppressive PIs.

Only one of the fifteen *B. carinata* PIs showed any suppressive activity (Fig. 2). The *B. carinata* PIs did contain AITC, but in lower concentrations than in the suppressive PIs of both *B. juncea* and *B. nigra* (Table 1). The *B. carinata* PI 197403 that had the highest concentration of AITC (0.087 mg/g of leaf tissue) also had the highest degree of suppressive activity.

All but two of the *B. napus* PIs tested significantly ($P < 0.05$) increased radial growth of *F. sambucinum* when compared with the unamended control (Fig. 2). None of the *B. napus* PIs produced detectable levels of AITC (Table 1).

Ten of the eleven *B. nigra* PIs tested were suppressive (Fig. 2). The *B. nigra* PIs also had the highest levels of AITC among all of the *Brassica* PIs. Among the PIs for which AITC data were available, inhibition of radial growth was correlated with AITC concentration. For example, the suppressive *B. nigra* PI 169067 (0% of the control) had 0.36 mg of AITC/g of leaf tissue, while the marginally suppressive PI 179860 (49% of the control) contained only 0.078 mg of AITC/g of leaf tissue (Table 1).

Detectable concentrations of AITC were found in *B. nigra*, *B. juncea*, and *B. carinata*. In contrast, the cultivars of *B. napus*, *B. campestris*, and *B. hirta* did not contain AITC (Table 1). Radial growth of *F. sambucinum*, in response to *Brassica* cultivars and PIs in experiments 1 to 5, was negatively correlated ($P < 0.05$) with the concentration of AITC in the plant tissue (Fig. 3).

Volatile compounds released by *B. juncea* cv. Cutlass completely suppressed the radial growth (0% of the unamended control) of *V. dahliae*, *V. albo-atrum*, *C. coccodes*, *P. ultimum*, and *R. solani*. None of the fungi grew when transferred to fresh media, indicating that the volatile compounds produced by the *B. juncea* tissue were fungicidal. In contrast, *B. napus* cv. Midas did not inhibit radial growth of any of the fungi (>100% of the unamended control). Fungal cultures exposed to volatiles from *B. napus* were also viable when transferred to fresh media.

DISCUSSION

Data on radial growth of *F. sambucinum* and AITC production from macerated tissues of *Brassica* species suggested that AITC could be responsible for the fungicidal activity of these plants in our assays. Volatile compounds from *B. juncea* cv. Cutlass were fungicidal to all the plant pathogenic fungi tested. This was consistent with the hypothesis that AITC is responsible for fungicidal activity, since this compound has broad spectrum activity. Lazzeri et al. (8) investigated the effect of selected glucosinolates and their by-products on the sugar beet cyst nematode *Heterodera schachtii* Schmidt. AITC was the most toxic of the compounds formed from glucosinolate hydrolysis, causing 100% mortality of nematodes after 96 h of exposure. McCloskey and Isman (10) reported that the growth and feeding preferences of Bertha armyworm, *Mamestra configurata* Walker, on various *Brassica* species were negatively correlated to AITC production in the tissue. *B. juncea* contained the highest concentration of AITC and was the least preferred of the plant species in the study, which included *B. napus*, *B. campestris*, and *B. hirta*.

The lack of volatile suppressive activity by *B. napus* cv. Midas, or any of the PIs of *B. napus*, was surprising since rapeseed has been reported to be effective as a biocontrol agent against some pathogens. Mojtahedi et al. (11) used green tissue of rapeseed (*B. napus* cv. Jupiter) as a soil amendment and reported that it reduced *M. chitwoodi* populations in soil. Chan and Close (3) found that *B. napus*, *B. oleracea* L., *Raphanus sativas* L. 'Resal', and *B. hirta* provided control of Aphanomyces root rot of pea. *B. hirta* reduced the number of infective propagules of *A. euteiches* in infested soil (12). We did not detect volatile fungicidal compounds from macerated leaf tissue of either *B. napus*, *B. hirta*, or *B. campestris* in our bioassays. Perhaps biologically active volatile compounds are produced from the degradation of *B. napus* in soil, or nonvolatile compounds are responsible for the activity observed in previous studies. Alternatively, fungal propagules in soil may be more sensitive to toxic volatile compounds than the cultures used in this study. It also is possible that the single cultivar of *B. hirta* or *B. campestris* tested in this study was not representative of this species as a whole.

Because glucosinolate production is genetically determined, the differences in AITC production within and between the *Bras-*

TABLE 1. Radial growth of *Fusarium sambucinum* (% of the unamended control) as affected by *Brassica* cultivars and accessions and allyl isothiocyanate (AITC) in the corresponding plant tissue

Species	Cultivars/PIs[a]	AITC[b]	Radial growth[c]
Experiment 1			
B. juncea	cv. Cutlass	0.18	0
B. carinata	cv. Dodolla	0.063	94
B. napus	cv. Midas	0	98
B. nigra	cv. Type 1	0.165	22
B. hirta	cv. Ochre	0	94
B. campestris	cv. Torch	0	98
Experiment 2			
B. juncea	PI 458942	0.123	13
B. juncea	PI 458934	0.165	11
B. juncea	PI 426298	0.039	99
B. juncea	PI 470241	0	92
B. juncea	PI 458928	0.261	28
Experiment 3			
B. carinata	PI 331378	0.063	109
B. carinata	PI 209203	0.078	108
B. carinata	PI 197403	0.087	63
B. carinata	PI 360887	0.048	116
B. carinata	PI 360886	0.057	111
Experiment 4			
B. napus	PI 469746	0	112
B. napus	PI 250135	0	114
B. napus	PI 311729	0	112
B. napus	PI 458971	0	110
B. napus	PI 251236	0	118
Experiment 5			
B. nigra	PI 169067	0.36	0
B. nigra	PI 169066	0.288	0
B. nigra	PI 179860	0.078	49

[a] PIs = plant introduction accessions.

[b] AITC concentrations (mg/g of tissue) were measured using gas chromatography.

[c] Radial growth was assessed in bioassays that measured the activity of volatile compounds released from macerated leaf tissue. Data are based on 40 g of tissue (experiments 1 to 4) or 25 g of tissue (experiment 5).

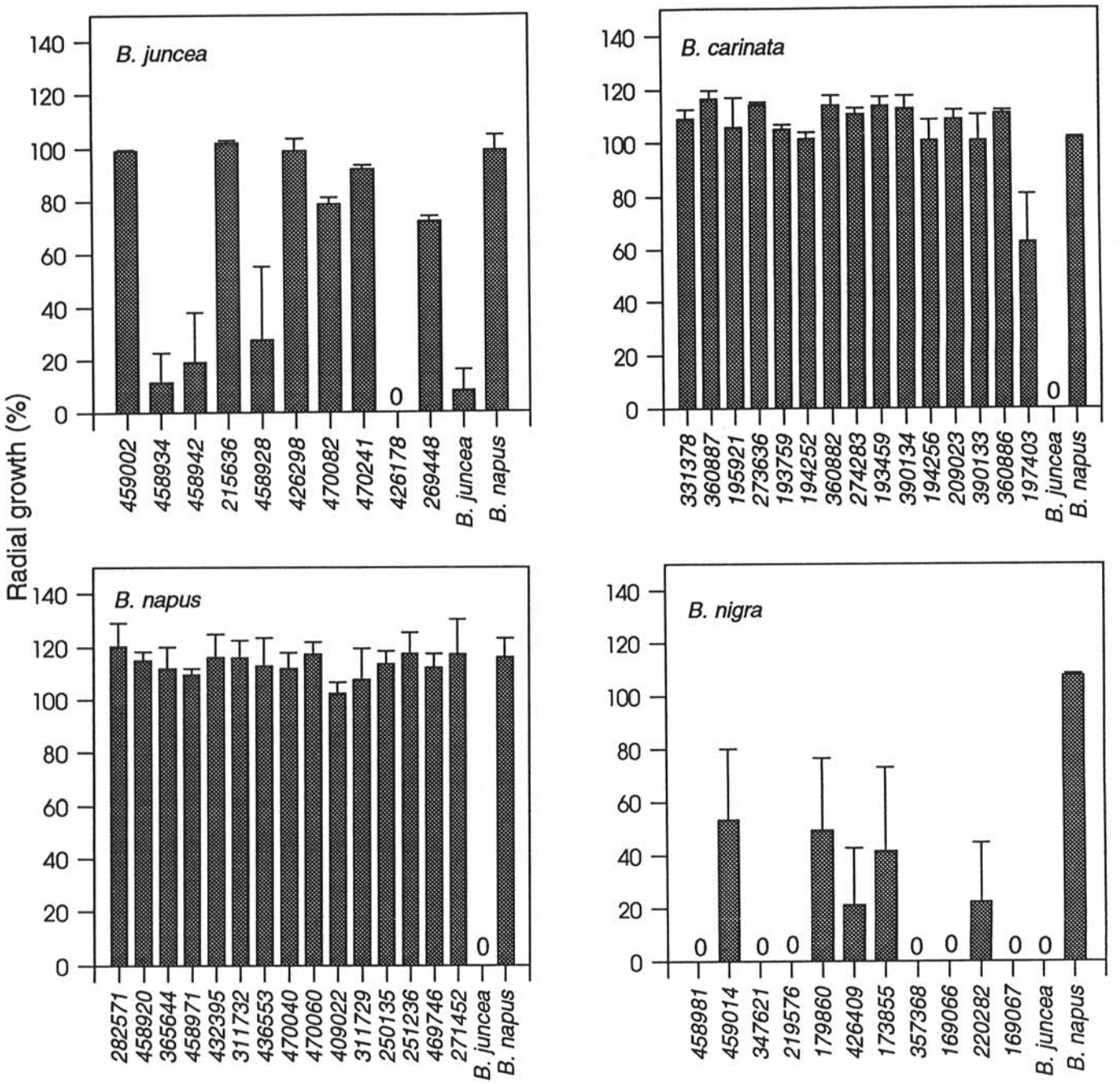

Fig. 2. Radial growth of *Fusarium sambucinum* (% of the unamended control) as affected by PIs of four *Brassica* species. *B. juncea* cv. Cutlass and *B. napus* cv. Midas were included as positive and negative controls in each experiment. Data are from bioassays that measured the fungicidal activity of volatile compounds released from macerated leaf tissue. Vertical bar indicates the standard error of the mean of three replicates. No error bar indicates no variation among replicates.

sica species tested may be because of the phylogenetic relationships among them. *B. nigra*, *B. campestris*, and *B. oleracea* are diploid species, while *B. juncea*, *B. napus*, and *B. carinata* are all natural amphidiploids. *B. juncea* is a naturally evolved amphidiploid with one set of chromosomes from *B. campestris* and the other from *B. nigra*. Allyl glucosinolate is the predominant glucosinolate found in *B. nigra*, while 3-butenyl and 4-pentenyl are two of the predominant glucosinolates found in *B. campestris* (17,21). Some *B. juncea* accessions had little or no AITC, while others contained significant amounts, possibly because of the segregation of genes for glucosinolate production. *B. napus* has chromosomes from *B. oleracea* and *B. campestris*, neither of which produce allyl glucosinolate in high concentrations. Some of the predominant glucosinolates in *B. oleracea* are 3-butenyl, 2-hydroxy-3-butenyl, 3-methylsulfinylpropyl, and 3-indolylmethy (17). *B. oleracea* cultivars produce allyl glucosinolate, but in smaller concentrations than *B. nigra*. Some *B. napus* species contain small quantities of allyl glucosinolate, but the most commonly found glucosinolates are 3-butenyl, 2-hydroxy-3-butenyl, 2-hydroxy-4-pentenyl, and 4-methoxy-3-indolymethyl (17,20,21).

B. carinata is a hybrid of *B. nigra* and *B. oleracea*. The *B. carinata* PIs tested consistently contained AITC, a breakdown product of allyl glucosinolate that is present in the diploid species *B. nigra* and *B. oleracea*. We found that AITC was present in all PIs tested, but not in the concentrations found in most *B. nigra* PIs and some *B. juncea* PIs. PIs of the diploid *B. nigra* showed less variability in suppressive activity and had the highest levels of AITC.

The ability of some *Brassica* genotypes to release volatile fungicidal compounds suggested that incorporation of green tissues of these plants as "green manures" could be useful for control of a wide range of soilborne fungal plant pathogens. This practice

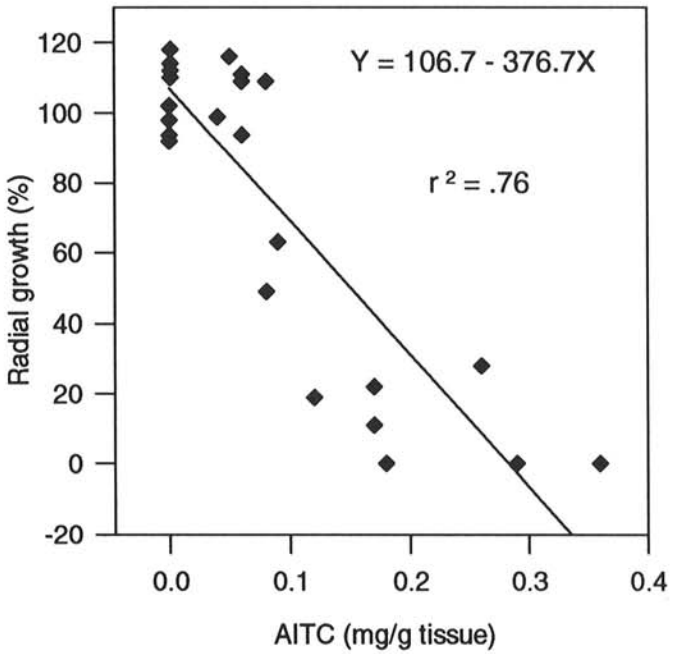

Fig. 3. Radial growth of *Fusarium sambucinum* (% of the unamended control) in response to allyl isothiocyanate (AITC) content of *Brassica* leaf tissue. Radial growth was assessed in bioassays that measured the activity of volatile compounds released from macerated leaf tissue. The AITC content (mg/g of leaf tissue) was measured using gas chromatography.

may substitute for soil fumigants in a variety of conventional agricultural systems and could also be used in organic crop production systems. The variability in both volatile suppressive activity and AITC concentration within and across *Brassica* species demonstrated that, in order to optimize biological control, *Brassica* genotypes will need to be individually selected. It should be possible to breed *Brassica* genotypes with very high AITC concentrations, and presumably high suppressive activity, along with agronomic characteristics appropriate for their use as cover crops in various crop production systems. However, reduction of population densities of fungal pathogens in soil due to the incorporation of *Brassica* tissue with high concentrations of allyl glucosinolate remains to be demonstrated.

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