

Mustards, mustard oils and mycorrhizas

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SUMMARY

Intact, living roots of *Brassica kaber* (DC.) Wheeler and *Brassica nigra*. L. (Brassicaceae) were inhibitory to the germination of spores of the mycorrhizal fungi *Glomus intraradices* Schenck & Smith and *Glomus etunicatum* Becker & Gerd. Roots from two similarly non-mycotrophic species, spinach (Chenopodiaceae) and *Amaranthus retroflexus* L. (Amaranthaceae), had no effect on the germination of spores of *G. intraradices*. The roots of two out of three mycotrophic species examined were stimulatory to germination of *G. intraradices*. To test the hypothesis that the inhibition of VAM fungus spore germination by the roots of *B. kaber* and *B. nigra* was due to isothiocyanates (mustard oils), lysine, arginine and glutathione were added separately to the nutrient solutions applied to the plants. These compounds were expected to react with isothiocyanates released into the rhizosphere. In one experiment, lysine, arginine and glutathione restored the germination of *G. intraradices* to control levels for *B. kaber*, and nearly to the control levels for *B. nigra*. In a second experiment employing lower concentrations of lysine, arginine and glutathione, germination of spores of *G. etunicatum* in the presence of *B. kaber* roots was restored nearly to the levels of controls. In the same experiment, these treatments had no significant effects on spore germination in the presence of *B. nigra* roots. Lysine and arginine also protected spores of *G. etunicatum* from *in vitro* reactions of *B. kaber* root glucosinolate extract with myrosinase. The involvement of isothiocyanates in the resistance of mustards to VAM fungi is discussed.

Key words: Vesicular-arbuscular mycorrhizas, isothiocyanates, glucosinolates, resistance, incompatibility.

INTRODUCTION

Mechanisms governing the incompatibility between non-mycotrophic plant species (non-hosts) and vesicular-arbuscular mycorrhizal (VAM) fungi are not well understood. It is unclear whether non-mycotrophic species fail to produce necessary signals or nutrients required by VAM fungi for successful invasion, or non-mycotrophic species are resistant to invasion by VAM fungi.

The presence of glucosinolates (mustard oil glucosides) in the Brassicaceae has been implicated in the resistance of this taxonomic group to VAM fungi (Tester, Smith & Smith, 1987 and references therein). Glenn, Chew & Williams (1985, 1988) examined the effects of *Brassica* species with varying glucosinolate levels on VAM fungus interactions and found no clear relationship between glucosinolate concentrations and the development of VAM fungi. While glucosinolates themselves appear to be non-

toxic (Holley & Jones, 1985; Mithen, Lewis & Fenwick, 1986; Vierheilig & Ocampo, 1990), isothiocyanates, produced by the action of myrosinase on glucosinolates, are toxic to a variety of organisms (Walker, Morell & Foster, 1937; McKay *et al.*, 1959; Larsen, 1981). It is clear that extracts from mustard plants are inhibitory to VAM fungi (Ocampo, Cardona & El-Atrach, 1986; Schreiner & Koide, 1992). Products of glucosinolate hydrolysis have also been shown to be inhibitory to VAM fungal spore germination *in vitro* (Vierheilig & Ocampo, 1990). However, we do not know what role glucosinolates may play in intact plants. Hydrolysis of glucosinolates is believed to occur primarily as a result of tissue damage, resulting in the mixing of glucosinolates with myrosinase (Luthy & Matile, 1984).

One purpose of this study was to investigate the effects of intact roots of mycotrophic and non-mycotrophic species on VAM fungus spore germination. Another purpose was to determine whether any negative effects of mustards on VAM fungus spore germination are due to isothiocyanates. Isothiocyanates have been shown to inhibit VAM fungus spore germination (Vierheilig & Ocampo,

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1990) and they can occur in the rhizosphere of intact roots (Tang & Takenaka, 1983; Schreiner, 1992). Two mustards were chosen for this investigation [*Brassica kaber* (DC.) Wheeler and *Brassica nigra* L.] because the predominant isothiocyanate from each species differs markedly from the other in its volatility.

METHODS

Source of materials

Seeds of the plant species were purchased from Valley Seed Co. (Fresno, CA, USA) or F&J Seed Service (Woodstock, IL, USA). Spores of *Glomus intraradices* Schenck & Smith and *Glomus etunicatum* Becker & Gerd. were purchased from Native Plants Inc. (Salt Lake City, UT, USA). Lysine, arginine, glutathione and myrosinase (thioglucosidase) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Glucosinolate extracts from the roots of *B. kaber* and *B. nigra* were obtained as described by Schreiner & Koide (1992).

Expt 1: In situ survey of the effects of plant roots on VAM fungi

Seeds of *Brassica kaber* DC. Wheeler, *Brassica nigra* L., *Spinacea oleracea* L., cv. America, *Amaranthus retroflexus* L., *Lactuca sativa* L., cv. Black Seeded Simpson, *Ambrosia artemisiifolia* L. and *Abutilon theophrasti* Medic. were sown in vermiculite and germinated in a growth chamber with a 14 h photoperiod ($PAR \approx 500 \mu\text{mol m}^{-2} \text{s}^{-1}$) and day and night air temperatures of 25 °C and 20 °C, respectively. Relative humidity was held constant at 50%. Seedlings were transplanted to soil after 7 d. The vermiculite attached to the seedlings was removed by gently washing the roots in distilled water.

The effects of living plant roots from mycotrophic and non-mycotrophic species on the germination of *G. intraradices* were examined using the filter 'sandwich' method described by Tommerup (1984). Thirty to 40 spores of the VAM fungus *G. intraradices* were placed on a 2 cm (diameter) nitrocellulose membrane with 0.45 μm pore size (Gelman Sciences Inc. Ann Arbor, MI, USA). The roots were placed on top of the VAM fungus spores and a second membrane was laid on top of the roots. The resulting 'sandwich' was then buried at a 45° angle in soil so that the sandwich would not separate during the experiment. The medium was an autoclaved mixture of sand and a low P silty loam soil (collected at the Rock Springs Agricultural Research Farm at the Pennsylvania State University) at a ratio of 3:1, respectively. Four replicates of each of the plant species and four control sandwiches without roots were placed in the growth chamber

under the conditions described earlier. The pots were fertilized with a one-fifth strength Hoagland solution (Machlis & Torrey, 1956) five times per week. All pots were amply supplied with additional water provided by automatic drip irrigators.

The spores within the membrane sandwiches were examined after 7 and 14 d incubation by retrieving them from the soil, separating each half and gently overlying them with trypan blue stain (0.01% in lactoglycerin). Any roots extending beyond the membranes were cut off before retrieving the sandwiches from the soil, so that the roots and VAM fungi inside the sandwiches were undisturbed. Germination of VAM fungal spores was measured by examining the stained samples under the microscope. Only spores with hyphae clearly attached were considered to be germinated. The length of roots within the sandwich was measured with a ruler to the nearest millimetre. VAM fungus spore germination data, expressed as percent germination, were analyzed using the analysis of variance procedure on Statgraphics version 5.0 (STSC, 1991). Mean contrasts were made at the 95% confidence level using Fisher's protected LSD method for all experiments except as noted.

Expt 2: In situ isothiocyanate reduction. I

We hypothesized that the inhibition of VAM fungus spore germination by mustard plants observed in expt 1 was due to isothiocyanates. Previous experiments demonstrated that we could not reduce the isothiocyanate concentrations in extracts of *B. kaber* roots grown in soil by sulphur limitation (Schreiner, 1992). Therefore, we tested the hypothesis that isothiocyanates were the active inhibitory agents by adding substances to the nutrient solutions that would be expected to react with any isothiocyanates before they could interact with VAM fungal spores. Lysine, arginine and glutathione were used because isothiocyanates are very reactive with primary amines. Isothiocyanates have been shown to react with lysine and arginine residues of proteins, converting them to thiourea derivatives (Kawakishi & Kaneko, 1987). Glutathione was also used because it has been shown to interact with allyl isothiocyanate (Kawakishi & Kaneko, 1985), and may be important in the ability of certain insects to feed on mustard species (Wadleigh & Yu, 1988). We employed a two-factor experiment using the sandwich method as in expt 1. The first factor had four levels [Hoagland solution control, Hoagland + 0.05% (w:v) lysine, Hoagland + 0.05% (w:v) arginine, Hoagland + 0.01% (w:v) glutathione] and the second factor (plant species) had three levels (no plant controls, *B. kaber*, *B. nigra*). There were four replicate pots per factor combination. All the pots were watered twice daily with the respective Hoagland solutions at one-fifth strength with the additions mentioned above.

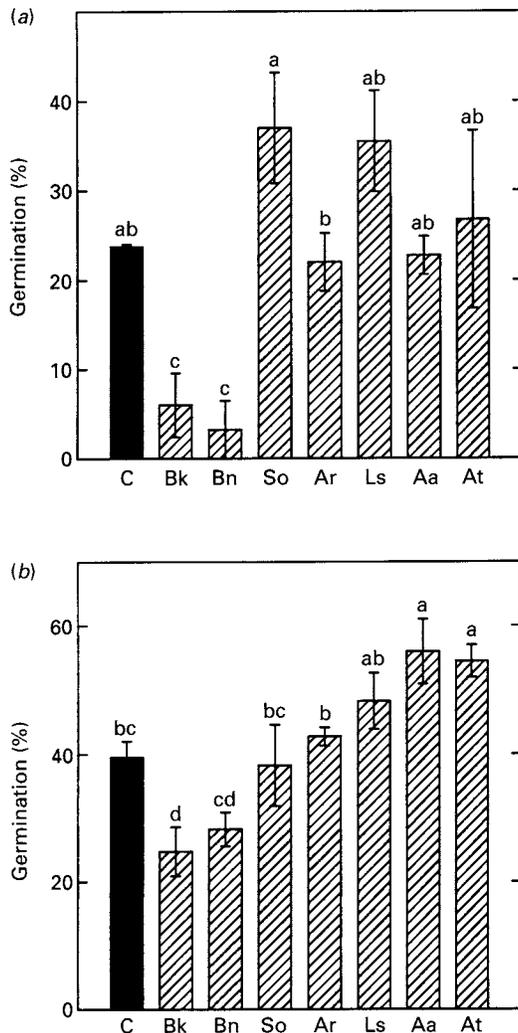


Figure 1. Effects of living roots from seven plant species on the percentage germination of *Glomus intraradices* spores in membrane sandwiches after (a) 7 d and (b) 14 d. Mean germination (%) \pm standard error for each species and the no-plant control are shown. Key: C, control; BK, *Brassica kaber*; Bn, *Brassica nigra*; So, *Spinacea oleracea*; Ar, *Amaranthus retroflexus*; Ls, *Lactuca sativa*; Aa, *Ambrosia artemisiifolia*; At, *Abutilon theophrasti*.

The membrane sandwiches were harvested after 10 d and examined as in expt 1. The shoots were cut off at soil level, dried and weighed to determine if the treatments were inhibitory to plant growth. Root lengths were measured as in expt 1.

Expt 3: In situ isothiocyanate reduction. II

This experiment was performed as was expt 2, except that lysine and arginine were added to nutrient solutions at 0.01% (approx. 500 μ M), and glutathione was added at a concentration of 0.005% (approx. 200 μ M). We expected that these treatments would not be as effective in arresting the negative effects of mustard roots on VAM fungus spore germination as the treatments used in expt 2. The experiment was repeated three times (3 blocks) with four replicate

pots per block. In the data analysis, each block was considered to be a replicate whose value was calculated as the mean of the individual pots within the block. The VAM fungus used was *G. etunicatum*.

Expt 4: In vitro isothiocyanate reduction

The ability of lysine, arginine and glutathione to arrest the inhibitory activity of isothiocyanates was independently tested by incubating VAM fungus spores in mixtures of root glucosinolates from *B. kaber* and myrosinase (in 20 mM final concentration MES buffer, pH 6.0), to which we added lysine, arginine or glutathione. Reaction mixtures of root glucosinolates and myrosinase alone served as the control (which was expected to inhibit VAM fungus spore germination). Reactions were made in a 5.0 mL volume with 1.0 unit of myrosinase (thioglucosidase, 1.0 unit = 1.0 μ mol glucose released per min at pH 6.0) and 1 mL of *B. kaber* root glucosinolate extract (previously adjusted to 1.0 g fresh wt per mL buffer). Spores of *G. etunicatum* were incubated in reaction mixtures for 30 min at 25 $^{\circ}$ C. After the incubation, spores were rinsed 10 times with distilled water and their germination was assessed after 10 d incubation on 'live' soil (unsterilized, see Schreiner & Koide, 1992). The maximum theoretical quantity of isothiocyanate(s) generated in reactions was 30 μ moles. Thus lysine, arginine and glutathione were added to give a final quantity of 100 μ moles.

Spores were also incubated in lysine, arginine, glutathione, root glucosinolate extract and myrosinase alone, in buffer at the same concentrations used in the reactions explained above. This was done to determine whether any reaction components had independent effects on VAM fungus spores. Incubation in buffer alone served as the control for the individual reaction components.

Expt 5: Soluble vs. volatile isothiocyanates

This experiment was to compare the abilities of soluble and volatile isothiocyanates generated from *B. kaber* and *B. nigra* glucosinolate extracts to inhibit the germination of VAM fungus spores. We tested soluble glucosinolate products as in expt 4 using extracts from both mustard species. Volatile glucosinolate products were tested by introducing reaction mixtures (root extracts+myrosinase) into small dishes that were placed inside the petri plates used to incubate the spores. Spores were incubated on nitrocellulose membranes on 'live' soil, but they did not have direct contact with the reaction mixtures. The extracts used in experiments with volatiles were at the same concentration as in the soluble test (1 mL of a 1.0 gm fresh wt per mL), but the enzyme concentration was reduced to 0.1 units of myrosinase. Fresh glucosinolate extracts and myrosinase

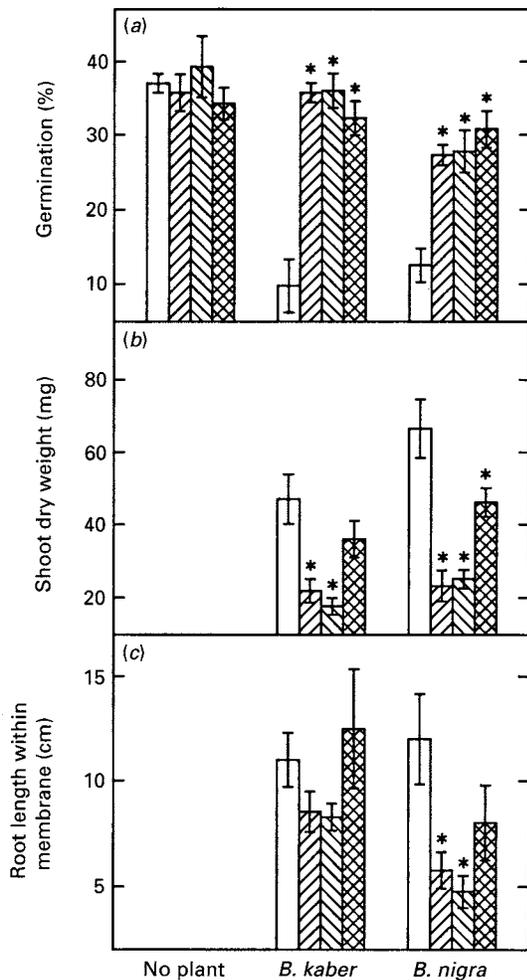


Figure 2. Effects of isothiocyanate-reducing treatments (I) on (a) percentage germination of *Glomus intraradices* spores, (b) shoot dry weight and (c) root length within the membrane sandwich. Means \pm standard errors (among individuals) are shown. Concentrations for lysine, arginine and glutathione in the nutrients solutions were 0.05%, 0.05% and 0.01%, respectively. The * above a bar indicates that the mean for that treatment is significantly different ($P \leq 0.05$) from the mean of the corresponding control based on Fisher's protected LSD. □, control; ▨, lysine, ▩, arginine, ▤, glutathione.

were added after 5 d in an attempt to maintain the concentration of volatile products within the petri plates throughout the 10 d incubation period. There were ten replicates per treatment.

RESULTS

Expt 1

The results from the *in situ* survey of the effects of living roots on VAM fungus spore germination are shown in Figure 1. After 7 d, the effects of plant species on the percentage germination of spores of *Glomus intraradices* were highly significant ($F = 5.76$, d.f. = 7,24, $P = 0.0005$). The roots of both mustards were significantly inhibitory to VAM fungus spore germination compared to controls. No

other plant species tested was significantly different from the control at 7 d (Fig. 1a). After 14 d, the effects of plant species on the percentage germination of spores were also significant ($F = 8.42$, d.f. = 7,24, $P \leq 0.0001$). *B. kaber* roots were still significantly inhibitory to the germination of spores (although less inhibitory compared to 7 d), while *B. nigra* roots were not significantly inhibitory at 14 d (Fig. 1b). The percentage germination of spores of *G. intraradices* in the presence of roots of the other non-mycotrophic species (*Spinacea oleracea* and *Amaranthus retroflexus*) was not significantly different from controls at either harvest. Two of the mycotrophic species (*Abutilon theophrasti* and *Ambrosia artemisiifolia*) were significantly stimulatory to the germination of VAM fungus spores at 14 d (Fig. 1b).

Expt 2

Both *B. kaber* and *B. nigra* reduced ($P \leq 0.05$) the germination of *G. intraradices* spores in the control treatments (nutrient solution alone), while the germination of spores in the presence of both mustards was increased by lysine, arginine and glutathione (Fig. 2). The percentage germination of VAM fungus spores within the *B. kaber* membrane sandwiches was restored to the no-plant control levels, while the percentage germination within the *B. nigra* sandwiches was restored nearly to the no-plant control levels by all of the isothiocyanate-reducing treatments (Fig. 2a). However, lysine, arginine and glutathione reduced the growth of both mustard species as compared to controls (Hoagland solution alone). Shoot dry weights were significantly reduced by the lysine and arginine treatments for *B. kaber* and were reduced by lysine, arginine and glutathione for *B. nigra* (Fig. 2b). The length of roots within the membrane sandwich was not significantly affected by treatment for *B. kaber*, whereas lysine and arginine treatments reduced the root lengths of *B. nigra* (Fig. 2c).

Expt 3

Isothiocyanate-reducing treatments were effective in restoring the germination rates of spores of *G. etunicatum* to, or nearly to, the no-plant levels for *B. kaber*, but not for *B. nigra*, when lower concentrations of lysine, arginine glutathione were used (Fig. 3a). None of the treatments (lysine, arginine, glutathione) had significant effects on the shoot dry weight or root length of either mustard (Fig. 3b, c).

Expt 4

The treatments used in expts 2 and 3 had similar effects on spore germination when spores of *G. etunicatum* were treated with isothiocyanates

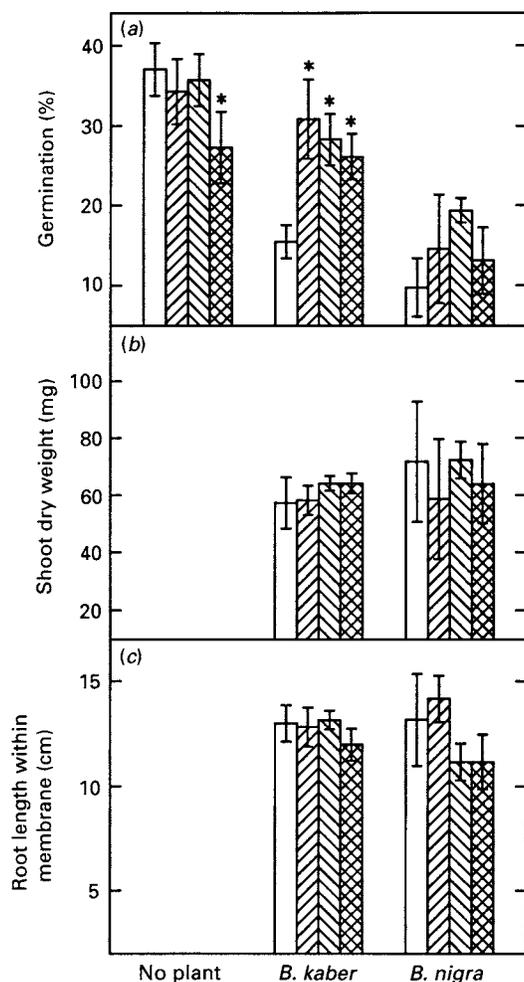


Figure 3. Effects of isothiocyanate-reducing treatments (II) on (a) percentage germination of *Glomus etunicatum* spores, (b) shoot dry weight and (c) root length within the membrane sandwich. Means \pm standard errors (among blocks, see materials and methods) are shown. Concentrations for lysine, arginine and glutathione in the nutrients solutions were 0.01%, 0.01% and 0.005%, respectively. The * above a bar indicates that the mean for that treatment is significantly different ($P \leq 0.05$) from the mean of the corresponding control based on Fisher's protected LSD. \square , control; ▨ , lysine, ▩ , arginine, ▧ , glutathione.

generated by reaction of glucosinolates with myrosinase *in vitro*. Lysine and arginine additions to reaction mixtures resulted in a significant restoration of the percentage germination of spores as compared to the reaction control with only glucosinolate extract and myrosinase (Fig. 4). Glutathione addition to reactions also increased the rate of germination of spores, but the value was not significantly different from the reaction control. Glutathione alone reduced the germination of spores (Fig. 4). Glucosinolate extract alone, myrosinase alone, and arginine alone were also shown to reduce ($P \leq 0.05$) the percentage germination of spores, as compared to buffer alone (control), but none of these reductions were as great as for glutathione alone and were certainly less than that resulting from extract plus myrosinase (Fig. 4).

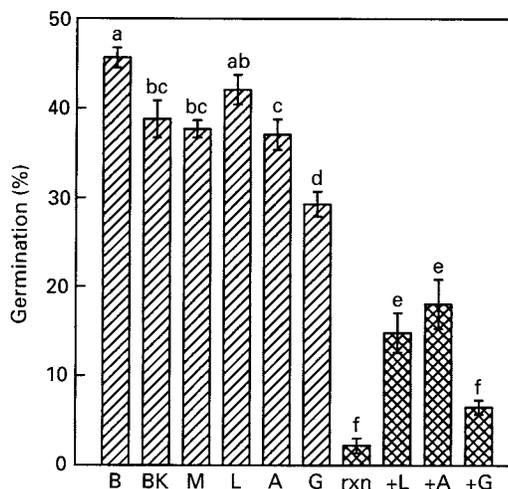


Figure 4. Effects of isothiocyanate-reducing treatments (III) on percentage germination of *Glomus etunicatum* spores treated with *in vitro* preparations of isothiocyanates. Single hatched bars represent control treatments of individual reaction components. Cross-hatched bars represent complete reactions (rxn) that produced isothiocyanates. Mean \pm standard errors are shown. Key: B, Buffer only; Bk, *Brassica kaber* extract only; M, myrosinase only; L, lysine only; A, arginine only; G, glutathione only; rxn, complete reaction; +L, rxn + lysine; +A, rxn + arginine; +G, rxn + glutathione.

Expt 5

A comparison of *in vitro* glucosinolate reactions between *B. kaber* and *B. nigra* root extracts showed a clear distinction between the two mustards. Soluble products from *B. kaber* root glucosinolate reactions were strongly inhibitory ($P \leq 0.05$) to the germination of spores, while soluble products from *B. nigra* reactions were not (Fig. 5a). In contrast, volatile products from reactions of glucosinolates from *B. nigra* roots were inhibitory ($P = 0.078$) to spore germination while those from *B. kaber* were not (Fig. 5b).

DISCUSSION

The results of this study clearly demonstrate that intact (undamaged) mustard roots can inhibit the germination of VAM fungus spores. These findings are consistent with the observations of Tommerup (1984). In contrast, the roots of spinach and *Amaranthus* had no significant effect on the germination of mycorrhizal fungal spores. This finding agrees with those of a previous study in which we showed that extracts of roots of spinach and *Amaranthus* did not have detectable antifungal activity (Schreiner & Koide, 1992). Others, however, have found some evidence for inducible defences against mycorrhizal fungi in the Chenopodiaceae (Allen, Allen & Friese, 1989).

We present three lines of evidence which are consistent with isothiocyanate release being the cause

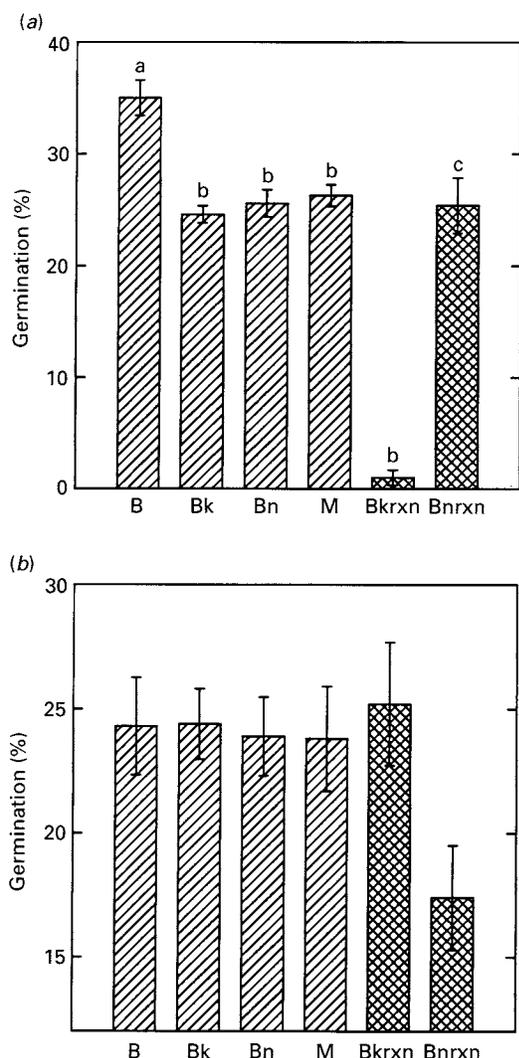


Figure 5. Comparison of the effects of (a) soluble and (b) volatile isothiocyanates from *Brassica kaber* and *Brassica nigra* on the percentage germination of *Glomus etunicatum*. Single hatched bars represent control treatments of reaction components. Cross-hatched bars represent complete reactions (rxn). Means \pm standard errors are shown. The mean separations in (b) were accomplished using an unprotected LSD method because the significant of treatment in this case was $P = 0.078$. Key: B, buffer only; Bk, *Brassica kaber* extract only; Bn, *Brassica nigra* extract only; M, myrosinase only; Bkrxn, *Brassica kaber* rxn; Bnrxn, *Brassica nigra* rxn.

of the inhibition of spore germination. Firstly, addition of compounds (lysine, arginine, glutathione) that were expected to react with isothiocyanates did diminish the inhibitory effects of intact mustard roots on the germination of spores. Consistent with this, the inhibition of germination by isothiocyanates produced *in vitro* from reactions of root extracts with myrosinase was also diminished by addition of the same compounds. Secondly, the *in vivo* effect of these hypothesized 'scavenger' compounds was better for *B. kaber* roots than for *B. nigra* roots, which is consistent with the non-volatile and volatile natures of the major isothiocyanates produced by these species, respectively. The major

glucosinolate produced by *B. kaber* roots has been shown to be sinalbin, which generates the relatively non-volatile 4-hydroxybenzyl isothiocyanate (Schreiner & Koide, 1992). The major glucosinolate occurring in *B. nigra* appears to be sinigrin, which generates the rather volatile allyl-isothiocyanate (Kjaer, 1960). Thirdly, in *in vitro* studies, the primary spore inhibitory activity was non-volatile for *B. kaber* and volatile for *B. nigra* extracts. This is, again, consistent with the known volatilities of the major isothiocyanates produced by the two species.

Although our results are consistent with the hypothesis that isothiocyanates were responsible for the inhibition of spore germination by intact mustard roots, we have not definitively established this. The most obvious alternative explanation for the effect of the 'scavenger' materials in expts 2 and 3 is that they altered the rhizosphere microbial populations, which could have had any one of a number of direct or indirect effects on VAM fungus spores. Further, we have not proven that the addition of lysine, arginine or glutathione did actually produce the respective thioureas, nor have we shown that the thioureas were necessarily less toxic than the isothiocyanate. However, in the *in vitro* expt, inhibition of spore germination occurred only when myrosinase was added to root extract. Thus, only when isothiocyanates could have been produced were spores inhibited. In that case, addition of the 'scavenger' materials did reduce the negative effects on spores. The most logical explanation for our findings is that the proposed scavenging compounds reacted with isothiocyanates in the rhizosphere of the mustard roots, converting them to less toxic compounds.

The mechanism responsible for the inhibition of spore germination by mustard roots may not be a direct effect of isothiocyanates. For example, it is possible that inhibition of spore germination could be a result of the interaction of isothiocyanates with chemical 'cues' important in initiating germination. The inhibition of spore germination by mustards may simply serve as a first line of defence against VAM fungi which functions to increase the time for roots to mature and become competent with other defences. Direct and indirect effects of isothiocyanates and the effects of anatomical and other chemical defences on subsequent steps in the infection process (adhesion, penetration, organ formation, etc.) also need to be carefully assessed. Regardless of the mechanism, however, it seems clear that isothiocyanates are capable of mediating some interactions between mustard roots and VAM fungi.

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