

# Antifungal compounds from the roots of mycotrophic and non-mycotrophic plant species

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## SUMMARY

The roots of twelve plant species from both mycotrophic (hosts of VAM fungi) and non-mycotrophic (non-hosts) families were examined for their ability to produce antifungal compounds. A TLC bioassay was used with the fungus *Cladosporium cucumerinum* Ellis & Arth. Of the non-mycotrophic species, five species from the Brassicaceae [*Brassica kaber* (DC.) Wheeler, *Brassica napus* L., *Brassica campestris* L., *Thlaspi arvense* L., *Rhaphanus raphanistrum* L.] had detectable antifungal compounds in extracts from roots, while two species from the Chenopodiaceae (*Spinacea oleracea* L., *Beta vulgaris* L.) and one species from the Amaranthaceae (*Amaranthus retroflexus* L.) did not. One mycotrophic species (*Daucus carota* L.) also had a detectable antifungal compound in root extracts, while three other mycotrophic species (*Lactuca sativa* L.K., *Abutilon theophrasti* Medic., *Sorghum bicolor* L.) did not. Chloroform extracts of *B. kaber* roots appeared to have the greatest quantity of extractable antifungal compounds as determined by the TLC bioassay. In a separate experiment, chloroform extracts from *B. kaber* roots greatly inhibited the germination of spores of *Glomus etunicatum* Becker and Gerd., while extracts from a number of other mycotrophic and non-mycotrophic plant roots were only marginally inhibitory. Three antifungal compounds in chloroform extracts from *B. kaber* roots were shown to be derived from glucosinolates. The predominant antifungal compound was identified as 4-hydroxybenzyl isothiocyanate, the isothiocyanate produced by the action of myrosinase on glucosinabin. The data provide evidence of a possible role for glucosinolates in determining the non-mycotrophic status in *B. kaber*.

Keywords: Antifungal compounds, vesicular-arbuscular mycorrhizas, glucosinolates, isothiocyanates.

## INTRODUCTION

Vesicular-arbuscular mycorrhizal (VAM) fungi are capable of forming functional mycorrhizas with a broad range of host plant taxa (Gerdemann, 1968). The non-mycotrophic plant species (incapable of forming VAM associations) make up only a small proportion of plant species and appear to be confined, for the most part, to particular families such as the Brassicaceae, the Chenopodiaceae and the Amaranthaceae (Tester, Smith & Smith, 1987). A notable exception is the non-mycotrophic genus *Lupinus* in the largely mycotrophic Fabaceae. The mechanisms governing compatibility between VAM fungi and the roots of plants are not well understood.

Some evidence suggests that non-mycotrophic plant species lack signals necessary for stimulating VAM fungi. Becard & Piché (1990) showed that exudates from transformed roots of carrot (a mycotroph) were stimulatory to hyphal growth of the VAM fungus *Gigaspora margarita*, while exudates from transformed roots of beet (a non-mycotroph) were not. Glenn, Chew & Williams (1988) proposed that *Brassica* species did not produce the stimulatory compounds necessary for VAM penetration of roots. Moreover, others have shown that limited mycorrhizal infections can be induced in non-mycotrophic species when they are grown in the presence of mycotrophic species (Hirrel, Mehravaran & Gerdemann, 1978; Ocampo, Martin & Hayman, 1980).

There may also be a role for fungal inhibitors in determining the non-mycotrophic status of some species. Compounds extracted from the non-mycotrophic *Lupinus* spp. induced abnormal infections

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in the mycotrophic white clover (Morley & Mosse, 1976). Aqueous root extracts from species in the Brassicaceae have been shown to inhibit VAM fungal spore germination (Ocampo, Cardona & El-Atrach, 1986). Since aqueous extracts from mustards would contain both glucosinolates and myrosinase, and since products of reactions between the glucosinolate sinigrin and myrosinase have been shown to inhibit VAM fungal spore germination (Vierheilig & Ocampo, 1990), the active substance(s) in aqueous extracts from mustards are most likely to be glucosinolate hydrolysis products. Glucosinolates themselves do not appear to be toxic to fungi (Holley & Jones, 1985; Mithen, Lewis & Fenwick, 1986), but the hydrolysis products of glucosinolates have for some time been known to be antifungal (Walker, Morell & Foster, 1937). Isothiocyanates (mustard oils) are the predominant hydrolysis products of glucosinolate reactions with myrosinase (Larsen, 1981). The glucosinolates and myrosinase appear to be compartmented in different regions of myrosin idioblasts. It is thus generally believed that hydrolysis occurs only when tissues are mechanically damaged (discussed in Luthy & Matile, 1984). However, some constitutive hydrolysis probably does occur (Tang & Takenaka, 1983) and thus it is possible that isothiocyanates may be important factors in the regulation of interactions between mustards and VAM fungi.

Glenn, Chew & Williams (1985) dismissed a role for glucosinolates in determining compatibility in *Brassica* species because no correlation was found between the glucosinolate content and the level of VAM fungal development in various *Brassica* cultivars. Indeed, while there are some reports that plants from the Brassicaceae can inhibit VAM infections in mycotrophic species growing nearby (Hayman, Johnson & Ruddlesdin, 1975; Powell, 1979; El-Atrach, Vierheilig & Ocampo, 1989), other reports have shown that VAM infections in mycotrophic species are unaffected by the presence of *Brassica* spp. (Black & Tinker, 1979; Powell, 1981).

The purpose of this study was to examine a number of mycotrophic and non-mycotrophic plant species for their ability to produce antifungal compounds. A greater number of species from the Brassicaceae were examined because of the possible role that glucosinolates play in determining their incompatibility with VAM fungi.

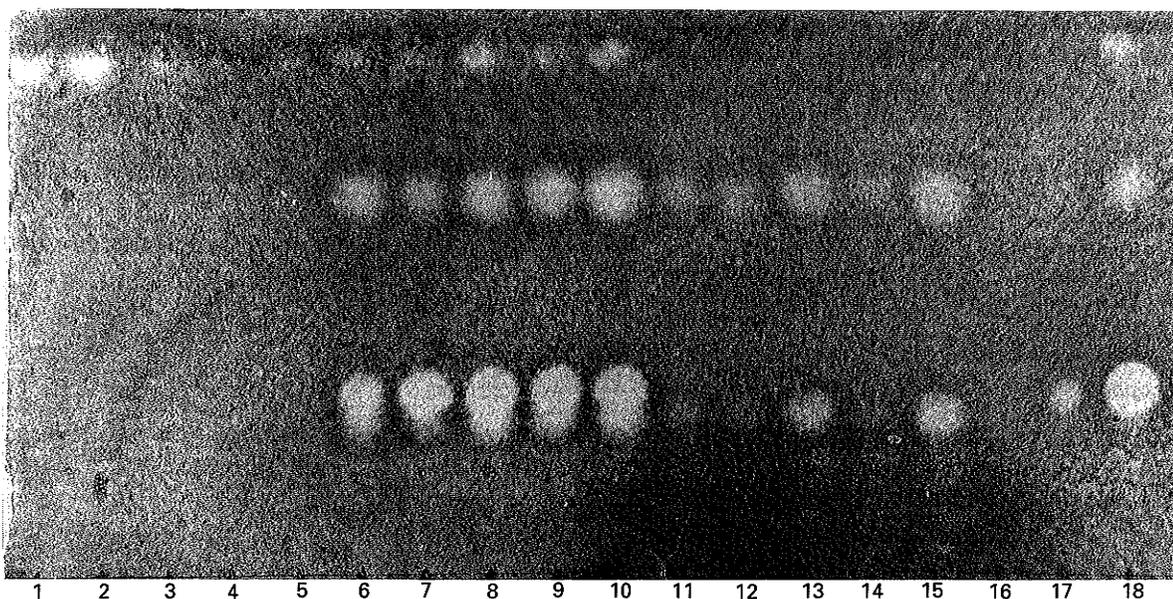
#### MATERIALS AND METHODS

Plant species were selected to represent a number of both mycotrophic and non-mycotrophic families. However, we examined a greater number of species from the Brassicaceae because of the possible role of VAM-inhibitory compounds produced by the members of this family. One-week-old seedlings of the twelve plant species listed in Table 1 were

transplanted from vermiculite into Turface, a calcined montmorillonite clay product (AMCOR, Deerfield, IL, USA). Turface was chosen as a growing medium because roots can be easily removed from the clay particles by gently washing in water. Fifteen individual plants from each species were planted in separate pots (150 mL ConeTainers, ConeTainer Nursery, Canby, OR, USA). Individual pots received approximately 4000 mycorrhizal fungal spores on attapulgite clay (NPI, Salt Lake City, UT, USA) that was mixed with the Turface before adding it to the pots. *Glomus etunicatum* Becker & Gerd. was the VAM fungal species used. Plants were placed in a growth chamber with a 14 h photoperiod at 500–600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR (400–700 nm) at bench top. Daytime air temperature was 25 °C and night air temperature was 20 °C. Relative humidity was held constant at 50%. Plants received one-fifth strength Hoagland solution without phosphorus five times per week (Machlis & Torrey, 1956). Plants were harvested 4 wk after transplanting and the roots were washed free from the growth medium, blotted dry, weighed, and extracted with either hexane, chloroform, or methanol for 48 h. Extracts were then decanted from the roots, concentrated by rotary evaporation and adjusted to a concentration of 1.00 g (f. wt) per mL solvent.

Root extracts were assayed for possible antifungal activity using a thin layer chromatography (TLC) bioassay method of Homans & Fuchs (1970). Twenty  $\mu\text{L}$  of each concentrated root extract from each plant (5 replicates for each solvent for each species) were spotted on silica gel 60 plates without fluorescence indicator (E. Merck Co., Darmstadt, Germany) and developed in 50:1 (v:v) chloroform:methanol. Standards of a *Brassica kaber* chloroform extract were also spotted on each species plants to account for variation among plates. Antifungal compounds were detected by spraying the plates with a suspension of *Cladosporium cucumerinum* Ellis & Arth. (obtained from M. Havey, USDA/ARS, Madison, WI, USA) and overlaying with half strength potato dextrose agar. The plates were incubated for 3 d in a humid chamber at 25 °C. Antifungal compounds appeared as white zones of inhibition of fungal growth against a dark background of fungal mycelium.

Root extracts were also tested for their effects on VAM fungal spore germination using spores of *G. etunicatum*. The extracts of each solvent from the five replicate plants were pooled. Forty  $\mu\text{L}$  of the pooled hexane or chloroform extracts from each species were transferred to 13 mm (diameter) nitrocellulose discs (0.45  $\mu\text{m}$  pore size, Gelman Sciences Inc. Ann Arbor, MI, USA) and the solvents were evaporated. Methanol extracts could not be tested on nitrocellulose since methanol dissolved the membrane. The methanol extracts were tested later using a different lot of *G. etunicatum* spores. The pooled



**Figure 1.** TLC bioassay plate for root extracts of *Brassica kaber*. Twenty  $\mu\text{L}$  of each extract (1.0 g f. wt per mL solvent) were spotted in each lane. Lanes 1–5, hexane extracts. Lanes 6–10, chloroform extracts. Lanes 11–15, methanol extracts. Lanes 16–18, standard *B. kaber* extracts equivalent to 0.2, 2.0 and 20 mg f. wt. root tissue applied. Four antifungal compounds detected in *B. kaber* were designated compounds 1, 2, 3 and 4, beginning at the bottom of the plate and increasing with the mobility of the compound.

methanol extracts were transferred to 13 mm nylon membrane discs (0.45  $\mu\text{m}$  pore size, MSI, Westboro, MA, USA). The root extracts and controls with hexane, chloroform, or methanol alone were allowed to evaporate. Approximately 25 spores of *G. etunicatum* were transferred to six replicate discs per treatment. The discs were then placed on the surface of wetted unsterilized (live) soil in petri plates and incubated in the dark at 25 °C for 5 and 12 d before staining. The soil used was a low P silt loam collected from the Rock Springs Agricultural Research Farm at the Pennsylvania State University. Discs were removed from the plates and gently overlaid with trypan blue stain (0.01% in lactoglycerin) to visualize the hyphae. Each disc was scored under 40X power for percentage spore germination. Only those spores that were clearly attached to darkly stained germ tubes were considered to be germinated. Spores were examined at 200X, if necessary, to see if hyphae were attached to a VAM fungal spore. Statistical analysis of percent spore germination was performed using an analysis of variance procedure on Statgraphics version 5.0 (STSC, 1991). Analyses were conducted separately for the harvests at 5 and 12 d, and for each solvent, since methanol extracts were assayed on a separate occasion. The factor analyzed was species. Mean contrasts were constructed using Fisher's protected LSD procedure at the 95% confidence level.

Glucosinolates were extracted from the roots of greenhouse-grown mustards by boiling fresh roots in 80% methanol for 10 min followed by grinding of the tissue with a mortar and pestle (Buchner, 1987). The methanol was removed by rotary evaporation.

The remaining solutions were centrifuged at 5000 g for 10 min to remove particulates. Glucosinolate extracts were then adjusted to 0.1 g (f. wt) per mL buffer [20 mM MES (2(*N*-morpholino)ethanesulphonic acid), pH 6.1].

To ascertain whether the antifungal compounds detected on TLC were derived from glucosinolates, 10 mL of glucosinolate extract were reacted with 0.20 units of myrosinase (Thioglucosidase, Sigma Chemical Co., St Louis, MO, USA). Control reactions were run with boiled enzyme. Reactions were extracted with chloroform after 15 min, after 1 h and after 5 h. The chloroform extracts were then spotted and developed on TLC plates using the procedure described previously.

Glucosinolates were also extracted from seeds and shoots of *B. kaber* using the same procedure as for roots. All three tissue samples from *B. kaber* were analyzed by HPLC for glucosinolate content following preparation of desulphoglucosinolates (Minchinton *et al.*, 1982) by Dr Allen Renwick (Boyce Thompson Institute, Ithaca, NY, USA).

Glucosinolate hydrolysis products in chloroform extracts of roots from *B. kaber* (Fig. 1) were purified by reversed-phase HPLC. Extracts were first fractionated employing a linear gradient of acetonitrile in water (5–95%) over a 30 min period. Further fractionations of components responsible for antifungal activity were carried out under isocratic conditions empirically determined to separate the components of interest. Ultraviolet spectral data were collected over the range 200–350 nm using a rapid scanning detector (Linear UVIS-206, Linear Instruments, Reno, NV, USA). Prior to mass

**Table 1.** TLC bioassay of root extracts from twelve plant species, some mycorrhizal (M), some non-mycorrhizal (NM). The presence of antifungal compounds is indicated by +. The solvent that best extracted the antifungal activity is indicated for each species

Family/Plant species <sup>1</sup>	VAM status	Antifungal compounds	Solvent	Rf ( <sup>2</sup> inhibition diam., cm)
Brassicaceae				
<i>Brassica kaber</i> (DC.) Wheeler	NM	+	Chloroform	0.28(0.6), 0.33(0.8), 0.71(0.7), 0.96(0.4) <sup>3</sup>
<i>Brassica napus</i> L.	NM	+	Chloroform	0.96(0.4)
<i>Brassica campestris</i> L.	NM	+	Chloroform	0.96(0.4)
<i>Thlaspi arvense</i> L.	NM	+	Chloroform	0.76(0.3), 0.96(0.6)
<i>Raphanus raphanistrum</i> L.	NM	+	Hexane	0.96(0.3)
Chenopodiaceae				
<i>Spinacea oleracea</i> L. cv. America	NM	—		
<i>Beta vulgaris</i> L. cv. Burpee's Red Ball	NM	—		
Amaranthaceae				
<i>Amaranthus retroflexus</i> L.	NM	—		
Compositae				
<i>Lactuca sativa</i> L. cv. Black Seeded Simpson	M	—		
Umbelliferae				
<i>Daucus carota</i> L. cv. Danvers Half Long	M	+	Methanol	0.17(0.4)
Malvaceae				
<i>Abutilon theophrasti</i> Medic.	M	—		
Gramineae				
<i>Sorghum bicolor</i> L.	M	—		

<sup>1</sup> Nomenclature according to Fernald (1970)

<sup>2</sup> The inhibition diameter values are the average diameters (for five replicates) of the areas without fungal growth for a particular antifungal compound.

<sup>3</sup> These compounds are herein referred to as compounds 1, 2, 3 and 4, respectively.

spectral analysis, the purity of individual components was assessed by examination of spectra taken at various points within each peak.

Mass spectral analysis was carried out on HPLC-purified components by the Penn State Chemistry facility employing electron ionization or chemical ionization (isobutane) as necessary.

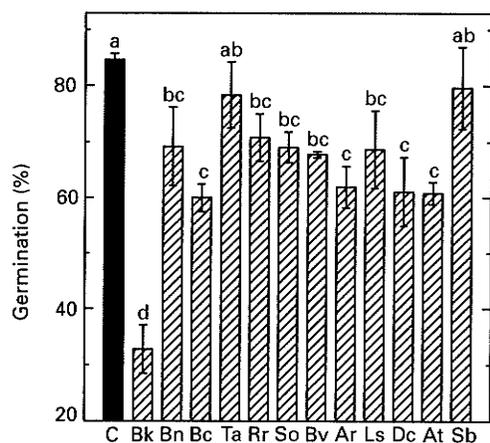
## RESULTS

Of the twelve plant species assayed by TLC, all five members from the Brassicaceae had detectable antifungal activity (Table 1). Chloroform was the best solvent for extracting the antifungal compounds from *B. kaber*, *B. napus*, *B. campestris*, and *Thlaspi arvense*, while hexane was the best solvent for extracting the *Raphanus raphanistrum* antifungal compound. Antifungal activity from all tested members of the Brassicaceae was detected near the solvent front. It is, therefore, unclear whether or not this represents a single compound since compounds running near the solvent front would not have been clearly separated. *Spinacea oleracea* and *Beta vulgaris* (Chenopodiaceae), and *Amaranthus retroflexus* (Amaranthaceae), did not have detectable levels of antifungal compounds in root extracts using any solvent. Three of the four mycotrophic plant species examined (*Lactuca sativa*, *Sorghum bicolor* and

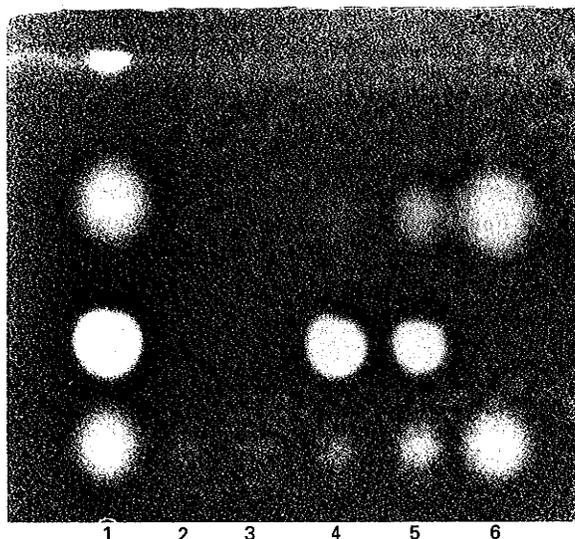
*Abutilon theophrasti*) similarly did not have detectable levels of antifungal compounds in root extracts using any solvent. However, the mycotrophic species *Daucus carota* had one distinct antifungal compound extracted by methanol (Table 1). Of all the plant species examined, *B. kaber* clearly had the greatest level of antifungal activity detected by this method (Table 1). *B. kaber* produced four distinct antifungal spots on the TLC bioassay plant (Fig. 1). These compounds were designated compounds 1, 2, 3 and 4 based on their relative mobilities from the bottom of the TLC plates.

The analysis of variance for the effects of the three root extracts (chloroform, hexane, methanol) from each species on the germination of spores of *Glomus etunicatum* at 5 and 12 d revealed that extracts from the various species were significantly ( $P \leq 0.05$ ,  $f = 7.2$ , d.f. = 12, 26) different from the controls only for chloroform at 5 d (Fig. 2). Chloroform extracts of *B. kaber* roots reduced the percentage germination of *G. etunicatum* to an average of 33%, as compared to 84% for the control treatment, after incubating for 5 d. Chloroform extracts from nine out of the remaining eleven species also significantly reduced the germination of *G. etunicatum* as compared to controls, but these effects were not as pronounced as for *B. kaber* (Fig. 2).

Compounds 1, 2 and 3 present in the chloroform



**Figure 2.** Effects of chloroform extracts of 12 species on the germination (%) of *Glomus etunicatum* spores after 5 d. Mean ( $\pm$ SE) germination for each species extract is shown. Control lane is for chloroform alone. Key: C, Control; Bk, *Brassica kaber*; Bn, *B. napus*; Bc, *B. campestris*; Ta, *Thlaspi arvense*; Rr, *Raphanus raphanistrum*; So, *Spinacea oleracea*; Bv, *Beta vulgaris*; Ar, *Amaranthus retroflexus*; Ls, *Lactuca sativa*; Dc, *Daucus carota*; At, *Abutilon theophrasti*; Sb, *Sorghum bicolor*.



**Figure 3.** TLC bioassay plate of glucosinolate hydrolysis products from the roots of *Brassica kaber*. Lane 1, standard *B. kaber* extract. Lanes 2 and 3, control reaction products using boiled myrosinase after 1 and 5 h, respectively. Lanes 4–6, reaction products after 15 min, 1 h and 5 h, respectively.

extracts from *B. kaber* were shown to be derived from glucosinolates, since reaction of glucosinolate extracts with myrosinase resulted in the production of compounds 1, 2 and 3, while control reactions did not produce these compounds (Fig. 3). Compound 2 was absent from the hydrolysis reaction after 5 h, suggesting it is unstable in aqueous solution. Similar reactions with glucosinolate extracts from the other four *Brassica* spp. also showed that the antifungal compounds detected in the TLC bioassay were glucosinolate derived (data not shown).

Compound 2 from *B. kaber* was identified as 4-hydroxybenzyl isothiocyanate on evidence from the mass spectrum, which gave a molecular ion of 165 and other fragment ions as would be expected from this compound. HPLC-purified compound 2 produced 4-hydroxybenzyl alcohol and thiocyanate ion when allowed to degrade in distilled water (data not shown), which is consistent with compound 2 being 4-hydroxybenzyl isothiocyanate. 'Compound 3' was shown to consist of two components, 8-methyloctylsulphonyl isothiocyanate and 9-methylnonylsulphonyl isothiocyanate; identification was based on the mass of molecular ions generated by chemical ionization. Daxenbichler *et al.* (1991) showed that these compounds are present in *B. kaber*.

HPLC analysis of desulphoglucosinolates from seeds, shoots and roots of *B. kaber*, showed that all three tissues contained glucosinabin (data not shown). There was a small amount of another unidentified glucosinolate in the root extracts from *B. kaber*.

#### DISCUSSION

The results of this study have shown that members of the Brassicaceae have the potential to produce significant quantities of antifungal compounds in roots. Most of the antifungal compounds detected in the mustards examined here have been shown to be derived from glucosinolates, which may indicate a role for a glucosinolate-mediated resistance to fungi in the roots of *Brassica* species. Ocampo *et al.* (1986) and Vierheilg & Ocampo (1990) have shown that extracts from the roots of various *Brassica* spp. and reactions of the glucosinolate sinigrin with myrosinase inhibited the germination of *Glomus mosseae* spores. Those reports, coupled with our observation that *B. kaber* extract containing isothiocyanates inhibited germination of *G. etunicatum*, provide evidence that isothiocyanates may inhibit germination of VAM fungal spores. The role of isothiocyanates in later stages of the infection process by VAM fungi (attachment, penetration, arbuscule formation, etc.) needs to be examined.

The fact that all species from the Brassicaceae produced antifungal compounds is not surprising because virtually all of the members of that family produce glucosinolates (Kjaer, 1960), and because some glucosinolate-derived compounds are known to be antifungal (Larsen, 1981). However, only extracts from *B. kaber* were strongly inhibitory to the germination of *G. etunicatum*. The lack of inhibition of VAM spore germination by extracts of the other mustard species may be due to a number of reasons. First, there is a wide range in toxicity of different isothiocyanates toward fungi (Drobnica *et al.*, 1967). Extracts from the roots of the other mustards did not appear to have as much antifungal activity on the TLC bioassay plates as the *B. kaber*

chloroform extracts (Table 1). This variation in biological activity may be associated with variation in the solubility of different isothiocyanates in aqueous media (Drobica *et al.*, 1967). Second, many isothiocyanates are fairly volatile and neither the TLC bioassay nor the spore germination tests employed here would be expected to detect volatiles with the same precision as non-volatile compounds. 4-hydroxybenzyl isothiocyanate produced by *B. kaber* is a relatively non-volatile isothiocyanate (Kjaer, 1960), and would be expected to remain on the membrane in contact with the VAM fungus spores for a longer period of time than volatile compounds. Third, the concentration of the antifungal compounds from the other mustard species examined here may have been below the concentration necessary to affect VAM fungus spore germination.

Variation in both the quantity and quality of glucosinolates may influence the interactions of different mustard species with VAM or other fungi. Thus, the ability or inability of mustards to affect VAM infections in mycotrophic species growing nearby (Hayman *et al.*, 1975; Black & Tinker, 1979; Powell, 1979, 1981; El-Atrach *et al.*, 1989) may be attributed to the range of toxicities observed for different isothiocyanates.

Since chloroform extracts of *B. kaber* were greatly inhibitory to the germination of spores of *G. etunicatum* at 5 d but not at 12 d, it appears that the inhibitory agent was unstable. 4-hydroxybenzyl isothiocyanate is known to be relatively unstable in aqueous conditions and appears to degrade to 4-hydroxybenzyl alcohol and thiocyanate ion (Kawakishi & Muramatsu, 1966). 4-hydroxybenzyl alcohol has been shown to be much less toxic to *Nematospora* than the parent isothiocyanate (Holley & Jones, 1985).

The absence of antifungal activity in root extracts from either *B. vulgaris* or *S. oleracea* from the Chenopodiaceae is consistent with observations in the literature that species from that family appear to have a higher incidence of VAM infections than species from the Brassicaceae (Tester *et al.*, 1987). In addition, species from the Chenopodiaceae appear to become infected to a greater degree by VAM fungi than species from the Brassicaceae when mycotrophic species are grown together with non-mycotrophic species (Hirrel *et al.*, 1978; Ocampo *et al.*, 1980). It would appear that resistance to VAM fungi is greater in species from the Brassicaceae as compared to the Chenopodiaceae, and may be related to the presence of the antifungal activity reported here.

The role of the antifungal compound extracted from *D. carota* roots in relation to VAM fungi is unclear. Carrot roots produced an amount of antifungal activity similar to that from three of five mustard species (*B. napus*, *B. campestris* and

*Raphanus raphanistrum*). However, extracts from roots of carrot and these mustard species did not reduce VAM fungal spore germination nearly to the extent that *B. kaber* extracts did at the same concentration. Moreover, the methods employed in this study were not likely to assess the activity of volatile compounds, and it is possible that volatile products of glucosinolates from these three mustard species may also be inhibitory to VAM fungi.

The data presented in this paper provide evidence that glucosinolate products have the potential to be involved in the resistance of *B. kaber* to VAM fungus infections. While glucosinolates are primarily believed to be deterrents to herbivory, there is increasing evidence that glucosinolate products may also play a role in resistance to various fungal pathogens (Schnug & Ceynowa, 1990). Whether any of these compounds, plays a role in determining the mycotrophic status of a plant hinges on its interaction with mycorrhizal fungi. It is not known to what extent antifungal activity in root extracts is related to antifungal activity *in vivo*.

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