Biol Fertil Soils (1996) 23:189–195 © Springer-Verlag 1996

ORIGINAL PAPER

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Mycorrhizae, biocides, and biocontrol. 4. Response of a mixed culture of arbuscular mycorrhizal fungi and host plant to three fungicides

Received: 27 June 1995

Abstract The effects of three commonly used fungicides on the colonization and sporulation by a mixture of three arbuscular mycorrhizal (AM) fungi consisting of *Glomus etunicatum* (Becker & Gerd.), *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe, and *Gigaspora rosea* (Nicol. & Schenck) in symbiosis with pea plants and the resulting response of the host-plant were examined. Benomyl, PCNB, and captan were applied as soil drenches at a rate of 20 mg active ingredient kg^{-1} soil 2 weeks after transplanting pea seedlings in a silty clay-loam soil containing the mixed inocula of AM fungi (AM plants). Effects of fungicides were compared to untreated plants that were inoculated with fungi (AM control). The effect of mycorrhizal inoculation on plant growth was also examined by including nonmycorrhizal, non-fungicide-treated plants (non-AM control). Fungicides or inoculation with AM fungi had only a small effect on the final shoot weights of pea plants, but had greater effects on root length and seed yield. AM control plants had higher seed yields and lower root lengths than the corresponding non-AM plants, and the fungicide-treated AM plants had intermediate yields and root lengths. Seed N and P contents were likewise highest in AM control plants, lowest in non-AM plants, and intermediate in fungicide-treated AM plants. All three fungicides depressed the proportion (%) of root length colonized by AM fungi, but these differences did not translate to reductions in the total root length that was colonized, since roots were longer in the fungicide-treated AM plants. Pea plants apparently compensated for the reduction in AM-fungal metabolism due to fungicides by increasing root growth. Fungicides affected the population of the three fungi as determined by sporulation at the final harvest. Captan significantly reduced the number, relative abundance, and relative volume of *G. rosea* spores in the final population relative to the controls. The relative volume of *G. etunicatum* spores was greater in all the fungicide-treated soils, while *G. mosseae* relative volumes were only greater in the captan-treated soil. These findings show that fungicides can alter the species composition of an AM-fungal community. The results also show that AM fungi can increase seed yield without enhancing the vegetative shoot growth of host plants.

Key words Fungicides · *Glomus* · *Gigaspora* · Populations · Sporulation · Mycorrhizae

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Introduction

Differential tolerance of particular species of arbuscular mycorrhizal (AM) fungi to fungicides has been observed among three different *Glomus* species treated with Bavistin (carbendazim), Tilt Turbo (propiconazole), or Calixin (tridemorph; Dodd and Jeffries 1989), between two *Glomus* species treated with benomyl or captan (Kough et al. 1987), and among three *Glomus* species treated with chloroneb, etridiazole, or triadimefon (Spokes et al. 1981). It is unclear how consistent such responses may be even under the same experimental conditions (Schreiner and Bethlenfalvay 1996), but different sensitivities among AM fungi to fungicides may lead to the selection of more tolerant isolates within AM-fungal communities after repeated use of particular chemicals.

Studies comparing different AM-fungal species or isolates have demonstrated that some fungi are more effective than others in promoting host plant growth (Bethlenfalvay et al. 1989), nutrient uptake (Sieverding 1991), heavy metal tolerance (Haselwandter et al. 1994), and soil aggregation (Miller and Jastrow 1992). Different species or isolates of AM fungi produce a range of mycorrhiza phenotypes that can be expressed within root and soil colonization patterns (Mc Gonigle and Fitter 1990; Jakobsen et al.

1992). Such inherent differences in AM-fungal species will affect their ability to compete within the soil community and dictate how environmental stresses influence them (Johnson and Pfleger 1992; Sylvia and Williams 1992). Recent studies have shown that cropping history (Johnson et al. 1991) and tillage (Douds et al. 1995) exert selective pressures resulting in changes in the AM-fungal community. Such population shifts may not be beneficial for future plant growth (Johnson et al. 1992) or soil conservation (Miller and Jastrow 1992).

Results from a previous study indicated that differences in the sensitivity of three AM fungi to the fungicides benomyl, PCNB, and captan occurred in association with pea plants in Chehallis silty clay-loam soil (Schreiner and Bethlenfalvay 1996). We suggested that such differences would give an advantage to the most tolerant fungus, thereby increasing its competitive ability in a community of AM fungi. The purpose of this study was to determine whether the fungicides benomyl, PCNB, or captan alter the population of AM fungi in a mixed culture of three isolates, whether such changes in mycosymbiont species composition are predicted by the apparent sensitivity of these fungi to fungicides as found previously (Schreiner and Bethlefalvay 1996), and whether host plant response to mycorrhizal infection would be altered.

Materials and methods

Experimental design

The experiment was a randomized block design with four fungicide treatments (benomyl, PCNB, captan, or no fungicide) applied to AM plants plus an additional control free of AM-fungal propagules (non-AM control). Ten replications per treatment were employed for a total of 50 experimental units (potted plants). Data were analyzed using analysis of variance (ANOVA) procedures on Stargraphics version 5.0 (STSC 1991). Mean contrasts were made at the 95% confidence level using the least squares method.

Biological materials

The host plant used was *Pisum sativum* L. cv. Little Marvel, which had been previously grown from a single seed to minimize variability. The AM-fungal inoculum consisted of a mixture of three isolates: *Glomus etunicatum* (Becker & Gerd.) INVAM#UT183-1, *Glomus mosseae* (Nicol. & Gerd) Gerd. & Trappe BEG#46 (Dodd et al. 1994), and *Gigaspora rosea* (Nicol. & Schenck) INVAM#FL105. Whole soil inoculum from each of the AM fungi containing spores, root fragments, and hyphae cultured in association with *Sorghum bicolor* L. was mixed into the experimental soil prior to placing soil in pots. The soil used was Chehallis silty clay-loam with a relatively high available P content (28 mg kg^{-1} $NaHCO_3$ extractable P). Other nutrient contents of this soil were described earlier (Schreiner and Bethlenfalvay 1996). Inoculum from each of the fungi was stored dry at 4 °C for 6 months prior to use to overcome any possible dormancy (Tommerup 1984).

The quantity of inoculum from each of the three species was adjusted so that each pot (1.5 l) received 200 infective propagules of each of the fungi. The number of infective propagules of each fungal inoculum was determined in a preliminary experiment with peas in the same soil used for the study. In this preliminary experiment, each fungal inoculum was individually mixed with soil, placed in pots (200 ml), and 5-day-old pea seedlings were transplanted into them. After 2 weeks of growth, the entire root system of ten replicate plants was washed from the soil, cleared, stained, and examined for the number of distinct infection units formed per plant by each fungus (Franson and Bethlenfalvay 1989). The mixed inoculum used in the experiment contained 920 *G. etunicatum* spores, 178 *G. mosseae* spores, 464 *G. rosea* spores plus infective hyphae and root fragments present in each inoculum, which yielded 200 infective propagules per species in the preliminary experiment.

Growth conditions and fungicide applications

Five-day-old pea seedlings were transplanted from vermiculite into 1.5-l pots filled with steam-pasteurized soil (75 °C, 45 min) to which AM-fungal inocula were added. Three fungicides [benomyl(methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate 50% WP), PCNB (pentachloronitrobenzene 75% WP), or captan (*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide 50% WP)] were applied as a soil drench (200 ml suspension) at the rate of 20 mg active ingredient (a.i.) kg^{-1} soil 2 weeks after transplanting seedlings, when the number of infection units should have been equal among the three fungi. The rate of fungicides applied was within the recommended application rate for each of the chemicals. Control pots received 200 ml water. Plants were grown in a greenhouse for 65 days after transplanting (March through May, 1993) until harvest. Supplemental lighting was not provided because it was an unusually sunny spring. Solar radiation on 43 of 65 days during this experiment exceeded 50% of the maximum daily value (full sun) during the growth period (Agri-Met 1993). The mean daily estimated photosynthetically active radiation (PAR) derived from solar radiation data by comparison to PAR measurements (Licor LI-185A, Quantum Photometer) made in the greenhouse on three sunny days in April 1993 was 19.4 mol m^{-2} day^{-1}. Temperatures were maintained between 20 and 28 °C during the photoperiod, and between 16 and 24 °C during the night. One week after transplanting, each pot received 200 ml of a one-half strength Hoagland's solution (Machlis and Torrey 1956) without phosphorus, 3 times per week.

Assays

Root samples were obtained by coring pots 65 days after transplanting when the majority of seed pods were dry. Total and AM-fungal colonized root lengths were determined by the grid-line intersect method of Newman (1966) after clearing and staining the washed roots from each soil core. Root samples were cleared in 5% (w:v) KOH (20 min, 90 °C), rinsed with water, acidified in 1% (w:v) HCl (10 min, 90 °C), stained with trypan blue (0.5% w:v in lactoglycerin, 20 min, 90 °C, and destained overnight in lactoglycerin. The percentage root length colonized was calculated from the total root length and colonized root length.

Shoot dry weights were obtained after removing seed pods and oven drying (70 °C, 3 days) plant tops cut off at the soil surface 65 days after transplanting. Seed pods were collected from each plant as they matured and the stem leading to the pod had turned yellow. Previous experience showed that some pods break open if left on the plants until all pods are mature. Seeds were allowed to dry in the pods for 1 week after the final harvest. The number of pods, seeds, seed weight (yield), and specific seed weight were determined for each plant. Seed N and P concentrations were estimated for each plant after acid digestion (18 *N* sulfuric acid: 30% H_2O_2, 400 °C, 1 h) of two randomly selected seeds with a dry mass between 0.18 and 0.23 g, followed by colorimetric determination of N (Nessler method, Jensen 1962) and P (Watanabe and Olsen 1965). Seed N and P contents were calculated by multiplying concentrations by yield.

The soil in each pot was removed, broken up, air dried, and mixed thoroughly before obtained 50-g subsamples for spore counts. The number of spores produced by each AM fungus was determined by wet sieving, collecting, and counting the number of spores of each fungus retrieved on sieves (500, 250, 125, and 75 μm). Spore volumes for each of the 3 AM fungi were calculated by multiplying the number of spores produced by each fungus with its mean spore vol-

ume, determined on 15 randomly selected spores from the inoculum of each fungus mounted in polyvinyl alcohol-lactic acid-glycerin (PVLG, Morton et al. 1993). The mean spore volumes for each fungus were: *G. etunicatum*, 0.45 nl spore^{-1}; *G. mosseae*, 4.19 nl spore^{-1}; *G. rosea*, 9.20 nl spore^{-1}. Relative abundance and relative volume for each AM fungus was calculated from the total number and total volume of spores for all three fungi combined. Spore numbers and volumes of the initial inoculum are presented with the final sporulation data for comparison.

Results

Shoot growth of pea plants was not greatly affected by the treatments. Captan significantly reduced shoot dry mass of AM plants as compared to the AM controls, but other comparisons were not significant (Fig. 1). Treatment effects were much larger for both root length and seed yield as reflected by the *P*-values from ANOVA (Fig. 1). Non-AM control plants had longer roots and lower seed yields than the corresponding AM control plants. All three fungicides reduced seed yield and increased root lengths in AM plants as compared to the untreated, AM controls (Fig. 1). Seed N and P concentrations were not affected by treatments, but total N and P contents of seeds were highest in AM control plants, lowest in non-AM plants, and intermediate in fungicide-treated AM plants (Table 1). The reduction in seed yield caused by fungicides was mostly due to a reduction in the number of seeds produced per plant (Table 1). Regression analysis across all treatments ($n=50$) showed that the number of seeds produced per plant accounted for 86% of the variation in yield (Table 2).

Fungicides reduced the proportion of root length colonized by AM fungi, but not the total root length colonized (Table 1), because the fungicide-treated plants had greater root lengths than the AM control plants (Fig. 1). The three AM fungi could not be accurately distinguished in the roots; an assessment of fungicide effects on colonization by each fungus could therefore not be made. The relative effects of fungicides on each of the fungi in the mix could only be inferred by examining spores produced by each fungus, which could be easily distinguished. Fungicides affected the production of spores by the three fungi expressed as either relative abundance or as relative spore volume (Fig. 2). Captan produced a significant decline in

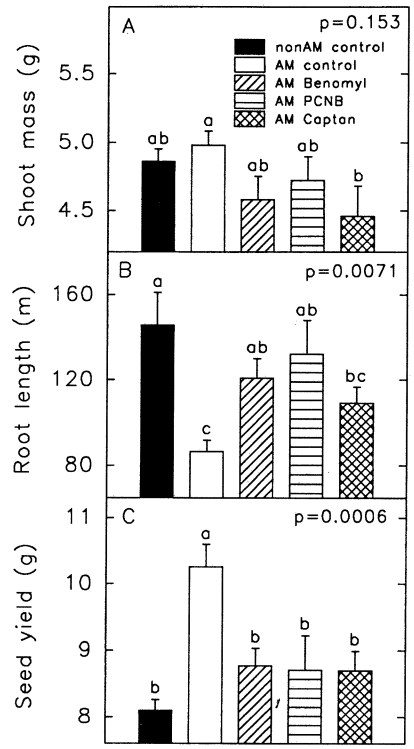

Fig. 1 Effects of three fungicides applied as a soil drench (20 mg a.i. Kg^{-1} soil) and AM-fungal inoculation with three AM fungi on *A* shoot dry mass, *B* root length, and *C* seed yield of pea plants. *Letters* designate significant groups at the 95% (LSD) confidence level; *P* values from ANOVA are indicated on each graph. Treatments were: non-AM control, nonmycorrhizal, untreated; AM control, inoculated, untreated; AM Benomyl, inoculated+Benomyl; AM PCNB, inoculated+PCNB; AM Captan, inoculated+Captan. Data represent means of ten plants+standard errors

Table 1 Effects of fungicides and AM-fungal inoculation on reproductive variables for pea plants and AM-fungal colonization of roots

	Non-AM control	AM control	AM Benomyl	AM PCNB	AM Captan
Pods/plant $P=0.404$[a]	15	15	15	13	14
Seeds/plant $P=0.073$	38b	45a	39b	38b	39b
Specific seed mass (mg) $P=0.215$	215b	228ab	224ab	232a	227ab
Seed P conc. (mg g^{-1}) $P=0.362$	2.25	2.25	2.39	2.43	2.51
Seed P content (mg) $P=0.0748$	18.2b	23.1a	21.0ab	21.2ab	21.8ab
Seed N conc. (mg g^{-1}) $P=0.306$	30.1	28.9	29.3	30.9	28.7
Seed N content (mg) $P=0.006$	243b	297a	256b	267ab	249b
% Root length colonized $P<0.001$	0d	77a	52c	56c	67b
Total root length colonized (m) $P<0.001$	0b	67a	62a	76a	73a

[a] *P* values from ANOVA; letters designate significant groups horizontally at 95% (LSD) confidence level

Table 2 Measured variables showing a significant linear regression to seed yield of peas for all data across all treatments

Variable	*P* value	Regression coefficient
Shoot dry mass (g)	0.0128	0.373
Pods/plant	0.0044	0.422
Seeds/plant	<0.0001	0.930
Seeds/pod	0.0001	0.564
Specific seed mass (mg)	0.0357	–0.317
Colonized root length (m)	0.0004	0.512
% Colonized root length	0.0007	0.493
G. etunicatum spores/g	0.0008	0.485
Relative abundance *G. etunicatum*	0.0159	0.361
Relative volume *G. etunicatum*	0.0442	0.305
G. mosseae spores/g	0.0003	0.522
G. rosea spores/g	0.0282	0.331
Relative abundance *G. rosea*	0.0483	0.299
Relative volume *G. rosea*	0.0180	0.355
Total number spores/g	0.0004	0.509
Total volume spores/g	0.0002	0.532

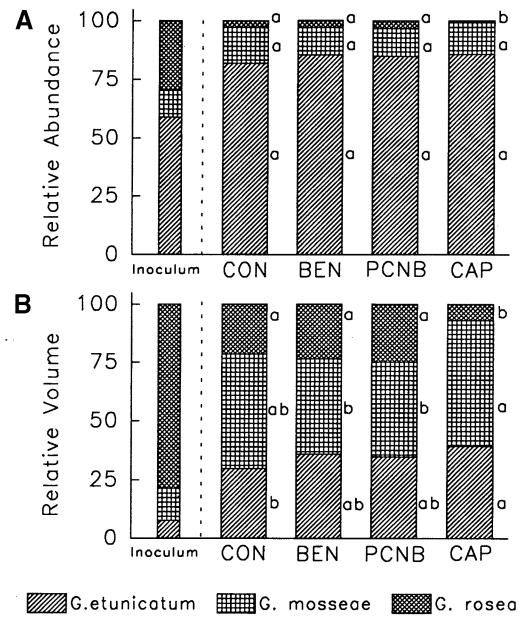

Fig. 2 Effects of three fungicides applied as a soil drench (20 mg a.i. kg^{-1} soil) on *A* relative abundance and *B* relative volume of spores produced by three AM fungi in association with pea plants. *Letters* designate significant groups horizontally at the 95% confidence level; *P* values from ANOVA for each fungus were: *G. etunicatum*, *A* 0.527, *B* 0.217; *G. mosseae, A* 0.534, *B* 0.066; *G. rosea, A* 0.001, *B* 0.0002. All treatments were inoculated with three AM fungi. Treatments were: CON untreated control, *BEN*+Benomyl, *PCNB*+PCNB, *CAP*+Captan. Data represent the means from ten replicates (soil from ten plants). Values for the initial inoculum are provided for comparison

the abundance and volume of *G. rosea* spores, indicating that development of this fungus was reduced by captan, but not by benomyl or PCNB relative to the other two fungi. The relative abundance of *G. etunicatum* and *G. mosseae* was unaffected by fungicides, but fungicide effects were significant when relative spore volumes were compared (Fig. 2). The reduction of *G. rosea* spore volume by captan was compensated by relative increases in both *G. etunicatum* and *G. mosseae* spore volumes. The relative volume of *G. etunicatum* was higher in all the fungicide treatments than in the untreated control, while *G. mosseae* relative volumes were highest in captan-treated soil, and lower in the benomyl and PCNB treatments. Captan therefore increased the relative populations of both *Glomus* species at the expense of *Gigaspora*, while benomyl and PCNB increased the relative population of *G. etunicatum* at the expense of *G. mosseae*, with *G. rosea* unaffected by these fungicides.

The absolute spore numbers and corresponding spore volumes of each of the three fungi per unit mass of soil also showed that *G. rosea* was inhibited by captan (Table 3), supporting the data expressed as the relative contribution *G. rosea* spores made to the total. The number (and corresponding volume) of *G. etunicatum* spores g^{-1} soil was unaffected by fungicides, while *G. mosseae* was reduced by PCNB in comparison to the AM control (Table 3). The total spore numbers and total spore volumes of the mix (accounting for all three AM fungi) were not different between the control and the fungicide treatments, but were higher in the benomyl treatment than with either PCNB or captan (Table 3). The number of spores of each of the three fungi increased over the course of the experiment, indicating that all three fungi were participating in the symbiosis. However, the number of spores for both *Glomus* isolates increased approximately 80- to 100-fold, while G. rosea only increased about 8-fold.

Table 3 Effects of fungicides on AM-fungal sporulation. Values represent absolute spore numbers (spores g^{-1} soil) and spore volumes (nl g^{-1} soil), and are the means of ten replicates

	Inoculum[a]	Control	Benomyl	PCNB	Captan	*P* value[b]
G. etunicatum spores	0.5	44	57	42	43	0.195
G. etunicatum volume	0.2	20	26	19	19	0.195
G. mosseae spores	0.1	7.8a	7.3a	4.6b	6.3ab	0.059
G. mosseae volume	0.4	33a	31a	19b	26ab	0.059
G. rosea spores	0.2	1.5a	2.0a	1.8a	0.4b	0.007
G. rosea volume	2.1	14a	18a	16a	3.6b	0.007
Total number spores	0.8	53ab	66a	48b	49b	0.136
Total spore volume	2.7	66ab	74a	55b	49b	0.037

[a] Initial inoculum levels
[b] *P* value from ANOVA, excluding the non-AM control treatment; letters designate significant groups horizontally at 95% (LSD) confidence level

Seed yield was significantly correlated with all the AM-fungal variables measured using data from all the treatments (Table 2). The best correlations between yield and AM-fungal parameters occurred for total spore volume, number of *G. mosseae* spores g^{-1} soil, total root length colonized by AM fungi, and the total number of spores produced (Table 2). Root length ($P=0.84$) was not correlated to yield across the entire data set, even though the means among different treatments were inversely proportional to yield (Fig. 1). However, shoot dry weights were correlated with yield (Table 2) even though the means among treatments did not show as strong a relationship to yield as root length (Fig. 1).

Discussion

Vegetative growth was not improved in AM plants in view of the relatively high soil P concentration. However, the significant increase in AM-plant seed yields showed that growing plants to maturity may be necessary to realize AM-fungal benefits to plant performance in soils containing P at moderately high concentrations. Changes in resource allocation by AM fungi within the plant are well known (Koide 1985) and are affected by both the host plant and the endophyte (Dhillon 1992). Increases in plant reproduction by AM fungi are largely a result of increased nutrient (especially P) uptake (Jakobsen 1983; Lu and Koide 1994), and our N and P data indicate that total uptake was improved by AM fungi comparison to non-AM roots and was decreased by fungicides in treated AM plants.

Seed N and P concentrations, however, were unaffected by treatments, and N and P contents changed in accordance with plant yield, suggesting that the mechanism responsible for yield reductions in fungicide-treated plants was not only due to decreased nutrient supply caused by the inhibition of AM fungi. The association of increased root growth with reduced AM-fungal colonization (percentage colonization) due to fungicide treatments suggested that plants compensated for the loss of nutrient uptake by AM fungi by increasing root length, confirming similar findings by Gnekow and Marschner (1989). Thus, the behavior of fungicide-treated AM plants was similar to that of the non-AM controls, whose longer roots were associated with lower yields. Apparently, the pea mycorrhiza was a more efficient supplier of nutrients for a given amount of carbon allocated to roots than both non-AM roots and fungicide-inhibited AM roots. Increased competition for reduced carbon between seed production and root growth in fungicide-treated plants was supported by reduced seed set as the primary cause of yield reduction. This response coupled with the lack of reductions in seed nutrient concentrations is best explained by a reduction in available sugars during early pod filling causing a greater abortion rate of seeds in fungicide-treated plants (Marschner 1995).

Direct effects of the fungicides on the plant cannot account for the increase in root length caused by fungicides, since prior results with the same soil, plant, and fungicide combinations resulted in either no effect or even reductions in root growth in non-AM plants (Schreiner and Bethlenfalvay 1996).

A single application of fungicides altered the spore populations of our AM-fungal community and showed that biocides are yet another agricultural practice that influences AM community dynamics. Shifts in AM-fungal spore populations in response to fungicides may have been due to: (1) direct effects on the fungi (Schreiner and Bethlenfalvay 1996), (2) indirect effects mediated through other soil microbes that are to some extent influenced by AM fungi (Meyer and Linderman 1986; Secilia and Bagyaraj 1987), or (3) effects mediated by the host plant that itself is modulated by the endophytes (Ocampo 1993). It is likely that a combination of these mechanisms contributed to our findings. It is important to note that relative spore volume was a more sensitive measure of AM-fungal dynamics than relative abundance: due to the large variation in AM-fungal spore sizes, volumes (as opposed to numbers) give a more accurate estimate of biomass allocated to spores by different fungi and may therefore be a better indicator of relative changes in AM-fungal populations.

The shift in the spore population of one of our fungi (*G. etunicatum*) confirmed previous findings using the same fungal isolates as individual inocula (Schreiner and Bethlenfalvay 1996). What appeared to be preferential fungicide tolerance by *G. etunicatum* relative to *G. mosseae* and *G. rosea* (applied as individual inocula and evaluated by root colonization) was now seen to be preferential reproduction relative to the same fungi (applied as a combined inoculum and evaluated as sporulation). Another finding consistent between this and the prior study was the sensitivity of our *G. mosseae* isolate to PCNB. The potent effect of this fungicide on both root colonization and sporulation by this fungus when growing alone, was now, in the combined culture expressed as a similar reduction in sporulation by this fungus. However, other findings were not consistent between the two studies. *G. rosea*, which was reduced by benomyl and PCNB both in terms of root colonization and sporulation before was not reduced by these fungicides in the present study. Instead, it was reduced by captan. These results present complex and little-understood interactions between AM fungi and fungicides, and indicate that fungicide effects may be modulated by the AM-fungal community structure.

The three fungi in our AM-fungal community did not develop equally within the roots of pea plants as inferred by their final spore populations. Our infectivity assay did not provide a good measure of the development of these three fungi over the lifetime of the plants when co-inoculated. Our interest was to produce an equal number of infection units by each of the fungi at the time of fungicide application. Nevertheless, if our community could have been more equal in terms of the increase in spore numbers for each of the fungi, the effects of fungicides and that of captan towards *G. rosea*, in particular, may have been different.

The selective action of captan towards *G. rosea* in our fungal community was the most striking effect of fungi-

cides on AM-fungal species composition observed. Both the relative and absolute sporulation data for *G. rosea* showed selective inhibition by captan under our experimental conditions. This effect was confirmed by other related work (Schreiner and Bethlenfalvay 1995, unpublished). Other shifts seen in the population of the three fungi relative to each other as a result of fungicide treatments were not as significant as the captan effect on *G. rosea* and were not always supported by absolute sporulation data.

Species of *Gigaspora* may be particularly vulnerable nontarget organisms, if the indications of the intolerance of this genus to agricultural practices (chemical and mechanical) are substantiated by further findings (Douds et al. 1993; Johnson and Pfleger 1992; Wacker et al. 1990). Should the loss of *Gigaspora* from the AM-fungal community of an agrosystem be indeed accelerated by fungicide use, the consequences for sustainability could be greatly compromised (Bethlenfalvay and Schüepp 1994) in view of the contribution of this genus to soil stability (Miller and Jastrow 1992).

Since the individual members of an AM-fungal community appear to have distinct effects on both plant and soil, changes in the composition of this community as affected by fungicides will affect the large-scale dynamics of the agrosystem. Further efforts to understand the specific interactions of biocides with mycorrhizal fungi and the integration of these effects into the emerging models of below-ground ecosystems (Moore 1988) will provide the framework for future sustainable practices that can continue to benefit plant growth.

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