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ORIGINAL PAPER

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Mycorrhizae, biocides, and biocontrol 3. Effects of three different fungicides on developmental stages of three AM fungi

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Abstract The effects of biocide use on nontarget organisms, such as arbuscular mycorrhizal (AM) fungi, are of interest to agriculture, since inhibition of beneficial organisms may counteract benefits derived from pest and disease control. Benomyl, pentachloronitrobenzene (PCNB) and captan were tested for their effects on the germination and early hyphal growth of the AM fungi *Glomus etunicatum* (Becker & Gerd.), *Glomus mosseae* (Nicol. & Gerd.). Gerd. and Trappe and *Gigaspora rosea* (Nicol & Schenck) in a silty-clay loam soil placed in petri plates. Application of fungicides at 20 mg active ingredient (a.i.) kg^{-1} soil inhibited spore germination by all three AM-fungal isolates incubated on unsterilized soil for 2 weeks. However, fungicides applied at 10 mg a.i. kg^{-1} soil had variable effects on AM-fungal isolates. Fungicide effects on germination and hyphal growth of *G. etunicatum* were modified by soil pasteurization and CO_2 concentration in petri plates and also by placing spores below the soil surface followed by fungicide drenches. Effects of fungicides on mycorrhiza formation and sporulation of AM fungi, and the resulting host-plant response, were evaluated in the same soil in associated pea (*Pisum sativum* L.) plants. Fungicides applied at 20 mg a.i. kg^{-1} soil did not affect the root length colonized by *G. etunicatum*, but both benomyl and PCNB reduced sporulation by this fungus. Benomyl and PCNB reduced the root length colonized by *G. rosea* at 48 and 82 days after transplanting. PCNB also reduced *G. mosseae*-colonized root length at 48 and 82 days, but benomyl only affected root length colonized by *G. mosseae* at the earlier time point. Only PCNB reduced sporulation by *G. mosseae*, consistent with its effect on root length colonized by this fungus. captan reduced the root length colonized by *G. rosea* at 48 days, but not at 82 days, and reduced colonization by *G. mosseae* at 82 days, but not at 48 days. Captan did not affect sporulation by any of the fungi. *G. rosea* spore production was highly variable, but benomyl appeared to reduce sporulation by this fungus. Overall, *G. etunicatum* was the most tolerant to fungicides in association with pea plants in this soil, and *G. rosea* the most sensitive. Benomyl and PCNB were overall more toxic to these fungi than captan. Interactions of AM fungi and fungicides were highly variable and biological responses depended on fungus-fungicide combinations and on environmental conditions.

Key words Arbuscular mycorrhiza · Fungicides · *Glomus* · *Gigaspora* · Benomyl · Captan · Pea · *Pisum sativum* · Pentachloronitrobenzene

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Introduction

Concern over the nontarget effects of biocides employed in agriculture has shifted the focus of pest and disease control towards integrated management techniques that employ combinations of cultural practices, biological control and the use of chemicals to combat pests and pathogens. Integration of the beneficial effects of arbuscular mycorrhizal (AM) fungi into such techniques will require continued efforts by researchers to further identify and define the beneficial roles that AM fungi play in agricultural production and conservation (Bethlenfalvay and Linderman 1992), but also to define how the AM-fungal community responds to the particular components of pest management strategies, like the use of biocides.

Trappe et al. (1984) concluded that our understanding of how pesticides affect mycorrhiza development had not improved since 1976. Numerous reports since that time have not greatly advanced the state of our knowledge concerning biocide effects on mycorrhizae, due in part to the complexity of the soil ecosystem where these beneficial fungi reside. However, it is clear that fumigation of soils with broad-spectrum biocides like methyl bromide and methyl isothiocyanate (Vapam) can greatly reduce arbuscu-

lar mycorrhiza formation (An et al. 1993; Nesheim and Linn 1969; Trappe et al. 1984). AM fungi appear to be at least as sensitive as soil-borne pathogens to methyl bromide, so that reducing rates will not offer selective kill of pathogens over AM fungi (Menge 1982).

Among the fungicides, two chemical classes are generally known to be harmful to AM fungi: the benzimidazoles and aromatic hydrocarbons (Johnson and Pfleger 1992). Of the benzimidazole fungicides, benomyl has been the most studied and has been shown to be an effective inhibitor of both AM-fungal spore germination (Carr and Hinkley 1985; Dodd and Jeffries 1989) and root colonization (Bailey and Safir 1978; Perrin and Plenchette 1993). Hence it has been used to investigate the contribution of AM fungi to plant development in the field (Bentivenga and Hetrick 1991; Carey et al. 1992; West et al. 1993). However, others have not had success in reducing AM-fungal infections in the field with benomyl (McGonigle and Fitter 1988). Among the aromatic hydrocarbon fungicides, pentachloronitrobenzene (PCNB) has been studied the most in relation to AM fungi and consistently inhibits root infections (El-Giahmi et al. 1976; Gnekow and Marschner 1989; Menge et al. 1979; Nesheim and Linn 1969; Parvathi et al. 1985). Numerous other fungicides have been reported to reduce AM fungi under some conditions, but not under others. In addition, some studies have shown positive effects of certain fungicides on AM-fungal development including captan (Sreenivasa and Bagyaraj 1989) and the anti-oomycete fungicides Aliette and metalaxyl (Nemec 1980; Groth and Martinson 1983; Jabaji-Hare and Kendrick 1985; Afek et al. 1990). However, others have reported negative effects on AM fungi after treatment with these fungicides (Jabaji-Hare and Kendrick 1987; Kough et al. 1987; Sukarno et al. 1993).

Recommendations for the use of fungicides that consider the agricultural benefits of AM fungi discourage the use of chemicals like benomyl and PCNB that typically inhibit AM fungi, and promote the use of those which do not (Schüepp and Bodmer 1991). However, fungicides detrimental to AM fungi will continue to be used under circumstances where less damaging fungicides will not control the particular disease-causing organisms. Therefore, increasing our knowledge of how particular fungicides affect different isolates of AM fungi with respect to the different stages of AM-fungal development will be useful in defining the best management strategies for fungicide use that will have the least impact on these beneficial fungi.

The majority of the information regarding fungicide effects on AM fungi has come from work with only a few fungal isolates confined to the genus *Glomus*. Little work has examined how different *Glomus* isolates respond to the same chemical under the same conditions. Dodd and Jeffries (1989), Kough et al. (1987) and Spokes et al. (1981) found significant differences in the responses of different *Glomus* species to various fungicides. Differential tolerances to particular fungicides among AM fungi may be exploited in prescribing the use of particular chemicals in soils where the endophytes have been identified and tested for tolerance to such chemicals. Further work to define such differences among AM fungi will provide necessary information for the eventual inclusion of AM-fungal benefits into integrated disease-management strategies.

The purpose of this study was to compare the responses of three AM fungi at different stages of AM-fungal development to three commonly used fungicides differing in their mode of action to establish if fungicide effects on AM fungi varied with the conditions of application and with fungus-fungicide combination.

Materials and methods

Spore germination and hyphal growth

Experimental design, fungicides and statistics

Four experiments were conducted to assess the effects of the fungicides benomyl [methyl 1-(butylcarbomoyl)-2-benzimidazolecarbamate, 50% wettable powder (WP)], PCNB (pentachloronitrobenzene, 75% WP) and captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide, 50% WP] on the germination and early hyphal growth of AM fungi. The first two experiments compared fungicide effects on three AM-fungal isolates. The third and forth experiments assessed the influence of soil treatments and the effect of burying spores on AM-fungal response to fungicides for *Glomus etunicatum*. The impact of fungicide treatments on spore germination and hyphal length per germinated spore was determined by employing an analysis of variance procedure on Statgraphics version 5.0 (STSC 1991). In the first two experiments, fungicide effects on the different AM fungi were analyzed separately, while in the third experiment the effects of each of three soil treatments were analyzed separately, since the non-fungicide-treated controls were significantly affected by the soil treatments. Mean contrasts were made at the 95% confidence level using Fisher's protected LSD method.

Biological materials

Spores of *Glomus etunicatum* (Becker & Gerd.), INVAM # UT183-1; *Glomus mosseae* (Nicol. & Gerd.) Ger. & Trappe, Banque Européene des Glomales (BEG) #46 (Dodd et al. 1994); and *Gigaspora rosea* (Nicol. & Schenck), INVAM # FL105 were obtained from pot cultures in association with *Sorghum bicolor* L. Pot cultures were produced in a low-P sandy loam soil described elsewhere (Bethlenfalvay et al. 1994) and soil inocula were stored dry at 4 °C until use.

The spores were prepared for germination tests by wet-sieving and decanting, removing root pieces and organic debris from the resulting suspension by aspiration, blending on high speed in a two-speed Waring blender for 15 s (to remove hyphae) and further wet-sieving and decanting until most of the soil particles were removed. The final spore suspensions were diluted to a concentration in distilled water so that 15–20 spores (*G. etunicatum* and *G. mosseae*) could be transferred in 50–100 µl water onto nitrocellulose membrane (Gelman Scientific, Ann Arbor, MI) disks. *G. rosea* treatments received only eight to ten spores per disk. Ten replicate disks (13 mm diameter) for each fungus and fungicide-treatment combination were incubated in two petri plates (five disks in each of two petri plates).

Soil, fungicide application and incubation

The soil was a Chehallis silty-clay loam (pH 6.4) containing 2.1% organic matter, macronutrients (g kg^{-1}): NH_4-N, 13.9; NO_3-N, 0.4; P (Olsen), 0.028; K, 363; Ca, 11.7; Mg, 4.8; S, 0.4; and micronutrients (mg kg^{-1}): B, 0.34; Cu, 2.7; Fe, 62.0; Mn, 168.0; and Zn, 9.4. Soil was collected at the Oregon State University Vegetable Research Farm north of Oregon Route 34, air-dried, passed through a 2-mm sieve and used unsterilized except where noted.

To test fungicide effects on AM-fungal spore germination and hyphal growth, 25 g soil was placed into the bottom of petri plates and wetted with distilled water beginning at the center of the plate and working towards the edges until only the edges (~3 mm) of the soil in each plate were dry. Benomyl, PCNB and captan suspensions prepared in distilled water or distilled water alone (controls) were then added to the soil at the center of the plate and allowed to diffuse into the soil for 5 min. Distilled water was then added to the edges of each plate until the first sign of standing water over the soil surface was observed to ensure that the fungicides were not added to dry soil, since this increases their adsorption to soil. The concentrations of fungicides employed were based on the quantity of active ingredient (a.i.) per mass of air-dry soil (10 or 20 mg kg^{-1}, so that 25 g soil in each petri plate received either 250 or 500 µg a.i., respectively). A large nitrocellulose membrane (47 mm diameter, Gelman, Ann Arbor, MI) was then laid on top of the soil in each plate and the smaller nitrocellulose disks containing the spores of the test fungi were laid on top of the large membrane. Plates were sealed with parafilm and incubated in the dark at 25 °C for 2 or 4 weeks.

Germination and hyphal length estimates

Germination of AM-fungal spores was determined after removing the small disks from the plates, placing each disk into its own small dish (to obtain accurate counts of total spores per disk, since some of them fall off) and staining with a 0.01% (w:v) trypan blue in lacto-glycerin. The number of germinated spores on each replicate disk were counted at X20 to X40 using a stereo microscope. Hyphal lengths were determined by a grid-line intercept method (Newman 1966) adapted to hyphal dimensions at the same magnification. Care was taken to count only hyphae that were clearly AM-fungal, distinguished from occasional saprophytic fungal hyphae by their size and growth pattern.

Specific experiments

The effects of three fungicides on the germination and hyphal growth of three AM fungi were tested in the first experiment at a concentration of 20 mg a.i. kg^{-1} soil and were examined after 2 weeks incubation. The second experiment was identical to the first, except that fungicide concentration was reduced to 10 mg a.i. kg^{-1} soil and the fungi were examined at 2 and 4 weeks. The soil employed in both experiments 1 and 2 was unsterilized.

The influence of different soil treatments on the effects of the three fungicides was further investigated with *G. etunicatum* only. In a third experiment, unsterilized soil incubated at ambient CO_2 was compared to that of steam-pasteurized soil (75 °C, 45 min) incubated at ambient CO_2 and to that of steam-pasteurized soil incubated in an atmosphere containing 2% CO_2. These conditions were chosen because most AM-fungal experiments are conducted in pasteurized soil and because previous germination trials in this soil revealed that AM-fungal spore germination is reduced as a result of pasteurization and that this effect is reversed by incubation at 2% CO_2 (Schreiner and Bethlenfalvay, unpublished results). Fungicides were employed at a concentration of 10 mg a.i. kg^{-1} soil and *G. etunicatum* spores were examined after 2 weeks incubation.

The fourth experiment tested the effects of three fungicides on spores that were buried in pasteurized soil in 600-ml pots incubated under greenhouse conditions. This experiment was conducted at the same time as experiment 3 using the same *G. etunicatum* spore preparation. The purpose was to compare the petri plate method used here to a method which more closely approximated conditions used for later greenhouse studies employing potted AM plants. Spores of the test fungus were incubated between two large nitrocellulose membranes making a "sandwich" that could be easily retrieved from the pots. Six replicate membrane sandwiches (47 mm) containing 20–25 spores were buried at a 45° angle in soil in the middle of the pots and each of three fungicides were drenched through the premoistened pots at 10 and 20 mg a.i. kg^{-1} soil. Pots were placed in the greenhouse, watered 2 times per week and spores were examined after 2 weeks. Day (24–28 °C) and night (20–24 °C) temperatures were under automatic control.

Mycorrhiza formation and sporulation

Experimental design

The experiment had a 4×4 factorial design with AM-fungal treatments (three isolates and a non-AM control) and fungicide treatments (three chemicals and an untreated control) as factors. Five replications per treatment combination were employed for a total of 80 experiemental units (potted plants). Pots were arranged in a randomized complete-block design. Data were analyzed using analysis of variance (ANOVA) procedures on Statgraphics version 5.0 (STSC 1991). The effects of fungicide and AM-fungal treatments on AM-fungal variables were analyzed for each AM-fungal isolate separately. Mean contrasts were made at the 95% confidence level using Fisher's protected LSD method.

Biological materials

The host-plant used was *Pisum sativum* L. (cv. Little Marvel), grown previously from a single seed to minimize variability. AM fungi and soil were the same as above.

Growth conditions and fungicide applications

Five-day-old pea seedlings were transplanted from vermiculite into 1.5-l pots filled with steam-pasteurized soil. A suspension containing 750 wet-sieved spores of either *G. etunicatum, G. mosseae* or *G. rosea* was placed on the roots of seedlings in the planting hole. Noninoculated control pots received background microbes collected from all three individual spore suspensions delivered to the planting hole. The background microbes were prepared from an aliquot of each AM-fungal spore suspension equivalent to one-third of the quantity used in each treatment, blending this resulting suspension on high speed for 1 min in a Waring blender and retrieving the sievate after passing it through a 45-µm sieve 6 times. After inoculating and transplanting the seedlings, fungicides were applied as a soil drench (200 ml suspension) at the rate of 20 mg a.i. kg^{-1} soil.

Plants were grown for 82 days (November 18, 1992, to February 8, 1993) on greenhouse benches. Temperatures were maintained between 20 and 28 °C during the photoperiod, and 16 and 24 °C during the night. Supplemental lighting was provided by five overhead 1000-W metal-halide lamps with phosphor-coated bulbs which supplied 450 µE $m^{-2}s^{-1}$ photosynthetically active radiation at soil level for a 16-h photoperiod. 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

Leaf areas were measured 35 days after transplanting (length and width of each individual leaflet and stipule). Pots were cored 48 and 82 days after tranplanting with a 2.0-cm borer to obtain root samples. Fresh soil was added to the holes produced by coring on day 48 and the location was noted to avoid coring again from the same spot. Total root length and AM-colonized root length were determined by the grid-line intersect method (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% (v:v) HCl (10 min, 90 °C), stained with trypan blue (0.05%, w:v, in lacto-glycerin, 20 min, 90 °C) and destained overnight in lacto-glycerin. The number of spores produced by each AM fungus was determined by washing the soil from each of the cores taken at 82 days onto a stack of sieves (500, 250, 125 and 75 µm) and counting the spores retrieved from each sieve or the number in appropriately diluted samples.

Shoot dry weights were obtained from oven-dried (70 °C, 3 days) plant tissue stripped of seed pods and cut off at the soil surface at

harvest. The total number of pods produced on each plant was counted and the mean fresh seed weight was determined for each plant using the seeds remaining in the pods collected at 82 days.

Results

Spore germination and hyphal growth

All three fungicides tested at a concentration of 20 mg a.i. kg^{-1} soil completely or almost completely (PCNB) inhibited spore germination by all three AM-fungal isolates incubated in unsterilized soil (Table 1). When tested at half this rate (10 mg a.i. kg^{-1} soil), benomyl completely inhibited spore germination by all three fungi for up to 4 weeks, PCNB reduced germination of *Glomus* spores, but not those of *G. rosea*, while captan had no effects (Fig. 1A, C). PCNB reduced hyphal growth by all three fungi at 2 weeks, but *G. mosseae* hyphal growth was no longer reduced by PCNB at 4 weeks (Fig. 1B, D). Captan did not reduce the hyphal growth of any of the three fungi in this experiment. Hyphal growth by *G. etunicatum* was

Table 1 Effects of three fungicides (applied at 20 mg a.i. kg^{-1} air-dry soil) on the germination of arbuscular mycorrhizal fungi after 2 weeks in unsterilized soil

AM species	Percentage germination (SE)[a]			
	Control	Benomyl	PCNB	Captan
Glomus etunicatum	39.5 (3.5)a	0b	0.5 (0.5)b	0b
Glomus mosseae	28.3 (2.3)a	0b	2.4 (1.0)b	0b
Gigaspora rosea	12.1 (3.5)a	0b	0b	0b

[a] Means of ten replicates; letters designate significant groups, horizontally, at the 95% confidence level

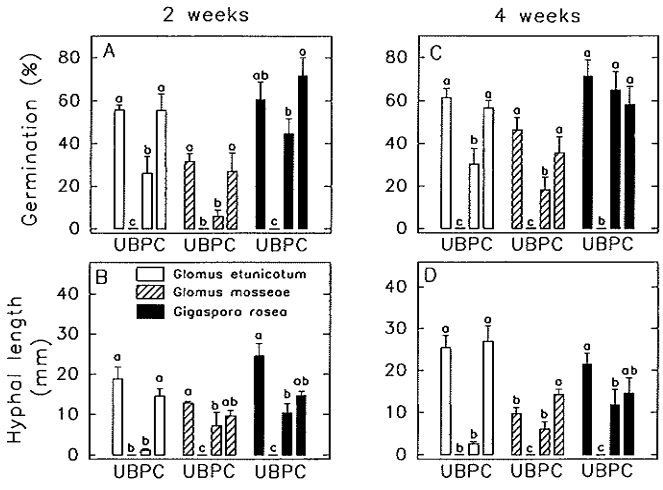


Fig. 1A–D Effects of benomyl, PCNB and captan applied at the rate of 10 mg a.i. kg^{-1} soil on **A, C** germination and **B, D** early hyphal growth of spores of *Glomus etunicatum, Glomus mosseae* and *Gigaspora rosea* after **A, B** 2 and **C, D** 4 weeks in unsterilized soil in petri plates. *U* untreated control, *B* benomyl, *P* PCNB, *C* captan

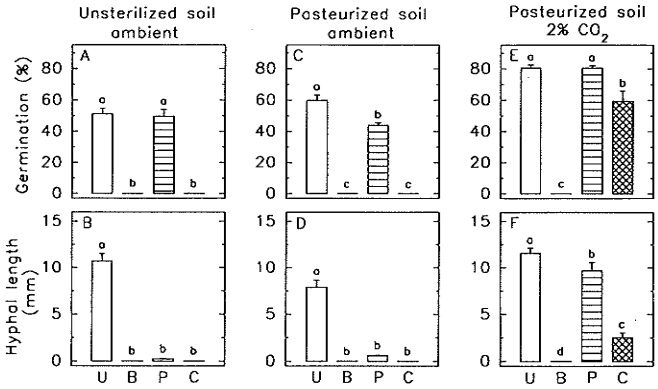


Fig. 2A–F Effects of benomyl, PCNB and captan applied at the rate of 10 mg a.i. kg^{-1} soil on **A, C, E** germination and **B, D, F** early hyphal growth of spores of *Glomus etunicatum* after 2 weeks in **A, B** unsterilized soil at ambient atmosphere, **C, D** pasteurized soil at ambient atmosphere and **E, F** pasteurized soil at 2% CO_2 in petri plates. *U* untreated control, *B* benomyl, *P* PCNB, *C* captan

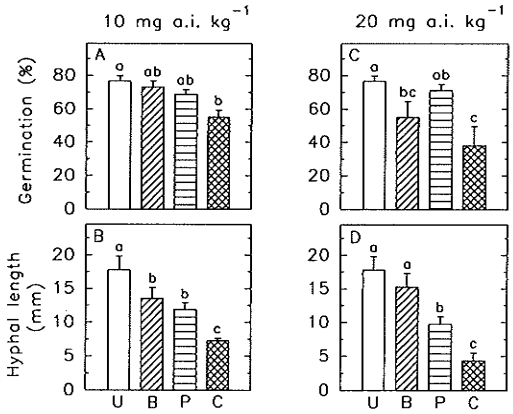


Fig. 3A–D Effects of benomyl, PCNB and captan on **A, C** germination and **B, D** early hyphal growth of spores of *Glomus etunicatum* after 2 weeks in pasteurized soil buried in pots. Fungicides were applied as soil drenches at the rates of **A, B** 10 mg a.i. kg^{-1} soil or **C, D** 20 mg a.i. kg^{-1} soil. *U* untreated control, *B* benomyl, *P* PCNB, *C* captan

more sensitive to PCNB, since it was more depressed than that of the other fungi compared to the untreated controls.

Different soil treatments modified the effects of fungicides on spore germination and hyphal growth by *G. etunicatum* in the third experiment, except for benomyl, which again completely inhibited germination in all soil treatments (Fig. 2). PCNB reduced germination in pasteurized soil, but not in unsterilized soil or in pasteurized soil incubated at 2% CO_2 (Fig. 2A, C, E). In addition, captan completely inhibited germination in both unsterilized and pasteurized soil, but this effect was also partially alleviated by high CO_2 in pasteurized soil. Hyphal growth from germinated *G. etunicatum* spores was reduced by PCNB in all treatments, but only slightly so in the elevated CO_2 treatment (Fig. 2B, D, F).

Benomyl applied as a soil drench to pots containing buried spores of *G. etunicatum* had no effect on spore germination at 10 mg a.i. kg^{-1} soil, and only a slight reduc-

tion in germination at twice the dose rate (Fig. 3A, C), while the opposite trend occurred with benomyl effects on hyphal growth of buried spores (Fig. 3B, D). PCNB did not reduce germination at either dose rate, but reduced hyphal growth of buried spores at both doses with greater effects at the higher rate (Fig. 3). Captan was the most inhibitory of the three fungicides tested on spores of *G. etunicatum* buried in soil, inhibiting both germination and hyphal growth more than the other two fungicides and with greater effects at the higher rate of application (Fig. 3).

Mycorrhizae formation and sporulation

Treatment effects on plant growth

Fungicides significantly affected the growth of pea plants (Table 2). PCNB and captan reduced early leaf development, an effect that persisted throughout the experiment so that the final shoot dry weights in these treatments were also lower. However, PCNB was more inhibitory to overall plant growth, as the root lengths at 48 days and 82 days and the number of pods per plant were also depressed. The effects of captan were more complex, increasing root length by the final harvest at the expense of shoot weight, but not of the number of pods produced. The outer edges of leaves of plants treated with captan showed chlorosis associated with iron deficiency between 2 and 4 weeks after transplanting. This symptom disappeared by 5 weeks after transplanting. Benomyl did not significantly influence the growth of pea plants (Table 2).

The AM fungi did not enhance shoot development or pod production compared to the non-AM plants. In fact, *G. rosea* had a negative effect on vegetative growth ($P=0.056$), reducing final shoot dry weight compared to non-AM plants, but without affecting pod production (Table 2). Colonization by *G. mosseae* and *G. rosea* significantly increased root length measured at 48 days. However, by 82 days the root lengths of the AM plants were not different from those of the non-AM plants, although differences in total root length occurred in the different AM-fungal treatments (Table 2). We ascribe the lack of response to AM fungi to the short growth cycle of peas and the relatively high phosphorus concentration of our soil.

Fungicide effects on root colonization and sporulation

Fungicides significantly affected the colonization of pea roots by *G. mosseae* and *G. rosea* at 48 and 82 days after transplanting (Fig. 4). Benomyl and PCNB reduced the root length colonized by *G. mosseae* measured at 48 days, while captan did not. By the final harvest, *G. Mosseae*-colonized root length in the benomyl-treated soil had recovered to the untreated control level, while colonized root length in PCNB-treated soil remained very low throughout the experiment. Captan did not reduce the root length colonized by *G. mosseae* at 48 days, but did so by the final harvest. PCNB reduced the root length colonized by *G. mosseae* to a much greater extent than either benomyl at 48 days or captan at 82 days. Both benomyl and PCNB reduced root length colonized by *G. rosea* to about the

Table 2 Effects of arbuscular mycorrhizal fungal inoculation and fungicide treatments on pea plant variables. Fungicides were applied at the rate of 20 mg a.i. kg^{-1}

Fungicide treatment	AM-fungal treatment	Leaf area 35 days (cm^2)	Root length 48 days (m)	Root length 82 days (m)	Shoot weight (g)	Pods per plant
Untreated	Non-AM control	497	140	163	6.52	19.0
	Glomus etunicatum	571	140	182	6.55	17.6
	Glomus mosseae	567	163	159	6.09	18.4
	Gigaspora rosea	522	180	165	6.02	16.2
	Mean	539	156	167	6.30	17.8
Benomyl	Non-AM control	574	125	145	6.34	18.2
	Glomus etunicatum	584	147	179	6.13	18.4
	Glomus mosseae	524	172	127	5.91	16.8
	Gigaspora rosea	546	175	202	5.33	16.6
	Mean	557	155	163	5.93	17.5
PCNB	Non-AM control	372	114	138	4.75	13.4
	Glomus etunicatum	370	137	177	4.73	16.0
	Glomus mosseae	284	148	111	4.18	12.6
	Gigaspora rosea	331	111	160	4.35	12.0
	Mean	339	128	147	4.50	13.5
Captan	Non-AM control	452	144	218	5.85	21.6
	Glomus etunicatum	429	168	213	5.46	18.8
	Glomus mosseae	463	190	161	5.82	20.8
	Gigaspora rosea	391	186	218	5.00	18.2
	Mean	434	172	203	5.53	19.9
ANOVA						
Factor	Significance levels					
Fungicide		<0.0001	0.0005	0.0085	<0.0001	<0.0001
AM fungi		0.1201	0.0019	0.0125	0.0559	0.1963
Interaction		0.0684	0.5201	0.8719	0.9498	0.8580

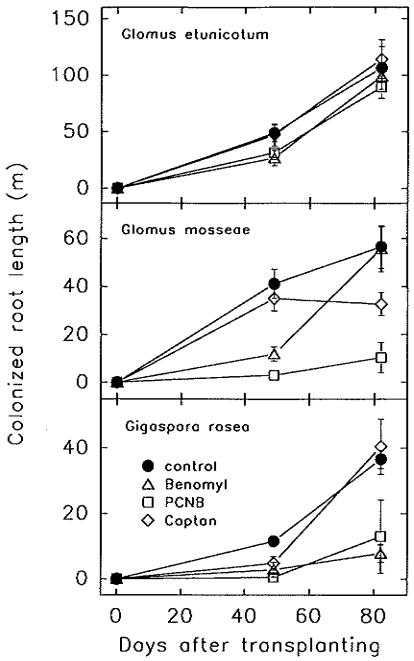


Fig. 4 Effects of fungicide treatments on the root length colonized by three arbuscular mycorrhizal fungi. *Data points* represent the means of five replicates±standard errors. ANOVA significance levels for fungicide treatment effects were: *Glomus etunicatum*, 48 days (P=0.145), 82 days (P=0.636); *Glomus mosseae*, 48 days (P<0.001), 82 days (P=0.002); *Gigaspora rosea*, 48 days (P=0.002), 82 days (P=0.011)

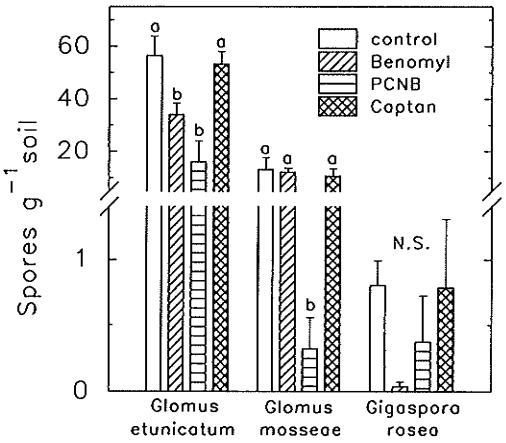


Fig. 5 Effects of fungicide treatments on the number of spores produced per gram of soil by three arbuscular mycorrhizal fungi. *Bars* represent the means of five replicates+standard errors. ANOVA significance levels for fungicide treatment effects were: *Glomus etunicatum* (P=0.001), *Glomus mosseae* (P=0.019), *Gigaspora rosea* (P=0.327)

same extent through the final harvest. Captan reduced colonized root length by *G. rosea* 48 days after transplanting, but it had recovered to the control level by the final harvest. Root colonization by *G. etunicatum* was not significantly affected by any of the fungicides at either time point (Fig. 4). The root length colonized by *G. mosseae* and *G. rosea* in the untreated controls by the final harvest was roughly half that of *G. etunicatum*-colonized root length, although *G. rosea* initially colonized pea roots at a much slower rate than the other two fungi. This was reflected by the large difference between *G. rosea*-colonized root length at 48 days (11.5 m) and the corresponding values for *G. mosseae* (41 m) and *G. etunicatum* (48.5 m).

Even though fungicide treatments had little effect on the colonization of pea roots by *G. etunicatum*, the number of spores produced by this fungus by the final harvest was significantly depressed by both benomyl and PCNB (Fig. 5). In addition, *G. etunicatum* sporulated within root tissues to a greater degree with PCNB than in the other treatments. We observed this phenomenon in the stained root samples taken at the final harvest, quantified the percentage root length containing spores within the cortex on pooled samples from each fungicide treatment, and found that 33% of the total root length contained spores for the PCNB treatment, while only 1–2% of the total root length contained internal spores in the other treatments. The root length containing hyphae, arbuscules and/or vesicles of *G. etunicatum* was between 51% and 62% of the total in all treatments.

Only PCNB reduced the sporulation of *G. mosseae* significantly (Fig. 5), mirroring the effects on colonization for this fungus. The variability of sporulation by *G. rosea* was large, leading to an insignificant main effect (P=0.326). However, benomyl reduced sporulation by *G. rosea* consistent with the persistent reduction in root length colonized by this fungus in the benomyl treatment (Fig. 4).

Discussion

The responses of our AM-fungal isolates confirm findings by others on AM-fungal sensitivity to fungicides. Spore germination experiments showed small but significant differences in the germination and/or hyphal growth responses of three AM fungi to different fungicides. Mycorrhiza formation by *G. etunicatum* was much less sensitive to fungicides than by *G. mosseae* or *G. rosea*. AM-fungal development is also differentially affected by fungicides at different stages, such that sporulation by *G. etunicatum* was reduced by benomyl and PCNB even though these fungicides had not affect on root length colonized by this fungus. However, we also found large variation in the germination response of a single fungal isolate to fungicides from one experiment to another employing the same soil and methods. These findings indicate that conflicting reports regarding the effects of specific fungicides on AM fungi (Trappe et al. 1984) can be due to different experimental conditions alone. The conditions that vary most are: the nature of the soil and its preparation, sterilization and storage, the methods of culturing the AM-fungal inocula and its placement in the experimental medium, the composition, activity and proliferation of the associated soil (micro)biota and even CO_2 concentration. Our findings suggest that the variation in AM fungus-fungicide interactions is due to variation in these conditions, as much as to the AM fungus-fungicide relationship itself.

All three fungicides completely inhibited spore germination of all three AM fungi in soil in petri plates at a rate of 20 mg a.i. kg^{-1} soil, but such strong effects did not occur for either germination or root colonization in potted soil at the same dose rate. Such differences were most striking with benomyl, which consistently inhibited spore germination in petri plates, but had not effect on spores buried in soil in pots. Our germination results with soil in petri plates confirm observations of the potent activity of benomyl towards AM fungi in agar (Carr and Hinkley 1985), but this effect cannot even be extended to soil in small pots. An explanation for this phenomenon is that benomyl (or carbendazim, its active breakdown product) was adsorbed to soil minerals and organic matter in the upper layer of soil (Aharonson and Kafkafi 1975; Helwig 1977) in both the germination experiment in pots and in the pea plant experiment and hence was less effective than in the small petri dishes. Carbendazim does not penetrate soil more than a few centimeters (Rhodes and Long 1974). The active ingredient could also have been leached from the soil in the pot experiment by watering, but this is unlikely, considering how strongly benomyl is adsorbed to soil (see Rhodes and Long 1974). Dodd and Jeffries (1989) found that benomyl inhibited root infection by AM fungi in sand (deeper penetration), but not in soil (adsorption). It is unlikely that enhanced degradation of benomyl or carbendazim by opportunist soil microbes (Aharonson and Katan 1993; Yarden et al. 1985) could account for our results, since benomyl was equally effective in unsterilized and in pasteurized soil in the petri plate experiments.

The effects of PCNB on spore germination and early hyphal growth were more consistent than those of the other two fungicides: Hyphal elongation was reduced in all cases by PCNB, even though spore germination was not. These findings are consistent with those of Esuruoso et al. (1968) and Priest and Wood (1961), who showed that PCNB did not interfere with germination of sensitive fungi, but rather suppressed hyphal elongation. The consistent inhibition of hyphal elongation by PCNB in petri plates was correlated with a persistent inhibition of root colonization by two of three fungi and a strong depression of sporulation by two of three fungi in association with pea plants. PCNB may therefore be a better fungicide than benomyl to depress AM fungi experimentally.

Captan produced the most variable results in our study with no effects in some petri plate experiments and complete inhibition of germination in others. Captan effects on root colonization were also the most variable. The persistence of captan in soils varies greatly, and is generally shorter than that of benomyl or PCNB (Sinha et al. 1988). The lack of captan effects on sporulation of AM fungi at 82 days, and the marginal effects on root colonization at 48 and 82 days as opposed to the inhibition of spore germination measured at 2 weeks, are most likely due to captan's shorter persistence in soils. Variation in the microbial composition or activity and in the physicochemical properties of our test soil as affected by storage or due to microsite heterogeneity may have affected the biological activity of captan towards AM fungi in different germination experiments. Such variation may also have affected the degree to which captan suppressed soil microbial respiration in different experiments.

The ability of CO_2 to counteract inhibition of spore germination and early hyphal growth by PCNB and captan is important, since CO_2 occurs at high concentrations in soils under natural conditions (Paul and Clark 1989). CO_2 is required to achieve maximal hyphal growth from AM-fungal spores in defined media (Bécard and Piché 1989) and CO_2 has been implicated in the stimulation of AM fungi by other soil microbes (McAllister et al. 1994). Fungicide effects on spore germination and hyphal elongation may therefore be partially due to a suppression of CO_2 production by the soil biota. Any condition that affects CO_2 concentration is of consequence in experimentation with AM fungi if the results are to have predictive value to field conditions. Both PCNB and captan have been reported to reduce soil respiration rates at moderate rates of application (Lockwood 1970; Vyas 1988).

From the diversity of responses here we discern at least three fungicide-related factors that affect the development of AM fungi and of mycorrhizae: (1) tolerance of the biocide by the fungus, (2) susceptibility at different stages of fungal development, and (3) secondary biocide effects mediated through changes in the activities of other non-target components of the soil microflora (Beare et al. 1993; Vyas 1988). Further factors that may affect symbiotic expression are the direct response of the host plant to the fungitoxicant (Table 2), and secondary effects of changes in host-plant physiology that may further affect endophyte development (Ocampo 1993). Phytotoxicity and dose-rate responses by the host plant itself to fungicides may also contribute to differences in root development and hence affect the development of the endophyte (Gnekow and Marschner 1989).

Our results confirm observations regarding the toxicity of the three fungicides tested towards AM fungi. Overall, benomyl and PCNB were found to be more effective inhibitiors of AM fungi than was captan. What remains unclear is whether (or how much) these different fungicides manifest their respective effects on AM fungi through direct inhibition or through mediation by the host-plant and by other soil inhabitants.

While it is unclear why AM-fungal isolates display differential sensitivities to particular fungicides (Spokes et al. 1981; Dodd and Jeffries 1989; Figs. 1, 4, 5), consistent responses by different AM fungi within a given population would give rise to shifts in the AM-fungal community due to the selective advantage impared by fungicides on the most tolerant isolates. Had our three isolates been native to this soil, *G. etunicatum* could in time have displaced the other two fungi under fungicide stress. The consequences of such shifts in the AM mycoflora are incalculable, since benefits of AM fungi to the plant-soil system are species dependent (Sieverding 1991) and range from increases in plant growth and reproduction (Smith 1980; Bryla and Koide 1990) to enhanced soil stability (Miller and Jastrow 1992). Fungicide-induced shifts in the AM-fungal community of agrosystems is therefore a factor in the sustainability of agriculture.

Further study employing the same fungi, soil and chemicals in the laboratory, greenhouse and field settings should reveal the sources of variation causing the effects observed here, and help define the best methods to assess biocide effects on AM fungi. At the present time, fungicide effects on AM fungi must be interpreted with caution as such effects only apply to the specific conditions in which observations were made.

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