Mycorrhiza (1997) 7:33–37

ORIGINAL PAPER

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Direct application of carbendazim and propiconazole at field rates to the external mycelium of three arbuscular mycorrhizal fungi species: effect on ^{32}P transport and succinate dehydrogenase activity

Accepted: 12 December 1996

Abstract The influence of the systemic fungicides propiconazole (Tilt 250E) and carbendazim (Bavistin) at field application rates on the functioning of three arbuscular mycorrhizal fungi was studied. Short-term fungal ^{32}P transport and succinate dehydrogenase (SDH) activity in external hyphae of *Glomus intraradices* Schenck and Smith, *G. claroideum* Schenck and Smith and *G. invermaium* Hall in symbiosis with pea (*Pisum sativum* L.) were measured. In the experimental system used, the hyphae grew into two root-free hyphal compartments (HCs). The fungicides were applied to each HC 24 days after sowing and ^{32}P was added to one HC of each pot. Four days later, the fungicide effect on fungal P transport was measured as the difference in ^{32}P content of treated and untreated plants. SDH activity in fungal hyphae was determined in the HCs given no ^{32}P. Carbendazim severely inhibited ^{32}P transport and SDH activity in external hyphae at an application rate of 0.5 μg g^{-1} soil. The ergosterol inhibitor propiconazole affected none of these parameters. The fungicides had similar effects on all three fungal species, although P transport efficiency and SDH activity differed markedly between the fungi.

Key words Arbuscular mycorrhiza · Enzyme activity · External hyphae · Fungicides · Phosphorus transport

Introduction

Arbuscular mycorrhizal (AM) fungi enhance plant growth and crop yield mainly by increasing the phosphorus supply to plants (Harley and Smith 1983). The rate and extent of fungal P transport depend on the plant and fungal species involved (Ravnskov and Jakobsen 1995) and on environmental factors, including the use of pesticides in agricultural practice (Menge 1982).

The harmful effect of many fungicides on AM fungi is well documented, but results are often inconsistent (Trappe et al. 1984). This inconsistency may be due to considerable variation in fungicide dose and AM fungi sensitivity (Spokes et al. 1981; Trappe et al. 1984; Dodd and Jeffries 1989). Most often fungicides have been applied as soil drenches to mycorrhizal root systems, and with this approach it is impossible to distinguish between a direct influence on the fungus and a phytotoxic reaction caused by a high fungicide dose.

Benomyl, a benzimidazole compound with carbendazim as the active degradation component, inhibits root colonization by AM fungi (Boatman et al. 1978), enzyme activity in intraradical hyphae (Kough et al. 1987; Thingstrup and Rosendahl 1994), growth and activity of external hyphae (Sukarno et al. 1993), P uptake by mycorrhizal root systems (Hale and Sanders 1982), and hyphal ^{32}P transport (Boatman et al. 1978; Larsen et al. 1996). However, the doses used in these investigations were 2–100 times higher than the recommended field rate, and the practical implications of the results are uncertain.

Propiconazole, one of the most frequently used systemic fungicides, is an ergosterol inhibitor and should have no major impact on AM fungi, which contain only small amounts of ergosterol (Schmitz et al. 1991; Frey et al. 1994). However, detrimental effects of propiconazole have been reported on root colonization (von Alten et al. 1993), plant growth and spore production (Nemec 1985), and ^{32}P uptake by mycorrhizal plants (Hetrick et al. 1988). The experimental doses in these studies were also several times higher than the recommended field rate.

The objective of the present work was to determine whether mycorrhiza functions are influenced by recom-

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mended field rates of carbendazim and propiconazole applied directly to the mycelium. Mycorrhiza functioning was measured as short-term ^{32}P uptake and SDH activity in external hyphae of three AM fungi in symbiosis with pea (*Pisum sativum* L. cv. Finale).

Materials and methods

Experimental design

The fungal species were *Glomus intraradices* Schenck and Smith (isolate 28 A), *G. claroideum* Schenck and Smith (isolate BEG-14), and *G. invermaium* Hall (isolate BEG-44). Pea plants were inoculated with each fungus or grown without an AM fungal inoculum. Carbendazim, propiconazole, or water was applied to root-free soil compartments in each of the four plant-fungus combinations. Each of the twelve treatments had three replicates.

Growth conditions

Pots were made of PVC tubes (Jakobsen 1994) with a vertical root compartment (RC) and two lateral hyphal compartments (HCs) (Fig. 1). Each HC was separated from the RC by a 36-μm nylon mesh which allowed the passage of hyphae but not roots.

The soil was a 1:1 (w:w) mixture of sandy loam (Rørrendegaard, Tåstrup, Denmark) and quartz sand, partially sterilised by irradiation (10 kGy, 10 MeV electron beam). The soil had a pH of 6.1 in 0.01 M $CaCl_2$ and contained 8.5 mg P kg^{-1} (0.5 M $NaHCO_3$, Olsen et al. 1954). Nutrients were uniformly mixed into the soil at the following concentrations (mg kg^{-1} soil): NH_4NO_3 86.0, KH_2PO_4 44.0, K_2SO_4 71.0, $CaCl_2 \cdot 2H_2O$ 71.0, $CuSO_4 \cdot 5H_2O$ 2.0, $ZnSO_4 \cdot 7H_2O$ 5.0, $MnSO_4 \cdot H_2O$ 10.0, $CoSO_4 \cdot 7H_2O$ 0.35, $MgSO_4 \cdot 7H_2O$ 45.0, $Na_2MoO_4 \cdot 2H_2O$ 0.18. The phosphorus supply corresponded to 10 mg P kg^{-1} soil dry wt.

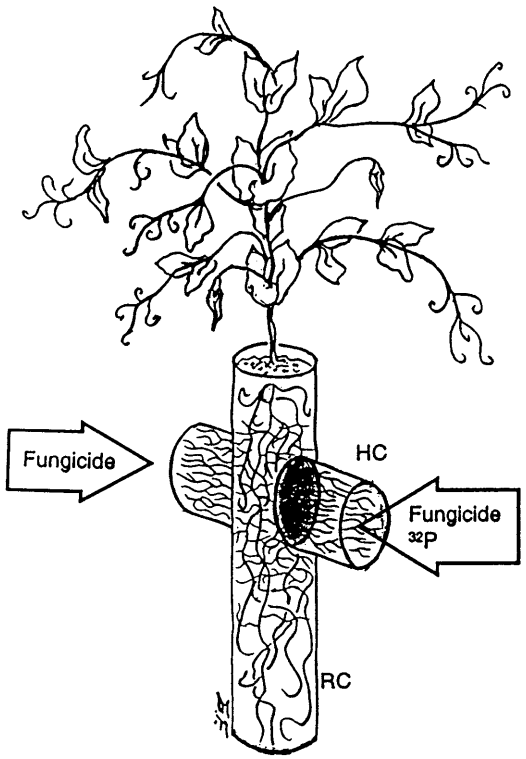

Fig. 1 Pot made of PVC tubes and divided into a three-compartment system by 36-μm nylon meshes (*RC* root compartment, *HC* hyphal compartment)

The root compartment contained 720 g soil, which included a 300-g layer of fungal inoculum-soil mixture placed between the HCs of the mycorrhizal treatments. The fungal inocula were propagated in soil-based pot cultures with *Trifolium subterraneum* L., air-dried, and stored at 5 °C until use. Inocula of *G. intraradices* (25 g), *G. claroideum* (50 g) or *G. invermaium* (75 g) were applied to obtain similar root colonization in all plant-fungus combinations. The quantities required were determined from an initial bioassay of inoculum potentials (data not shown). Each HC was filled with 60 g of soil corresponding to a 25-mm column. The HC soil was held in place by plastic vials which fitted inside the PVC tubes. Each pot received 40 ml of an inoculum filtrate to provide a similar background microbial population in all pots (not including the AM fungi). The inoculum filtrate were prepared by filtration through a double layer of 36-μm meshes of a suspension of 25 g of *G. intraradices*, 50 g of *G. claroideum*, and 75 g of *G. invermaium* inoculum in 1500 ml water. The soil was watered to 60% of water holding capacity (WHC), and the pots were incubated at room temperature for 1 week before one pregerminated pea seed was sown per pot. After seedling emergence, the pots were placed in a completely randomized pattern in a growth chamber with a 16-h photoperiod (500 μE m^{-2} s^{-1} at 21/16 °C day/night). The plants were watered daily to maintain 60% WHC and repositioned in rotation. Nitrogen was added (80 mg per pot in total) as an aqueous solution of $Ca(NO_3)_2$ on days 11, 14, 17, and 22 after sowing.

Fungicide treatment and isotope labelling

The fungicides tested were Bavistin (carbendazim, 50%) and Tilt 250E (propiconazole, 25%) at rates recommended for field use: 0.5 kg Bavistin ha^{-1} (0.5 μg a.i. per g soil dry wt.) and 0.5 l Tilt ha^{-1} (0.25 μg a.i. per g soil dry wt.). Only the HC soil (60 g) was considered when calculating fungicide concentration. The plastic vials holding the soil in each HC were temporarily removed 24 days after sowing and 3 ml of a suspension of either fungicide or water was applied to each HC (two per pot). After 30 min, 2 ml of a carrier-free aqueous solution of $H_3{}^{32}PO_4$ (74 kBq ml^{-1}) was added to the soil surface of one HC in each pot.

Harvest and analysis

The plants were harvested 4 days after the fungicide treatment and isotope labelling. Roots were washed and weighed, and subsamples were taken for determination of root colonization. Total and mycorrhizal root length were measured by a line intersect method (Newman 1966) after clearing and staining (modified after Kormanick and McGraw 1982). Shoots and roots were dried at 80 °C for 24 h, weighed, ground, and digested in nitric acid:perchloric acid (4:1). The activity of ^{32}P in the digest was measured by Cerenkov counting using a Packard 1900 TR liquid scintillation analyser. Counts were corrected for background values, counting efficiency, and isotopic decay. The total phosphorus content in the digest was analysed by the molybdate-blue method (Murphy and Riley 1962) in a Technicon Autoanalyzer II.

The HCs were removed at harvest and the RC distal 1-cm section of each soil column was used for hyphal measurements. A previous study showed that the ^{32}P was adsorbed in this outermost 1-cm section, which was considered to be the site of hyphal isotope uptake (Ravnskov and Jakobsen 1995). Soil from the ^{32}P-labelled HCs was dried at 40 °C for 24 h and used for determination of total hyphal length. The mycelium density (m g^{-1} soil dry wt.) was measured by an aqueous extraction and membrane filtration technique (Jakobsen et al. 1992). Soil from the unlabelled HCs was used for measurements of the SDH-active hyphal length. Duplicate 2-g samples of fresh soil were transferred to vital-staining solutions and incubated overnight at room tempera-

Table 1 Dry weight, root length, and phosphorus content of pea (*Pisum sativum* L.) plants uninoculated or grown in symbiosis with an arbuscular mycorrhizal (AM) fungus. Different letters in the same column indicate significant differences between means, n = 9, $P < 0.05$

AM fungus	Dry weight (g)		Root length (m)		P content (mg/plant)
	Shoot	Root	Total	Colonized	
Uninoculated	1.39c	0.89c	76.4b	0.0c	2.40c
Glomus intraradices	2.27a	1.46a	92.4a	72.7a	7.18a
Glomus claroideum	2.35a	1.48a	91.5a	68.7a	7.39a
Glomus invermaium	2.01b	1.33b	90.3a	30.8b	4.33b

ture (Kough et al. 1987). The mycelium from the incubated soil samples was collected by the membrane filtration technique as above but without the staining step. Hyphal lengths were calculated using a gridline intersect method (Tennant 1975) after counting 25 fields at ×160 magnification. The background hyphal length in the HC soil of nonmycorrhizal plants was measured and subtracted from the lengths recorded in the mycorrhizal treatments.

Statistics

The data were analysed by two-way analysis of variance with the GLM procedure (SAS Institute Ltd.) for treatment effects, interactions, and mean comparisons. Log transformation was used for the ^{32}P data in order to obtain variance homogeneity.

Results

Mycorrhiza formation and plant growth

The proportions of root length colonized were 78%, 75% and 34% in pea plants inoculated with *G. intraradices*, *G. claroideum* and *G. invermaium*, respectively. Uninoculated plants remained nonmycorrhizal. All three fungi increased the growth parameters measured (Table 1), but the increases in shoot and root dry weight and total phosphorus content were less with *G. invermaium* than with the other two AM fungi. The short-term exposure to the fungicides had no effect on either growth parameter.

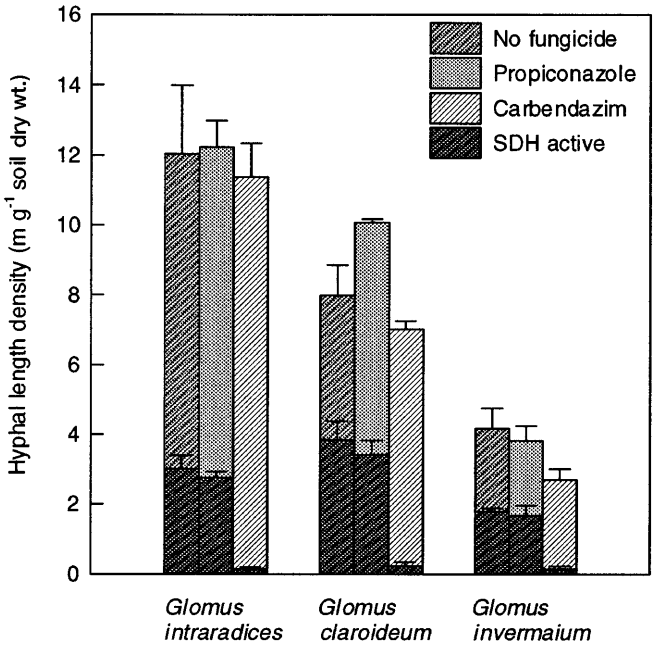

Fig. 2 Total and succinate dehydrogenase (SDH)-active mycelium density of three *Glomus* species in symbiosis with pea (*Pisum sativum* L.) 4 days after fungicide treatment; *bars* SEM

Hyphal length and SDH activity

The three fungal species differed in their mycelium production. The mycelium density was highest in HCs of the *G. intraradices* association and lowest in HCs of the *G. invermaium* association (Fig. 2). The 4-day exposure to the fungicides did not significantly affect mycelium density except for a negative effect of carbendazim on *G. invermaium* ($p < 0.01$). The hyphal length in nonmycorrhizal treatments was 1.9 m g^{-1} soil.

There was no correlation between SDH activity and mycelium density. The proportion of total hyphal length showing SDH activity was 48% of *G. claroideum* and 45% of *G. invermaium*, whereas only 25% of the *G. intraradices* hyphae were active. Carbendazim severely reduced the SDH-active hyphal length of all fungal species. No significant effect of propiconazole on SDH activity was detected (Fig. 2).

Fungal ^{32}P transport

The hyphal transport of ^{32}P from soil to plants amounted to 23% of the total supplied in treatments with *G. intraradices* or *G. claroideum* in the absence of fungicide. The transport was 40-fold less in the *G. invermaium* treatment (Table 2). *G. claroideum* appeared to be the most efficient fungus, with 33% of ^{32}P in shoots of the *G. claroideum* symbiosis, compared with 25% in shoots of the *G. intraradices* and *G. invermaium* symbioses. The length specific ^{32}P transport was higher with *G. claroideum* than with *G. intraradices*, and the efficiency of ^{32}P transport per unit hyphal length with *G. invermaium* was very low (Fig. 3).

Carbendazim application resulted in a nearly complete inhibition of ^{32}P transport to the plants (Table 2). In the carbendazim treatments plants contained only 3% of the ^{32}P present in untreated plants grown in association with *G. intraradices* or *G. claroideum*. The corresponding figure was 8% in the *G. invermaium* symbiosis, but here the ^{32}P content of untreated plants

Table 2 Transport of ^{32}P to pea (*Pisum sativum* L.) plants (Bq/plant) by three AM fungi during a 4-day period after treatments with field rates of propiconazole or carbendazim. The background ^{32}P content in nonmycorrhizal plants was 30 Bq per plant. Different letters indicate significant differences between means, n = 3, $P < 0.01$

AM fungus	Fungicide treatment		
	None	Propiconazole	Carbendazim
Glomus intraradices	32158a	29611a	875b
Glomus claroideum	34873a	41136a	1107b
Glomus invermaium	815b	423b	39c

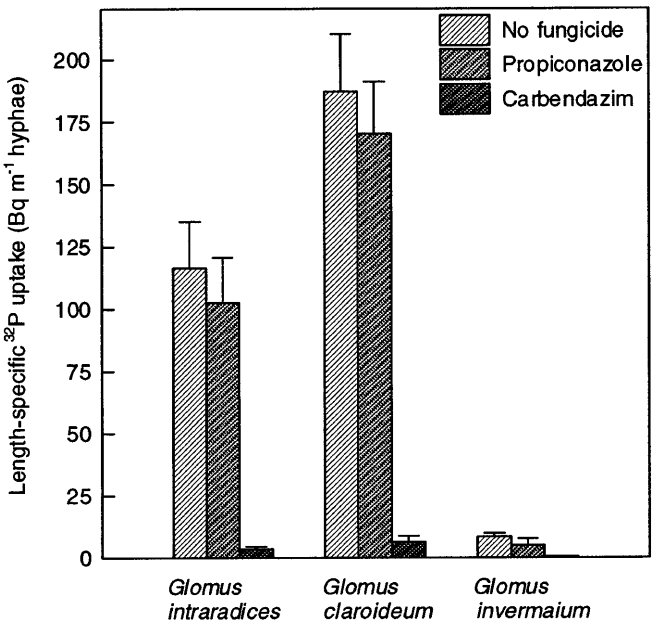

Fig. 3 Length-specific ^{32}P uptake into pea plants by three *Glomus* species during a 4-day period after fungicide treatment; *bar* SEM

was much lower. Application of propiconazole did not reduce ^{32}P transport by any fungus.

Discussion

In most previous studies of fungicide effects on AM, it is difficult to distinguish between direct effects on the AM fungi and indirect effects caused by changes in host plant performance. This problem was solved in the present work by applying the fungicide directly to the fungal hyphae. In this way, we demonstrated that a calculated field rate of carbendazim could impair the functioning of AM fungi, whereas propiconazole had no effect.

Carbendazim, which prevents microtubule formation and inhibits mitosis in fungal cells (Richmond and Phillips 1975; Davidse 1986), severely reduced ^{32}P transport and SDH activity of the AM fungi examined. This is consistent with previous studies (Boatman et al. 1978; Kough et al. 1987; Sukarno et al. 1993; Larsen et al. 1996), but the effects reported here were caused by only 0.5 μg carbendazim g^{-1} soil (0.25 kg ha^{-1}). This is the lowest effective dose yet reported in AM studies, and was derived from the recommended rate in Scandinavia. The short time between fungicide application and ^{32}P labelling, as well as the short period until harvest and analysis, showed that carbendazim acted immediately on AM fungal metabolism. Carbendazim is highly persistent in the soil with a half-life of 3 years (KemI 1992). Repeated field applications of this fungicide may produce a high residual concentration in the soil, leading to even more severe detrimental effects on AM. Negative effects of carbendazim have been shown to persist throughout a 5-month experimental period after application (Spokes et al. 1981). Thus the use of carbendazim in agricultural soils should be avoided to maintain optimal conditions for mycorrhizas.

The ergosterol inhibitor propiconazole was reported to influence AM fungi (Nemec 1985; Hetrick et al. 1988; von Alten et al. 1993), but in the present study propiconazole at standard field rates had no direct impact on mycorrhizal function. This is in accordance with the detection of only minute amounts of ergosterol in AM fungi, and the subsequent assumption that ergosterols play no important role in these fungi (Schmitz et al. 1991; Frey et al. 1994). Indirect effects of propiconazole on AM fungi were demonstrated by von Alten et al. (1993), who reported decreased colonization rates after propiconazole application to the plant shoots. The authors considered the effect to be a result of fungicide-induced changes in host plant metabolism. Phytotoxic effects of propiconazole on nonmycorrhizal cotton has been observed at rates of 0.56 kg a.i. ha^{-1} (Whitson and Hine 1986), which is about four times higher than the calculated field rate used in our study. This phytotoxicity may explain the inconsistencies between our results and those from previous propiconazole studies, where the fungicide was applied either as a soil drench or foliar spray at rates 4- to 75-fold higher than in our study. Obviously, there is a need for dose-response studies of effects of propiconazole and other fungicides on AM fungi. Such studies should be designed to distinguish between effects on the plant and effects on the fungus in order to fully understand the influence of a fungicide on the functioning of AM.

The fungicides had similar effects on all three fungal species despite the large differences in mycorrhizal P transport efficiency between the species. These differences were not related to rate of mycorrhiza formation, the amount of external hyphae in the soil, or the proportion of the mycelium showing SDH activity. However, hyphal P transport showed some correlation with the total length of SDH-active hyphae, which can be regarded as the amount of metabolically active mycelium in the soil. These results are consistent with the simultaneous reduction of the activity of external hyphae and total P uptake after benomyl treatment found by Sukarno et al. (1993, 1996). The reason for the low

^{32}P transport by *G. invermaium* is not clear, but may be the result of functional incompatibility with the pea plant. Effective P transport by *G. invermaium* was previously observed with only one out of three host plant species tested (Ravnskov and Jakobsen 1995).

Hyphal ^{32}P transport and SDH activity appear to be sensitive parameters for measuring effects of environmental stress on the symbiotic effectiveness of AM fungi. Both methods are suitable for the detection of short- and long-term effects of pesticides. The application of the pesticide to root-free soil ensures that a measured response reflects a direct effect on the fungal symbiont and not an indirect effect on the plant. This may provide detailed information on the targets of pesticides, and shed light on the physiological effects on AM fungi of present-day agricultural practices.

Acknowledgements We wish to thank Anette Olsen for assistance with the phosphorus analysis and Birgitta Vegerfors for help with the statistics.

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