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Limitations to using benomyl in evaluating mycorrhizal functioning

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Abstract Arbuscular mycorrhizal (AM) grasses compete for nutrients with ectomycorrhizal (EM) pine in the southeastern United States. Our objective was to determine if benomyl could be used to selectively inhibit the function of AM and thereby reduce grass competition in the field. The effects of Benlate (active ingredient: benomyl) in the greenhouse and field were evaluated. No effect was observed on pine inoculated with *Pisolithus tinctorius* in the greenhouse. Colonized root length of benomyl-treated *Zea mays* L. plants inoculated with *Glomus* sp. in the greenhouse remained static over time and the response was not dose dependent at concentrations of 0, 20, 60 and 150 kg benomyl ha^{-1} equivalent. In contrast, colonization of nontreated plants increased over time. In the field, a minimal reduction of grass colonization was observed following four applications of benomyl ranging from 5 to 20 kg ha^{-1}. We conclude that benomyl can successfully inhibit development of AM fungi under controlled conditions in the greenhouse with no inhibitory effects on the EM fungus *P. tinctorius*; however, in the field several factors may interfere with the effect of benomyl on AM fungi. These factors include: (a) the presence of ground cover which obstructs penetration of the fungicide to the soil, (b) timing of application in relation to mycorrhizal development, and (c) the application method of benomyl, a soil drench being preferable to a foliar spray.

Key words Arbuscular mycorrhiza · Ectomycorrhiza · Benomyl · Colonization · External hyphae · Field study · Glomales · *Zea mays* · *Pisolithus tinctorius*

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Introduction

A limitation to mycorrhizal field research is the difficulty of obtaining an appropriate nonmycorrhizal control, since plants in nature are normally colonized. Soil fumigation has been used to control mycorrhizal fungi; however, the broad biocidal effects limit the usefulness of this technique. Fungicides are more specific and alter fewer biological soil processes. Paul et al. (1989) summarized the ideal properties of a fungicide used to chemically exclude an organism from an experiment. The fungicide properties should include: (a) moderate persistence to reduce mechanical disturbance from the application process, (b) an appropriate activity spectrum that targets selected organisms only, and (c) no direct physiological effects on plants.

The systemic fungicide benomyl, a benzimidazole, has been used frequently to reduce AM activity in experimental treatments (Jalali and Domsch 1975; Kough et al. 1987; Fitter and Nichols 1988; Hartnett et al. 1994; Nelson and Allen 1993; Newsham et al. 1995; West et al. 1993). Benomyl's lack of direct effects on plants and somewhat selective effects against AM fungi (Zygomycetes) currently make it a better choice compared to other fungicides (Paul et al. 1989; Sukarno et al. 1993). Nonetheless, the amount of mycorrhizal control achieved with benomyl has varied. Reduction of colonization or biomass of mycorrhizal plants has been observed in several cases (Trappe et al. 1984; Evans and Miller 1988; Fitter and Nichols 1988; Nelson and Allen 1993; Merryweather and Fitter 1996), but these results are not always achieved (Koide et al. 1988; Fitter 1986; Trappe et al. 1984). Much of this variability is likely attributable to experimental conditions such as soil type, method and timing of fungicide application and potentially more complex interactions occurring within the soil microbial community. For example, benomyl can inhibit nematodes (Elamayem et al. 1978) and different fungi that do not form mycorrhizas (Edgington et al. 1971), thereby indirectly altering mycorrhizal effects.

Several studies have addressed the effects of arbuscular mycorrhizas on plant interactions (for reviews see Miller

and Allen 1992; Newman et al. 1992; Pedersen and Sylvia 1997). Some have utilized benomyl (Hetrick et al. 1989; Carey et al. 1992; Hartnett et al. 1993; Merryweather and Fitter 1996; Nelson and Allen 1993; Newsham et al. 1995) or other fungicides (Gange et al. 1993) to create control treatments. Only one study has addressed the influence of EM fungi on plant competition (Perry et al. 1989). To our knowledge, none have addressed the role of mycorrhizas in the interactions between AM and EM plants. Benomyl's putative selective effect against AM fungi and neutral effects on EM fungi (Trappe et al. 1984) could be valuable in sorting out the individual benefits of these two types of mycorrhizal symbioses to different host plants competing for the same nutrients.

As part of a larger plant competition study between AM and EM plants, the usefulness of benomyl as a tool to selectively control mycorrhizas was tested. The main objectives were to (a) compare the efficacy of benomyl in controlling mycorrhizas in the greenhouse to that in the field, (b) differentiate effects of benomyl on external hyphae from those on the internal mycorrhizal structure, and (c) determine if the intensity and longevity of the fungicide's effect was dose dependent.

Materials and methods

Field study

The site was located 21 km northwest of Gainesville, Florida, and was part of a larger plant competition study involving slash pine (*Pinus elliotti* Engelm. var. *elliottii*) and various weeds. Slash pine had been planted in April 1990 in beds approximately 26 cm in height and about 2 m in width with rows spaced 213 cm apart. The soil was a Pomona fine sand (sandy, siliceous, hyperthermic Ultic Haplaquod). The surface 10 cm of soil had 7 μg P g^{-1} (extracted in 2 m*M* $CaCl_2$) and a soil solution pH of 3.9. Approximately 3.3% weight was lost upon ignition. The dominant weeds were *Panicum chamaelonche, P. aciculare, Andropogon* spp., *Paspalum* spp., *Rubus* sp. and *Serenoa repens.* In December 1991, we observed less than 1 AM spore g^{-1} field soil. Subsequent trap cultures on *P. chamaelonche* originating from the field and grown in field soil yielded two AM isolates: *Gigaspora rosea* (INVAM FL224) and *Scutellospora heterogama* (INVAM FL225).

Two areas (each 18.4×11 m) containing slash pine and weeds were selected randomly for this study. The control plot received no fungicide sprays. Benlate 50 DF (E.I. du Pont de Nemours & Co., Wilmington, DE) was applied to the second area with a CO_2-pressurized backpack sprayer by covering the area once and then making a second application perpendicular to the first. The first spray (2 April 1991) was applied at the rate of 5 kg benomyl ha^{-1} using the equivalent of approximately 150 ml water m^{-2}. Subsequent sprays (30 May, 11 July and 19 September 1991) were applied at a rate of 20 kg benomyl ha^{-1}.

Panicum chamaelonche was chosen as the indicator plant of AM fungal activity because it was a dominant weed species at the site. Samples were taken on 2 April, 16 April, 30 May, 10 June, 2 July, 22 July, 13 August and 10 October 1991. At each sampling, three plants were selected randomly and removed from each plot. The roots were washed and cut into lengths of 1–2 cm. To determine fungicide effects on colonization and metabolic activity, 1- to 2-g subsamples of roots were stained at room temperature for 8 h in a solution containing 0.2 *M* TRIS HCl (pH 7.4), 1 mg ml^{-1} iodonitrotetrazolium violet (INT) and 3 mg ml^{-1} NADH (Sylvia 1988). This was followed by clearing the roots in a boiling, saturated solution of chloral hydrate for 10 min and subsequent counterstaining overnight in 0.5% aniline blue in lactoglycerol. The chloral hydrate treatment proved unnecessary and was eliminated in samplings collected after May. The roots were destained in lactoglycerol and a minimum of 25 l-cm-long root segments per plant were placed on a slide. The percentage of root segments with arbuscules and the percentage of arbuscules that were active (i.e., positive stain with INT) were estimated using bright-field microscopy at ×600 magnification. The effect of benomyl on mycorrhizal development was evaluated using the relationship of time to either arbuscule abundance or activity. The slopes of linear regression of benomyl-treated versus nontreated plants were compared using the General Linear Model procedure (SAS Instiute, 1989).

Greenhouse studies

Both of the following experiments had completely randomized factorial designs (two mycorrhizal treatments × four benomyl levels) with seven replications each. To maintain a uniform daylength of approximately 12 h, extra light (800 μmol m^{-2} s^{-1} from 17:00 to 20:00 hours) was provided. Plants in all experiments were fertilized semiweekly with 3.2 μ*M* NH_4NO_3, 7.5 μ*M* $Ca(NO_3)_2$, 7.7 μ*M* KCl, 1.0 μ*M* $MgSO_4$, 20 n*M* NaFeEDTA, 5.0 n*M* $CuSO_4 \cdot 4H_2O$, 240 n*M* H_3BO_4, 20 n*M* $MnCl_2 \cdot 4H_2O$, 5 n*M* $Na_2MoO_4 \cdot 2H_2O$ and 20 n*M* $ZnSO_4 \cdot 7H_2O$. The nutrient solution for corn or pine contained, respectively, 3.2 n*M* H_3PO_4 or 0.32 n*M* H_3PO_4. All data were analyzed by analysis of variance using the General Linear Model procedure (SAS Institute, 1989). Both experiments were repeated once under similar environmental conditions.

Benomyl effects on pine

Slash pine seeds were disinfested for 2 min in a 5.25% sodium hypochloride solution with 0.2 ml Liqui-Nox surfactant (Alconox, New York, NY) and then rinsed thoroughly with tap water. Plants were raised from seed for 12 days in a growth chamber [29/23 °C (day/night), with a 15-h light period and irradiance of 1000 μmol m^{-1} s^{-1}] in a vermiculite/sand (1:1) mix. They were then transplanted into sand in 50-ml pots (5 cm^2 surface area) grown in the greenhouse for 6 weeks where they received water only. *Pisolithus tinctorius* (Pers.) Coker & Couch (isolate S106) was grown with no shaking in a modified Melin-Norkrans liquid medium (Marx 1969) containing glucose instead of sucrose. Just prior to use, fungal mats were washed with tap water, added to a food processor with water and chopped (Rousseau and Reid 1990). Eight-week-old pines were inoculated with the fungus by immersing the washed roots in the suspension and then grown in the greenhouse in 500 ml sand in Deepots (McConkey, Sumner, WA). Six weeks after inoculation, 10 ml of a suspension of Benlate 50 WP in deionized water was applied once at 0, 20, 60 or 150 kg benomyl ha^{-1} equivalent (based on a pot surface area of 28 cm^2). Plants were grown from January to March 1993 under a mean photosynthetic photon flux density of 535 μmol m^{-2} s^{-1} and 17/30 °C (min/max) temperature regime.

Groups of plants were harvested before, and then 2 and 4 weeks after benomyl application. Prior to harvesting the plants, a soil core (15.5 mm in diameter by 15 cm deep) was removed from each pot. Hyphal length and activity were evaluated by a slightly modified procedure of Sylvia (1988). A thoroughly mixed, 10-g, wet-mass subsample of soil was added to 500 ml water and chopped in a Waring blender at the high setting for 20 s. The resulting suspension was allowed to settle for 20 s before a 25-ml portion was removed and filtered through a 0.45-μm-pore size membrane (GN-6 Metricel; Gelman, Ann Arbor, MI). The hyphae on the membrane were stained for 6 h with INT solution, destained with tap water, counterstained for 30 min with 0.1% trypan blue in lactoglycerol and destained again with tap water. Using a gridline-intercept method, total and active hyphal lengths were determined microscopically at ×400 from 20 randomyl selected fields on the filter.

Pine needles were removed from seedlings and dried overnight at 65 °C, and P content was determined colorimetrically (Murphy and Riley 1962). Egosterol (a sterol found in fungal, but not plant, membranes) content in the root was used to provide a relative estimate of total fungal biomass present (Salmanowicz et al. 1989; Martin et al. 1990). Fresh

roots were washed, ground in liquid nitrogen and thoroughly mixed. A 0.1- to 0.3-g subsample was extracted overnight at room temperature with 5 ml 100% ethanol. This sample was filtered through a 0.45-µm syringe filter and then assayed for free ergosterol by high-performance liquid chromatography (Waters 715 Ultra WISP, Gilson 115 UV detector). Separation was achieved in a C-18 column (Supelcosil LC-18; Supelco, Bellefonte, PA) at 40 °C using a methanol-water mobile phase (92:8) flowing at 2 ml min^{-1} with detection at 282 nm.

Benomyl effects on corn

The effect of benomyl on colonization of corn (*Zea mays* L. cv. Silver Queen) by the AM fungus *Glomus* sp. (INVAM FL329, formerly FL906) was studied. Corn was used as a substitute for *P. chamaelonche* due to lack of native, nonmycorrhizal plant material. Germinated seed was planted in sand in Deepots with 5 g soil inoculum (83 spores g^{-1}) placed 2–3 cm below the seedling. Control plants received a 5-ml suspension of inoculum filtrate obtained by mixing 60 g soil from a pot culture with 1.2 l water and then filtering this through a 10-µm membrane filter. Benlate 50 WP was applied, 19 days after planting, to the soil surface at rates of 0, 20, 60 and 150 kg benomyl ha^{-1} equivalent. Plants were grown from March to May 1993 under a mean photosynthetic photon flux density of 608 µmol m^{-2} s^{-1} and 18/35 °C (min/max) temperature regime.

The plants were sampled before, and then 2, 4 and 6 weeks after benomyl application. The harvest procedures were the same as for pine, with the exception of estimation of root colonization. Washed root segments (1–2 cm) were cleared with 10% KOH for 30 min at 80 °C, rinsed several times with tap water, acidified for 30 min in 5% HCl and stained overnight in 0.05% aniline blue in lactoglycerol. Colonization was determined using a gridline-intersect method (Giovannetti and Mosse 1980). Although fungi other than AM existed in this particular system, the differentiation of saprophytic from characteristic AM fungal hyphae was possible based on gross morphological differences. Arbuscular mycorrhizal fungi generally had a somewhat larger hyphal diameter (4 µm compared to <2 µm), stained darker with aniline blue, were not dematiacious, lacked septation or clamp connections and demonstrated a less angular growth pattern compared to other fungi present. Prior to statistical analysis, percentage colonization data were transformed using the arcsine, square root procedure.

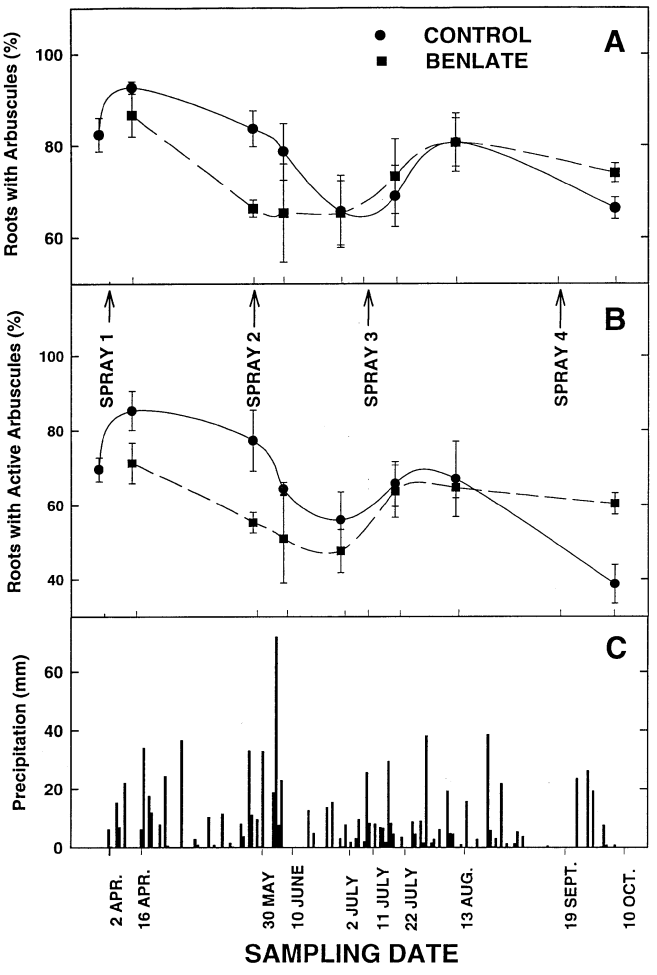

Fig. 1 A–C Assessment of arbuscular activity in *Panicum chamaelonche* roots from the field site in 1991: **A** Percentage of root length with arbuscules in benomyl-treated and nontreated plots, **B** percentage root length with metabolically active arbuscules in benomyl-treated and nontreated plots and **C** precipitation. *Each symbol* represents the mean of three replicates ±SE

Results

Field study

Initial AM colonization of *P. chamaelonche* in the field was high, indicating that root growth and mycorrhizal development commenced earlier than the first fungicide application on 2 April (Fig. 1A). Over the entire growing season, both the proportion of roots with arbuscules (slope = –0.012) and the activity of arbuscules (slope = –0.010) for benomyl-treated plants did not change significantly, whereas samples from the control plots had significantly ($P \leq 0.01$) negative slopes with time for both arbuscule abundance (–0.104) and activity (–0.164). Early in the season ground cover was sparse and the spray was applied directly to the soil. This was paralleled by a short-term decrease in the proportion of roots with arbuscules (Fig. 1A) as well as metabolic activity (Fig. 1B). As ground cover increased through the growing seasaon, more of the spray was intercepted by foliage, leaving less to penetrate into the soil. Concomitant with this, the differences between treated and nontreated plots disappeared. In late summer, as the plants started to senesce, roots of benomyl-treated plants had more arbuscules and arbuscule activity than nontreated plants. In an adjacent study area receiving the same benomyl treatments, there was no effect of benomyl on shoot-P status of samples collected in June and August (unpublished data). Furthermore, there was no apparent relationship between precipitation, application of benomyl and mycorrhizal response (Fig. 1C).

Benomyl effects on pine grown in the greenhouse

There were no significant effects of benomyl on inoculated or noninoculated pine biomass (Fig. 2A). Phosphorus content of the needles increased over time for all treatments from a mean of 320 mg to 450 mg per plant, but this was not related to the benomyl treatments (data not shown). Similarly, benomyl had no effect on the length or viability of external hyphae of the ectomycorrhizal fungus (Fig. 3A). There was a difference in EM colonization, as measured by ergosterol concentration, at 4 weeks between

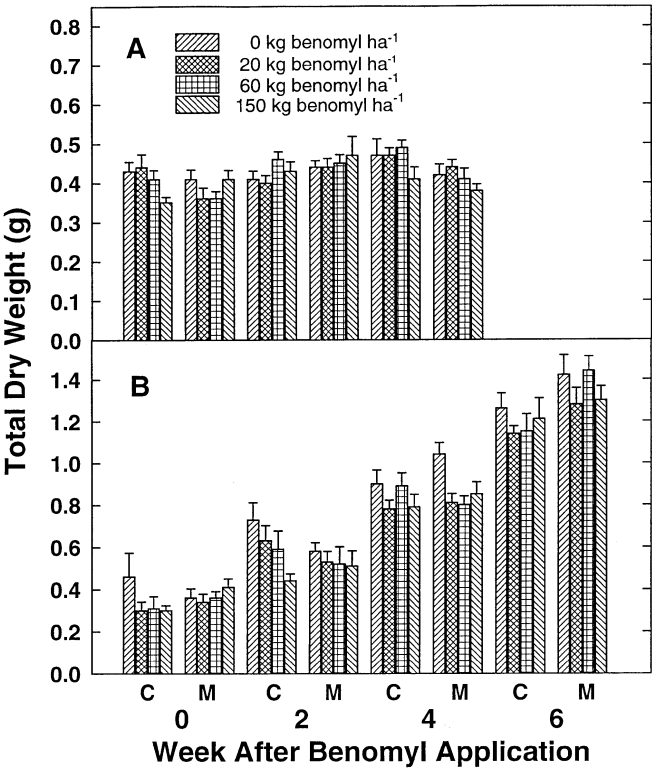

Fig. 2 A, B Total dry weight of nonmycorrhizal (*C*) and mycorrhizal (*M*) plants: **A** *Pinus elliotti,* inoculated with *Pisolithus tinctorius* in the M treatment, and **B** *Zea mays,* inoculated with *Glomus* sp. in the M treatment, in response to 0, 20, 60 or 150 kg benomyl ha^{-1} in the greenhouse. *Each symbol* represents the mean of seven replicates ±SE

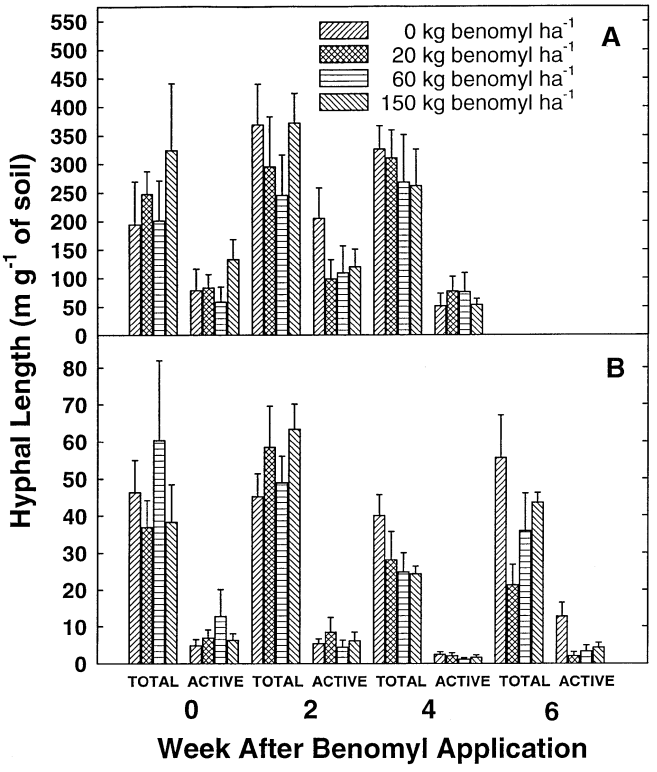

Fig. 3 A, B Soil hyphal length (total) and activity (active), as measured by iodonitrotetrazolium violet, of mycorrhizal **A** *Pinus elliottii* inoculated with *Pisolithus tinctorius* and **B** *Zea mays* plants inoculated with *Glomus* sp. in response to 0, 20, 60 or 150 kg benomyl ha^{-1} in the greenhouse. *Each symbol* represents the mean of seven replicates ±SE

the 60 and 150 kg benomyl ha^{-1} treatments (Fig. 4A); however, this was not repeatable.

Benomyl effects on corn grown in the greenhouse

Benomyl, at all concentrations, arrested further root colonization by the AM fungus, whereas colonization in the treatment receiving no benomyl continued to increase over the 6-week period (Fig. 4B). There was no dose-related response in colonization. Noninoculated plants remained noncolonized. Total biomass of mycorrhizal and nonmycorrhizal plants was approximately 12% lower in benomyl-treated corn (Fig. 2B); however, this was unrelated to the fungicide concentration applied. The length of external hyphae of AM fungi or their viability was not affected significantly or consistently by the different rates of benomyl (Fig. 3B). The P concentration of corn leaves decreased steadily throughout the experiment from 3.64 to 0.62 mg P g^{-1} without any evidence of a benomyl effect (data not shown).

Discussion

Benomyl arrested AM development in the greenhouse experiment. This fungicide is known to inhibit nuclear division by binding to tubulin (Davidse 1986) and has been reported previously to suppress AM development (Trappe et al. 1984). We did not observe a dose-dependent response by the AM in the greenhouse – all the concentrations tested were above the threshold required to obtain maximum inhibition of mycorrhizal development. The sand substrate used in the experiment would minimize sorption phenomena that normally occur in field soils. Not only was the fungicide readily available, but it was also well above the manufacturer's recommended application rate. These factors presumably caused the decrease in plant biomass unrelated to plant mycorrhizal status. In agreement with most previous literature (Trappe et al. 1984), we observed no effect of benomyl on mycorrhizal pine, although sometimes an increase in growth has been reported (De la Bastide and Kendrick 1990; Pawuk and Barnett 1981).

Arbuscules were quantified in the field since they are the distinguishing character of AM and, more importantly, they are the site of active nutrient exchange. The initial decrease in arbuscule activity we observed in the field following benomyl application was also documented in a greenhouse study (Sukarno et al. 1993). However, the response to 5 kg benomyl ha^{-1} was minor compared to total colonization so the benomyl application rate was increased for the remaining applications. The lack of AM response to benomyl application in the field during most of the

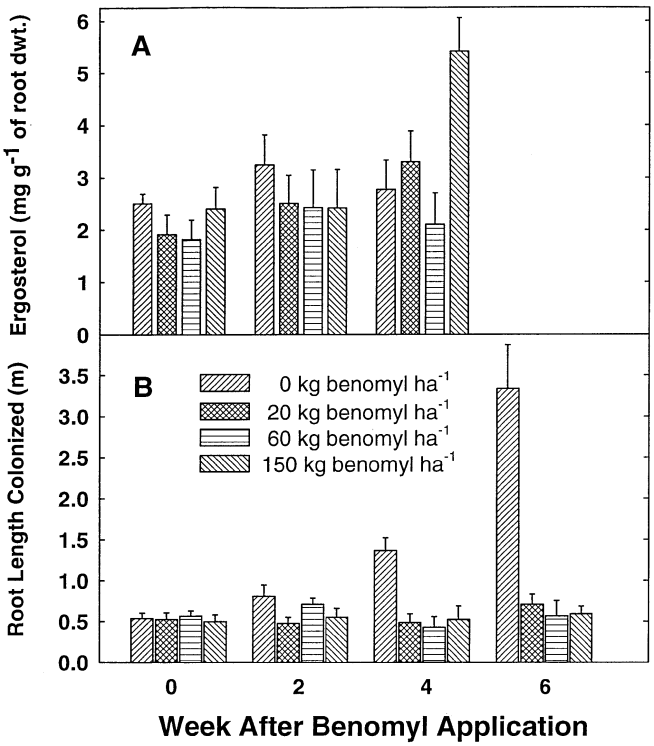

Fig. 4 A, B Mycorrhizal colonization of **A** *Pinus elliottii* inoculated with *Pisolithus tinctorius* and **B** *Zea mays* plants inoculated with *Glomus* sp. and grown in the greenhouse with 0, 20, 60 or 150 kg benomyl ha^{-1}. *Each symbol* represents the mean of seven replicates ±SE

growing season may be attributed to the increased interception of the fungicide by ground cover. Although benomyl can enter through leaves, systemic translocation is not as efficient as direct application to the target site (Hassall 1990; Larsen et al. 1996), in this case, the roots.

Larsen et al. (1996) determined that benomyl applied directly to the leaves of cucumber had little effect on mycorrhizal efficiency; yet when benomyl was applied to the soil, complete inhibition of P uptake by hyphae occurred within 5 days. Although no fungicide effect on fungal alkaline phosphatase activity was found inside the root, the rapid response, nonetheless, suggests some direct influence on uptake or transport mechanisms. Kough et al. (1987) and Thingstrup and Rosendahl (1994) have observed suppressive effects of benomyl on internal fungal enzyme activity in mycorrhizal plants. Although benomyl had no significant effect on external hyphal length or viability in this study, an inhibitory response has been found in another system (Sukarno et al. 1993). At the last sampling in our field study, plants were beginning to senesce and the increase in arbuscule number and activity of plants treated with benomyl may be due to a reduction in the impact of nonmycorrhizal fungi on plant growth and subsequent mycorrhizal functioning.

Benomyl can be an effective tool for inhibiting AM activity in the field; however, researchers need to be aware of the limitations of this approach. The timing of root colonization and initial nutrient contribution to mycorrhizal-dependent seedlings can be critical to their survival (Hetrick et al. 1989; Plenchette and Perrin 1992; Hartnett et al. 1994). Fungicide applications in the field should be timed according to the plant's optimal benefit from mycorrhizas, which, correspondingly, would provide the full impact of the fungicide treatment on mycorrhizal functioning (Gange et al. 1993; Newsham et al. 1995). Furthermore, the frequency of application is determined by fungicide persistence in the soil, which is variable (Ware 1992) due to degradation and sorption in different soil environments. In sandy soils where sorption is low persistence may be longer, assuming leaching does not occur, so that an application every 5–6 weeks may suffice. Soils with higher levels of organic matter or clay require more frequent applications or higher concentrations.

The method of application is also critical. Although benomyl is considered a systemic fungicide, translocation from leaves to the active site in the roots appears to be minimal. A soil drench is the optimal method of application (Fitter and Nichols 1988; Hassall 1990; Perrin and Plenchette 1993). Appropriate preparations should be made to accommodate the increasing ground cover as treatments are applied later in the growing season. Tall ground cover may be compensated for by applying a large volume of water to wash the active ingredient to the soil. Benomyl concentrations applied experimentally have ranged from 0.5 to 300 kg benomyl ha^{-1} (Trappe et al. 1984). Treatments of as little as 3 kg ha^{-1} biweekly in a short turf grass setting have been adequate to reduce AM colonization by 80% (Rhodes and Larsen 1981). It is only after careful consideration of benomyl concentration, frequency of application, and volume of water used in balance with environmental conditions that one can achieve the desired reduction of mycorrhizal activity.

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