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Nitrogen limitation of production in a Californian annual grassland: The contribution of arbuscular mycorrhizae

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Abstract. Nutrient availability limits plant production across a wide range of terrestrial ecosystems. In this study of a Californian annual grassland community, the influence of arbuscular mycorrhizal (AM) associations on plant nutrient acquisition was investigated using factorial combinations of nitrogen (N), phosphorus (P) and benomyl fungicide. N additions resulted in a significant increase in shoot biomass demonstrating that plant productivity in these soils was N-limited. The effect of P additions consistently depended on fungicide treatment. In the absence of benomyl, shoot P accumulation was unaltered by P addition. In the presence of benomyl, P addition significantly increased shoot P accumulation and was associated with a consistent trend towards enhanced shoot biomass. The induction of P-deficiency with benomyl application suggests that effective P acquisition by AM may contribute to the strong N-limitation of production observed in many terrestrial ecosystems.

Introduction

Primary production in terrestrial ecosystems is most frequently limited by nitrogen (N) supply (Vitousek & Howarth 1991). Distinctive biogeochemical characteristics of N favour the development of ecosystem N-limitation (Vitousek & Howarth 1991). First, N is readily returned to relatively inaccessible gaseous source pools by volatilisation and denitrification in terrestrial ecosystems. Second, litter produced by N-deficient plants tends to have a high carbon:nitrogen ratio that restricts decomposition activity "further promoting" ecosystem N-limitation. Third, N-limitation to primary production may be the net result of a suite of component processes, some of which are limited by other factors. For example, ecosystem N-limitation may be the result of restricted N inputs by N_2-fixing plant/bacterial symbioses whose

activity is constrained by phosphorus (P) supply. We suggest that plant symbioses with mycorrhizal fungi can also influence the development of ecosystem N-limitation. The hyphal network emanating from roots colonised by arbuscular mycorrhizae (AM) could enhance N supply and thus alleviate N-limitation. Alternatively, AM may accentuate N-limitation to primary production by elevating plant P supply above a threshold of incipient P-limitation. Here, we describe a direct field test of the influence of AM-plant associations on N-limitation to primary production.

Plant production in Californian annual grasslands during the wet season is limited by the supply of available nutrients (Jones 1974; Woodmansee & Duncan 1980; Jackson et al. 1988). Budgets of annual N fluxes (Woodmansee & Duncan 1980) and N addition experiments (Jones 1974; Huennecke et al. 1990) indicate that plant growth is most commonly limited by N availability. AM frequently colonise the roots of grasses and forbs in this system (Hopkins 1986; Whitbeck 1993). However, the nature and degree of AM influence on plant nutrient acquisition have proven difficult to characterise under field conditions. Microcosm ^{15}N-labelling studies suggest that AM hyphal translocation of N may enhance plant N acquisition (Ames 1983; Johansen et al. 1992). Glasshouse studies have repeatedly demonstrated that AM association can elevate plant phosphorus (P) status (Harley & Smith 1983; Allen 1991). Nevertheless, field studies of natural plant communities infected with AM often fail to show any effect of the fungal associate on host P status, P uptake rates or plant fitness (Fitter 1991; Brundrett 1991; Sanders & Fitter 1992; Jakobsen 1994). Furthermore, those field studies that have demonstrated a community response to fungicide application are best explained in terms of enhanced growth following eradication of root pathogenic fungi rather than disruption of AM activity (Koide et al. 1988; Carey et al. 1992). In this paper we address the paradox that most plants growing in natural communities are infected with AM and yet frequently show no response to fungal association. Our aim was to investigate AM functioning in a plant community where growth is primarily limited by soil N availability in order to test the following hypotheses: (1) AM associations alleviate ecosystem N-limitation by enhancing plant N status; (2) AM associations accentuate ecosystem N-limitation through increases in plant P supply that overcome incipient P-deficiency.

Materials and methods

We conducted the field study in ungrazed oak savannah at the University of California Sierra Foothills Research Station in N.E. California (39°15′N, 121°17′W). The site had a blue oak (*Quercus douglasii*) overstory with c. 70% cover and a continuous understory dominated by the exotic annual

grasses *Bromus mollis, Hordeum hystrix* and *Avena barbata* (Jackson et al. 1988). The soil is a shallow Argonaut silt loam (mollic haploxeralf) in which 87% of the grass root biomass is contained within the upper 10 cm (Jackson et al. 1988).

On 21 January 1995, we established a 3-way factorial experiment with additions of N, P and benomyl using a randomised block design containing 10 replicate plots (0.75 × 0.75 m) per treatment. We applied benomyl ('Benlate' supplied by Du Pont Inc., Wilmington, Delaware {50% benomyl: methyl 1-[butylcarbamoyl] – 2-benzimidazole carbamate}) as a surface drench on the vegetated soil at the rate of 5 g active ingredient m^{-2} in an aqueous solution (4.8 l m^{-2}). We repeated benomyl applications every 4 weeks. On 11 February, we fertilised with N as NH_4NO_3 at a rate of 20 g N m^{-2} and P as superphosphate (P_2O_5) at a rate of 20 g P m^{-2}. On 8 April, as the grasses were entering the flowering stage, we harvested the central 0.2 × 0.2 m zone of each plot. Total aboveground biomass was collected and dried at 65 °C for 96 hours prior to weighing. Soil cores (7 cm in depth and diameter) were taken from the center of each plot and washed to extract all roots. A sub-sample of the roots was preserved in 50% alcohol and later stained with Trypan Blue (Koske & Gemma 1989) to reveal AM infection. We used a dissecting microscope (×40) to measure percentage of the root length colonised with vesicles by the grid line intersect method (Giovannetti & Mosse 1980). We also used the magnified root intersection method to determine frequencies of arbuscules and vesicles at ×400 (McGonigle et al. 1990). In both methods, a minimum of 100 intact root intersections were examined, and the samples were observed in random order by a single operator. For N and P tissue determinations, a sub-sample of the above-ground biomass of 4–6 replicates was ground in a Wiley mill, digested (micro-Kjeldahl) and analysed colorimetrically by auto-analyser using the nitroferricyanide procedure for N (Lachat 1988) and the phosphomolybdate procedure for P (Kedrowski 1983; Lachat 1990).

To test for any effects of benomyl on plant growth that were not linked to fungicidal disruption of AM activity, we grew 20 *Avena fatua* plants for 12 weeks in pots containing partially sterilised soil/sand. The soil was obtained from the Sierra Foothills field site and autoclaved for 2 hours at 15 psi (130 °C). In an attempt to re-introduce bacterial components of the soil microbial community to the sterilised soil, we added a filtrate (40 microns) of Sierra Foothills soil to all pots in the second week of the experiment. Benomyl fungicide at 12.5 micrograms active ingredient per gram dry weight of soil (which is a rate similar to that used in the field study) was added at planting and after 5 weeks. Nutrients were supplied as weekly additions of 1/12th Hoagland's solution. No AM-colonised roots were observed in either benomyl-treated or control plants at the end of the glasshouse study indi-

Table 1. Statistical significance of treatment effects on above-ground biomass, above-ground tissue nutrient concentrations and above-ground nutrient pools. Treatments are nitrogen (N), phosphorus (P) and benomyl fungicide (B). Data were analyzed by 3-way ANOVA, $n = 10$ replicate plots per treatment for biomass and 4–6 replicate plots per treatment for nutrient analyses. Symbols for significance levels: — $p > 0.1$ (not significant); † $p < 0.1$; ∗ $p < 0.05$; ∗∗ $p < 0.01$; ∗∗∗ $p < 0.001$.

	N	P	B	NP	NB	PB	NPB
Above-ground biomass	∗∗∗	—	∗	—	—	∗	—
Above-ground P conc.	—	∗∗∗	∗	∗∗	—	†	—
Above-ground P pool	∗∗∗	∗∗	—	—	†	∗∗	—
Above-ground N conc.	∗∗∗	—	—	—	—	†	—

cating that the sterilisation and re-inoculation treatment had been effective in eliminating AM fungi.

Treatment effects on shoot and root biomass, percent root colonisation and shoot nutrient concentrations were assessed by analyses of variance (ANOVAs) using Systat 5.2 (Systat Inc. IL). Homogeneity of variances was tested using Cochran's test (Winer 1971). In many cases, there were significant interactions between factors in the ANOVAs. Significant interactions occur when the effect of one factor is not independent of the presence of a particular level of another factor (Zar 1996). Where significant interactions occur, interpretation of ANOVA results should focus not on absolute differences between means of either factor, but rather on differences between means of one factor at each level of the other factor (and vice-versa) (Zar 1996; Underwood 1997). Percent root colonisation data were transformed (arcsine-square root) prior to analysis (Mc Gonigle et al. 1990).

Results

Above-ground biomass showed 2 major responses to manipulation of nutrient supply and fungal activity in the field: (1) The increase in shoot biomass in response to N addition (Table 1, $p < 0.001$; Figure 1) clearly demonstrated N-limitation of production in this Californian grassland. (2) The increase in above-ground biomass in response to the fungicide (Table 1, $p < 0.05$) suggests that the activity of benomyl-sensitive fungi (either AM and/or root pathogens) restricted plant growth. However, the presence of a significant benomyl-P interaction within this ANOVA (Table 1, $p < 0.05$) indicates that the effect of the fungicide was dependent on the level of applied P. In the

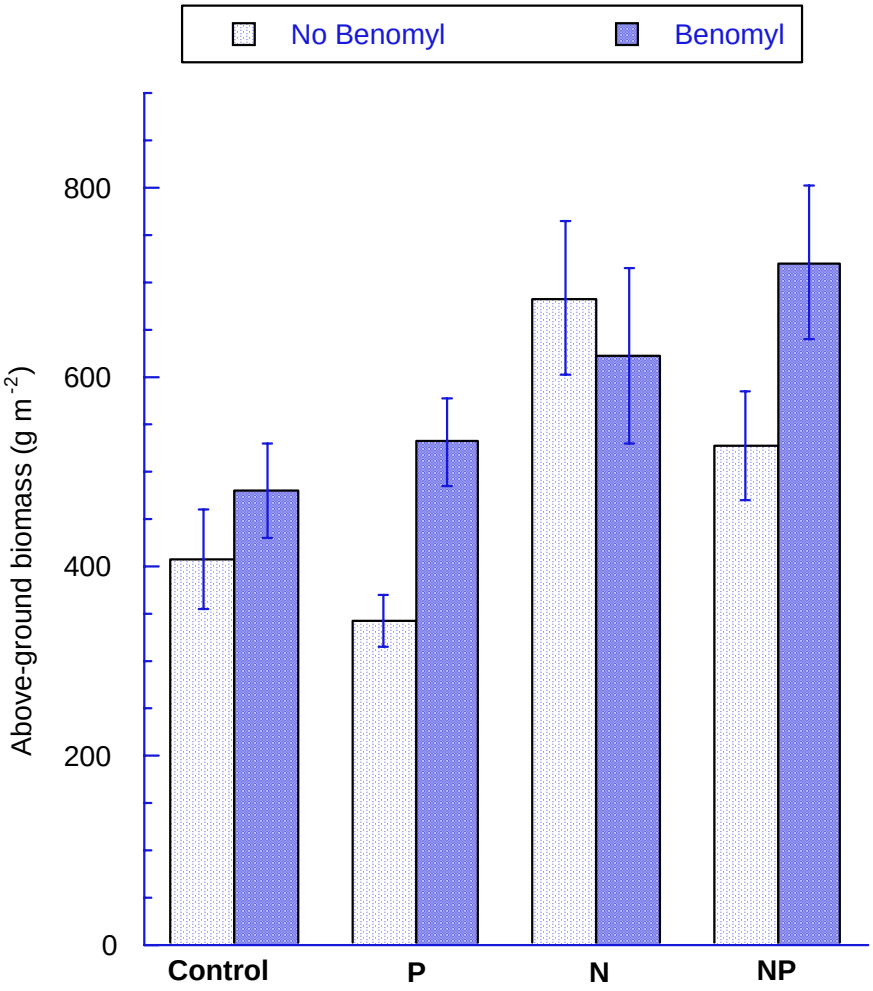

Figure 1. Above-ground plant biomass of a Californian annual grassland treated with factorial combinations of nitrogen (N), phosphorus (P) and benomyl fungicide (n = 10; bars = SEs).

absence of benomyl (light bars, Figure 1), growth was enhanced by N additions but tended to be inhibited by P additions. In the presence of benomyl (dark bars, Figure 1), growth was enhanced both by P and N additions. The consistent trend towards a P-fertilisation effect on above-ground biomass in the presence of the fungicide suggests that plant growth became limited both by P and N supply in the benomyl-treated plots.

Measurements of nutrient concentrations in above-ground biomass also indicated that the benomyl treatment disrupted plant P acquisition. Tissue P concentrations were lowered by benomyl (Table 1, $p < 0.05$; Figure 2)

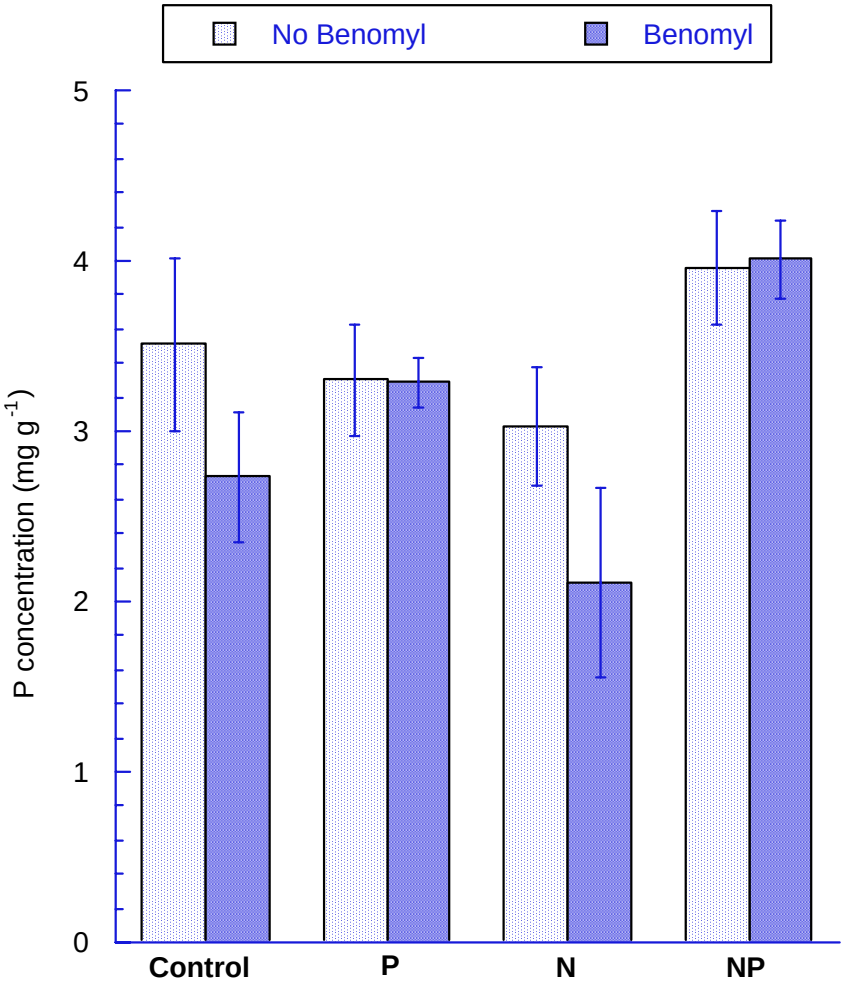

Figure 2. Above-ground tissue phosphorus concentrations in a Californian annual grassland treated with factorial combinations of nitrogen (N), phosphorus (P) and benomyl fungicide (n = 4–6; bars = SEs).

but the effect of the fungicide was again dependent upon the P treatment (benomyl-P interaction $p < 0.10$, Table 1). The influence of benomyl on shoot P concentrations was confined to plots without P addition (Figure 2) indicating that under natural conditions of low P availability in this grassland, shoot P concentrations were enhanced by the presence of benomyl-sensitive fungi.

Shoot P pool sizes were also explained by a strong interaction between the benomyl and P treatments (benomyl-P interaction $p < 0.01$, Table 1; Figure 3). The nature of the interaction once again indicates the import-

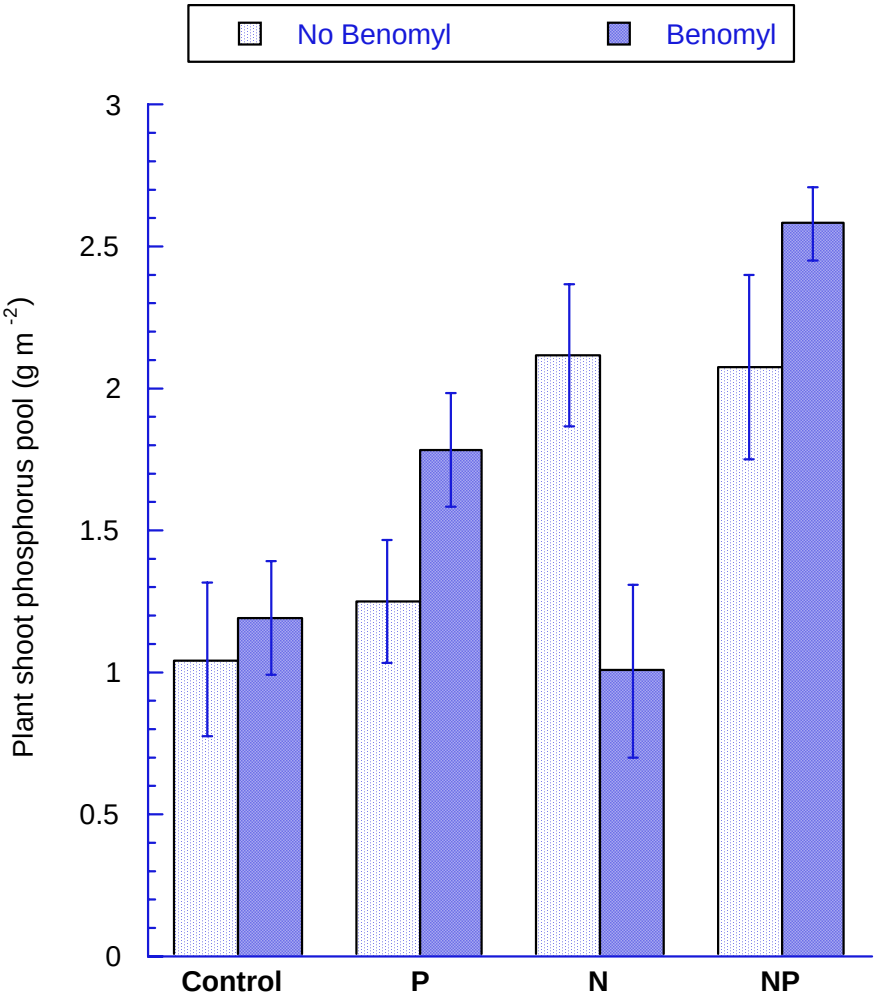

Figure 3. Above-ground plant phosphorus pools in a Californian annual grassland treated with factorial combinations of nitrogen (N), phosphorus (P) and benomyl fungicide (n = 4–6; bars = SEs).

ance of benomyl-sensitive fungi in supplying P to plants in this community. In the absence of benomyl (light bars, Figure 3), shoot P accumulation was unaffected by P additions. By contrast, in the presence of the fungicide (dark bars, Figure 3), shoot P pools responded strongly to P addition. When N-limitation was alleviated for this grassland (the N-fertilised treatment), elimination of benomyl-sensitive fungi reduced shoot P pools by 50% (Figure 3). Together, the consistent pattern in these results strongly suggests that benomyl-sensitive fungi were critical to the P nutrition of plants in

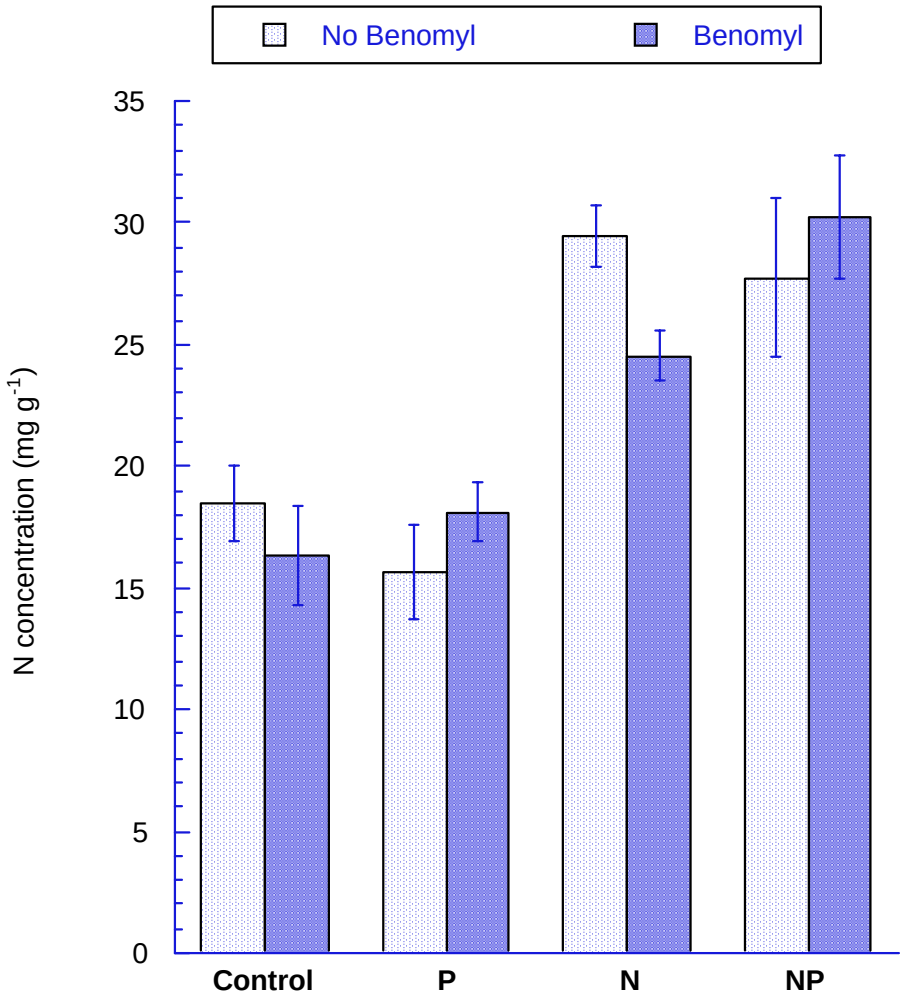

Figure 4. Above-ground tissue nitrogen concentrations in a Californian annual grassland treated with factorial combinations of nitrogen (N), phosphorus (P) and benomyl fungicide (n = 4–6; bars = SEs).

this ecosystem, perhaps explaining why the grassland is more limited by N than P.

As expected, N addition increased tissue N concentration (Table 1, $p < 0.001$; Figure 4), indicating luxury uptake when N was supplied in excess of plant requirements. Furthermore, N addition stimulated tissue P concentrations in the elevated P treatment (N–P interaction $p < 0.01$, Table 1; Figure 2). Consequently, the stimulation of above-ground biomass with N addition resulted in a strong N effect on shoot P pools ($p < 0.01$, Table 1; Figure 3).

Table 2. Statistical significance of treatment effects on fungal colonization levels within roots as measured on arbuscules by the magnified root intersection method (Mc Gonigle et al. 1990) and on vesicles by the grid-line intersect method (Giovannetti & Mosse 1980). In the latter case, samples from the nutrient treatments were confined to full (NP) and no nutrient controls. Treatments and significance level symbols as in Table 1. Data were analyzed by 3-way ANOVA, $n = 30$ for the magnified root intersection method and by 2-way ANOVA, $n = 13$ for the grid line intersect method. All data was transformed (arcsine-square root) prior to analysis.

	N	P	B	NP	NB	PB	NPB
Arbuscular frequency	*	—	—	†	*	—	—
Vesicular frequency			†	—			—

To assess more directly the impact of our treatments below-ground, we quantified roots and determined percentage root length in which arbuscules and vesicles occurred. There were no treatment effects on root biomass. Arbuscular mycorrhizal colonisation levels were significantly reduced in N-treated plots ($p < 0.05$, Table 2; Figure 5). However, the significance of this effect was dependent on the benomyl treatment (benomyl-N interaction $p < 0.05$, Table 2). In the absence of benomyl, arbuscular colonisation was markedly reduced by N additions whereas N-fertilisation had no effect on AM association in fungicide-treated plots. Benomyl-treated plants tended to have reduced vesicular colonisation levels with the grid-line intersection method ($p < 0.10$, Table 2; Figure 6), indicating that benomyl reduced the presence of either endomycorrhizae or pathogenic fungi, both of which can form vesicles.

The glasshouse study suggested that benomyl does not influence plant growth in soils free of AM fungi. Application of the fungicide at rates equivalent to those used in the field had no effect on biomass of *Avena fatua* (data not shown) grown in nutrient-limited partially sterilised soils. No AM-colonised roots were observed in the presence or absence of benomyl. These results suggest that the responses to benomyl addition observed in the field were mediated by its effects on benomyl-sensitive fungi rather than by any direct chemical effect on plant growth.

Discussion

Our results suggest that arbuscular mycorrhizae (AM) are critical to the nutrition and productivity of this Sierra Foothills grassland. In the absence of benomyl, shoot P accumulation was unaltered by P additions and growth was

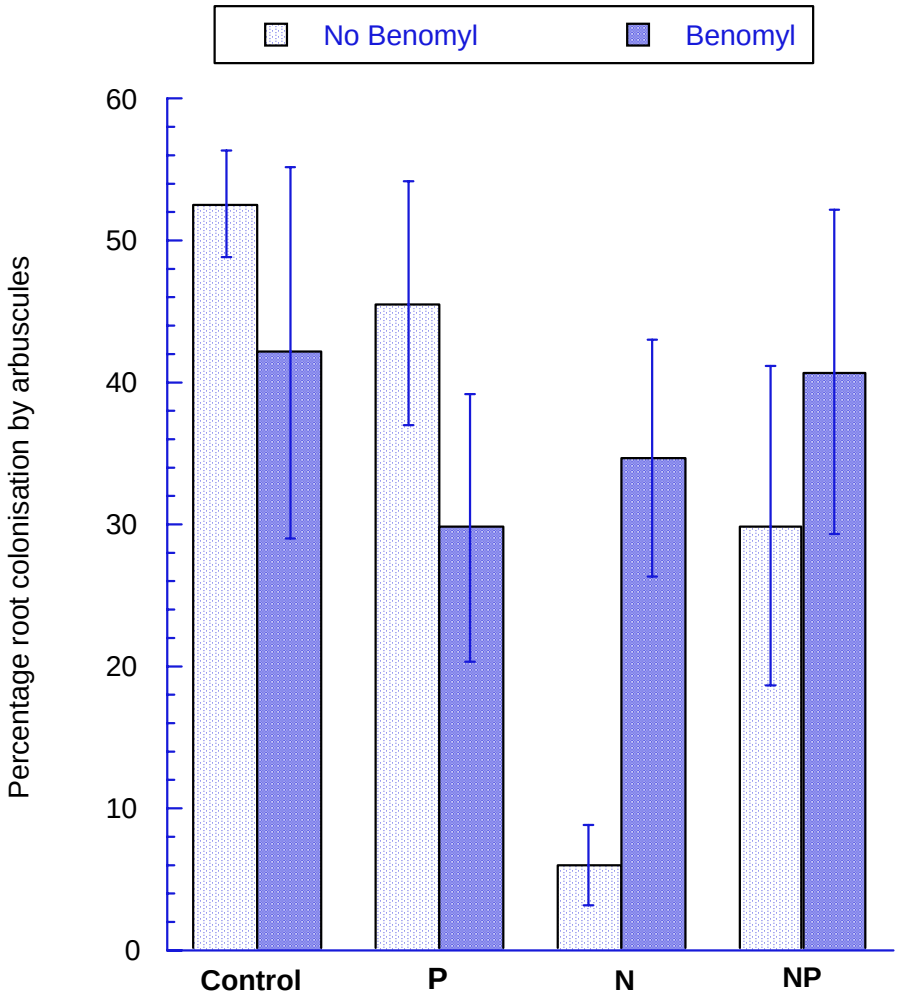

Figure 5. Root colonization by arbuscules in a Californian annual grassland treated with factorial combinations of nitrogen (N), phosphorus (P) and benomyl fungicide (n = 3–4; bars = SEs).

limited by N supply. However, when benomyl was added, reducing vesicular infection levels, P accumulation responded strongly to P addition and there was a consistent trend in shoot growth towards limitation by both N and P supply. This induction of P-deficiency with addition of benomyl is important in two respects. First, it strongly suggests that the major effect of the fungicide was to disrupt plant P supply at our site. Second, it suggests that effective P acquisition by benomyl-sensitive fungi may alleviate incipient P-limitation and contribute to the strong N-limitation commonly observed in

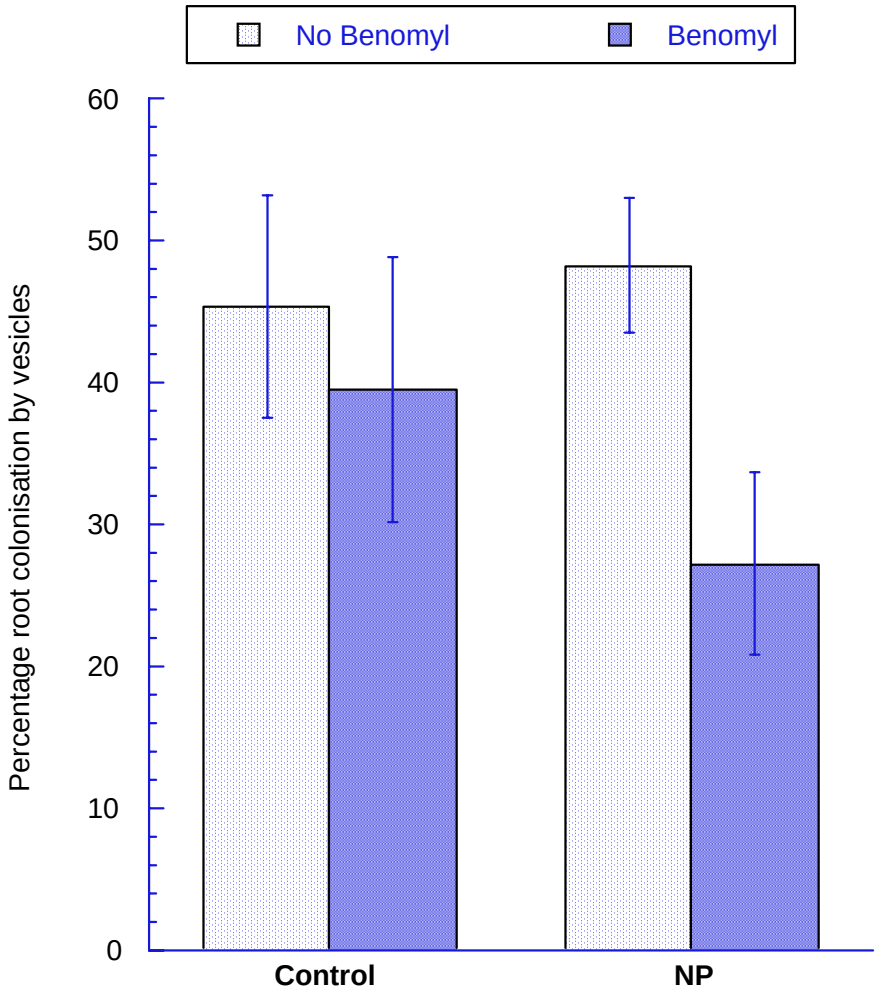

Figure 6. Root colonization by vesicles in a Californian annual grassland treated with factorial combinations of nitrogen plus phosphorus (NP) and benomyl fungicide (n = 2–4; bars = SEs).

temperate grasslands (Jones 1974, Huenneke et al. 1990; Vitousek & Howarth 1991).

Our study is one of the few field experiments whose results are consistent with a strong benomyl effect restricting the ability of plants to take up P. Previous studies of AM activity under natural field conditions have produced a complex set of results. Benomyl applications have: (a) reduced AM colonisation of roots but had no effect on growth (West et al. 1993; Newsham et al. 1994); (b) reduced shoot P concentrations and total plant density without any observed effect on AM colonisation (Koide et al. 1988); (c) increased or

decreased plant production depending on the presence of site-specific pathogens (Carey et al. 1992); and (d) decreased plant P acquisition in an obligately mycorrhizal species (Merryweather & Fitter 1996). It appears that facultatively mycorrhizal species may or may not respond to benomyl depending on the fungicide's relative impact on mycorrhizal as opposed to plant pathogenic fungi (Newsham et al. 1995). Finally, it is also possible that the fungicide may influence other components of the rhizosphere soil community that mediate phosphorus availability to plants. In our study, tissue P concentrations were reduced by benomyl only under natural conditions of low P availability. In these same plots, there was no effect of benomyl on shoot biomass indicating that the over-riding response to the fungicide at our site was reduced plant P uptake rather than a growth-stimulatory effect due to the elimination of pathogenic fungi. Furthermore, the significant benomyl-P interactions indicate that both growth and plant P accumulation became P-limited with the addition of benomyl. Together, these results strongly suggest that the major effect of the fungicide was in modulating plant P nutrition by disrupting AM activity.

The effects of benomyl on vesicular and arbuscular frequencies are somewhat complicated to interpret. Unfortunately, it is not possible to directly measure fungicidal effects on the hyphal network emanating out from root systems that is probably most critical in regulating AM influences on plant nutrient acquisition. The extra-radicle component is the part of the fungus most vulnerable to soil drenches with fungicide. We contend that the absence of a benomyl effect on arbuscular colonisation within the roots does not imply that there was no effect on the AM hyphal network radiating out from those roots. We did observe a reduction in internal root colonisation by vesicles with benomyl applications (Figure 6). Vesicles are a good indicator of long-term plant-fungal association since they persist even after root death (Allen 1991). Arbuscular frequency is the parameter most definitive of endomycorrhizal colonisation, but arbuscules persist for approximately 10 days (Allen 1991) and hence are short-term indicators of AM presence immediately prior to sampling. In our study, the 4-week interval between benomyl drenches may have been too long to continuously inhibit arbuscule formation. Furthermore, our sampling for colonisation may have missed a fungicidal effect because the root samples were harvested 4 weeks after the final benomyl drench. The continuous cover of vegetation at the site may have limited penetration of the fungicide drench into the soil. In seedling pot studies, there is abundant evidence for disruption of AM activity by benomyl drenches (Boatman et al. 1978; Nemec 1979; Menge 1982; Hale & Sanders 1992; Trappe 1984; Paul et al. 1989). Benomyl has been most effective in field studies where vegetation cover was sparse (Fitter 1986) or where soil blocks had been completely immersed in benomyl suspensions (Merryweather &

Fitter 1996). Nevertheless, our growth and tissue nutrient results are entirely consistent with a fungicidal effect that disrupted plant P supply. These results indicate that the benomyl treatment did effectively penetrate the soil at the site. Although we did not observe a reduction in arbuscular colonisation with benomyl at harvest, we have outlined several reasons why this might have occurred and contend that the most likely explanation of the responses to fungicidal treatment was a disruption of AM activity.

Our results indicate that benomyl-sensitive fungi did not influence plant N supply in this grassland. The absence of any effect of benomyl or benomyl-N interaction on shoot N concentration suggests that AM association did not enhance plant N status under field conditions. Second, these results indicate that N contained within benomyl did not act as a source of N-fertiliser at our study site (Cade-Menun & Berch 1997). The soil inorganic N pool in Californian annual grasslands is dominated by NO_3^- (Jackson et al. 1988), which is highly mobile in soil solution and can readily move to the root by diffusion (Chapin 1980). By contrast, $H_2PO_4^-$ is relatively immobile which may explain the benefit for plant P uptake of an enhanced surface area for absorption provided by the AM hyphal network radiating out from roots.

We have demonstrated under natural field conditions that benomyl-sensitive fungi can enhance above-ground tissue P concentrations with negligible impact on the productivity of a plant community. Our results suggest that the principal effect of benomyl applications was to disrupt the activity of AM fungi. The factorial nutrient additions indicate that there may be discrete phases in the nature of the symbiotic relationship between plant communities and AM fungi. Under P fertilisation, tissue P concentrations were unaffected by benomyl. However in these same plots, above-ground plant biomass was significantly lower in the absence of benomyl suggesting that AM association occurred under conditions of high soil P availability even though it represented a carbon drain on plant growth. We conclude that there may be a dynamism in AM symbiotic associations with wild plant communities that can range across varying degrees of mutualism depending on the balance between soil P availability and plant P demand. This dynamism has important implications for our understanding of the functioning of AM associations because of the spatial and temporal variability in soil P cycling and plant P uptake rates across natural ecosystems.

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