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Influence of liming, inoculum level and inoculum placement on root colonization of subterranean clover

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Abstract Arbuscular mycorrhizal (AM) fungi differ in their response to soil pH. Thus, change in soil pH may influence the relative abundance of mycorrhizal fungi inside roots. Root colonization by two AM fungi was studied in relation to addition of lime ($CaCO_{3)}$, quantity of inoculum and inoculum placement. Addition of $CaCO_3$ to an acid soil decreased the colonization of roots by *Acaulospora laevis* but increased colonization by *Glomus invermaium* when both fungi were present. In acid soil (pH 4.7), almost all roots were colonized by *A. laevis*, while *G. invermaium* was dominant when soil pH was increased to pH 7.3. This occurred regardless of whether the inoculum was banded or mixed throughout the soil. There was no effect of $CaCO_3$ on the relative abundance of fungi inside roots at intermediate rates of $CaCO_3$ application (pH 5.3–6.3) when both fungi were inoculated together. In this experiment, both fungi colonized roots at all levels of $CaCO_3$ when inoculated alone, except for *A. laevis* at the highest level of $CaCO_3$. We conclude that soil pH affects the competitive ability of these two AM fungi during mycorrhiza formation primarily by affecting hyphae growth in soil and thus the relative abundance of hyphae at the root surface and subsequently inside the root.

Keywords Subterranean clover · pH · *Glomus invermaium* · *Acaulospora laevis* · Interactions

Introduction

Field soils contain diverse communities of arbuscular mycorrhizal (AM) fungi (Smith and Read 1997). Although root systems usually become colonized by more than one AM species (Daft and Nicolson 1974; Abbott and Robson 1982; Merryweather and Fitter 1998; Jacquot et al. 2000), little is known of the interactions between species during mycorrhiza formation (Juniper et al. 1998). The quantity of mycorrhizas formed by species of AM fungi in field soils will depend on both the amount of infective hyphae and their relative competitive ability (Abbott and Robson 1984). The length of root colonized by AM fungi at early stages of root growth is related to inoculum density (Carling et al. 1979; Abbott and Robson 1984; Wilson 1984; Giovannetti and Avio 1986) and species of AM fungi differ in their competitive ability (Wilson and Trinick 1983; Abbott and Robson 1984; Wilson 1984; Lopez-Aguillon and Mosse 1987; Smith et al. 2000). The differences in competitive ability to form mycorrhizas may be based on antagonism (Hepper et al. 1988), depletion of nutrients within roots (Wilson and Trinick 1983) and carbon demand (Pearson et al. 1993). The compounding effects on colonization of both inoculum level (Abbott and Robson 1984) and rate of hyphae growth from inoculum to root surface have not been clarified.

AM fungi clearly differ in their response to soil pH (see review by Robson and Abbott 1989). Increasing the pH of an acid soil may result in marked changes in the species of AM fungi present as assessed by spores in the soil (Siqueira et al. 1990). *Acaulospora laevis*, for example, is generally found more in acid than in neutral soils (Davis et al. 1985; Porter et al. 1987a; Young et al. 1985) and germination of spores was limited at >pH 6 (Hepper 1984; Porter 1987b). On the other hand, an isolate of *Glomus invermaium* appeared less affected by soil pH, colonizing roots of subterranean clover (*Trifolium subterraneum* L.) over the soil pH range of 5.3 to 7.5 (Abbott and Robson 1985).

We investigated the role of soil pH and inoculum quantity on the relative competitive ability of *A. laevis* and *G. invermaium*. In one experiment, we varied soil pH by adding $CaCO_3$ and the soil was inoculated throughout with propagules of *A. laevis*. Subsequently,

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inoculum of *G. invermaium* was introduced in a band and/or dispersed throughout the soil. This simulated inoculation into a field soil with a known background of AM fungal propagules. Variation in placement of *G. invermaium* was used to vary the quantity of inoculum at the root surface. In the second and third experiments, we varied the quantities of both inoculum and $CaCO_3$ in an attempt to alter the competitive advantages of the AM fungi.

Materials and methods

General procedures

A heathland soil (Brennan et al. 1980) was collected from the top 10 cm of the profile from Lancelin, Western Australia, dried and sieved through a 4-mm mesh sieve, mixed thoroughly and steamed twice at 100°C for 1 h with a 24-h interval. The soil was air dried and placed in plastic-lined pots (1.5 kg soil). Nutrients were added in solution to the surface of each pot and allowed to dry. The soil, $CaCO_3$ (where appropriate) and dry inocula (except the inoculum for the banded treatment) were then added to a large plastic bottle and shaken thoroughly.

The inocula consisted of dried soil containing spores and roots of subterranean clover from stored pot cultures prepared as described by Abbott and Robson (1979). Two isolates of *Acaulospora laevis* Gerd. and Trappe [WUM 11(4) from Badgingarra, WA (experiment 1) and WUM 11(6) from Brookton, WA (experiment 2)] and *Glomus invermaium* Hall [WUM 10 (1) from Merredin, WA (experiments 1 and 2)] were used.

Phosphorus was added at a level adequate for approximately 60% maximum growth of non-mycorrhizal subterranean clover (Abbott and Robson 1979). For experiment 1, nutrients were added to each pot at the following rates (mg kg^{-1} soil): KH_2PO_4 35.3; K_2SO_4 160; $CuSO_4.5H_2O$ 5; $ZnSO_4.7H_2O$ 5; $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.1. In experiments 2 and 3, the above nutrients were added at the same rates, except for K_2SO_4. Additional nutrients (mg kg^{-1} soil) were added as follows to overcome discoloration symptoms (red-brown margins on some old leaves) observed at the second harvest in experiment 1 that were unrelated to the treatment (Snowball and Robson 1983): $MgSO_4.H_2O$ 10; $MnSO_4.7H_2O$ 20; $CoSO_4.7H_2O$ 0.36; $CaCl_2$ 71; and K_2SO_4 71.

The soil was watered to field capacity (10% w/w) 14 days prior to sowing and maintained at this level by watering as required. Pots were kept in temperature-controlled tanks at 20°C in a glasshouse.

Seeds of subterranean clover (*T. subterraneum* L. cv Seaton Park) were germinated in aerated deionized water, sown at a depth of 1.5 cm and inoculated with 1 ml of *Rhizobium leguminosarum* biovar *trifolii* (TA1) suspended in a 1% sucrose solution. Ten seeds were sown per pot and thinned to four plants after 10 days.

At each harvest, shoots were cut at the soil surface, weighed and dried. Roots were washed free of soil, weighed, cleared and stained overnight at room temperature with Trypan blue (0.05%) in lactoglycerol (1:1:1.2/lactic acid:glycerol:water) and destained in lactic glycerol (Abbott and Robson 1981). Fungi in the roots were identified as described by Abbott (1982). Root length and root length colonized by AM fungi were measured using the grid intercept method (Giovannetti and Mosse 1980). A grid of 0.5 cm was used for the first harvest and 1.27 cm for the second harvest for both experiments as suggested by Tennant (1975).

The data were subjected to analysis of variance using "Genstat" (Rothamsted Experimental Station). Each harvest was analysed separately. The percentages of colonization were first arcsin transformed for comparison between treatments using the *F* test, and means were separated by Least Significant Difference at $P<0.05$.

Experimental details

Experiment 1

This experiment was designed to test the hypothesis that the concentration of inoculum by banding and addition of lime would enhance mycorrhiza formation by *G. invermaium* [isolate WUM 10 (1)] in the presence of *A. laevis* [isolate WUM 11(4)].

The experimental design was a complete factorial with two levels of $CaCO_3$ (0 and 1.33 g kg^{-1} soil) and three fungal treatments (*A. laevis* alone; *A. laevis* + *G. invermaium* (mixed throughout the soil); *A. laevis* (mixed throughout) + *G. invermaium* (banded). There were two harvests (at 14 and 30 days from sowing) and three replicates of each treatment.

Addition of 1.33 g of $CaCO_3$ per kg soil increased the pH from 4.9 to 7.3 when measured in 1:5 w/v 0.01 M $CaCl_2$. Inoculum of *A. laevis* was added at 100 g per kg of soil and mixed throughout all pots, and that of *G. invermaium* (66.6 g per kg of soil) was either banded in a layer at a depth of 2.5 cm or mixed thoroughly throughout the entire soil. Inoculum of *A. laevis*, plus soil cultivated with clover without mycorrhizal fungi, plus a soil solution of *G. invermaium* inoculum filtered through Whatman paper no. 10 (5 ml per pot from 1:10 w/v of soil/water) were added to each control pot. Inocula and $CaCO_3$ were added to soil prior to watering.

At harvest, each root system was stained separately as described above. One root system per pot was selected at random and examined at ×100 magnification. The presence of each fungus within the roots was recorded at each intercept between a root and a 0.5-cm grid marked onto the slide.

Experiment 2

We assessed the effect of colonization by each fungus separately on plant growth at five levels of $CaCO_3$ 28 days after sowing. *A. laevis* [isolate WUM 11 (6)] and *G. invermaium* [isolate WUM 10(1)] were used at 25g and 10 g per kg soil, respectively. The inocula of each species were equivalent in terms of the number of spores present in the soil. The treatments were replicated three times. All inocula were mixed thoroughly throughout the soil prior to watering and incubation. Addition of $CaCO_3$ at 0.16, 0.33, 0.50 and 1.33 g $CaCO_3$ per kg soil increased soil pH from 4.7 to 5.3, 5.7, 6.3 and 7.3, respectively. At the harvest after 28 days, mycorrhiza formation by each fungus was assessed as described in experiment 1 after cutting all roots into 1- to 2-cm pieces.

Experiment 3

This experiment examined the effect of inoculum levels on mycorrhiza formation by *A. laevis* and *G. invermaium* when inoculated together over a range of soil pH values intermediate to those in experiments 1 and 2. It was predicted that increasing the inoculum of *A. laevis* would overcome the advantage of *G. invermaium* in colonizing roots at higher pH levels. This experiment was a full factorial design of three levels of $CaCO_3$ (0.16, 0.33, 0.50 g per kg soil), which increased soil pH from 4.7 to 5.3, 5.7, and 6.3, respectively, and three mixed fungal communities (A1G1 25:10, A2G1 100:10, A1G2 25:25) replicated three times. All inocula were mixed thoroughly throughout the soil prior to watering and incubation. Mycorrhiza formation by each fungus was assessed at 14 and 28 days as in experiments 1 and 2.

Results

Liming increased soil pH at each level of $CaCO_3$ added and the pH remained constant in each treatment throughout both experiments.

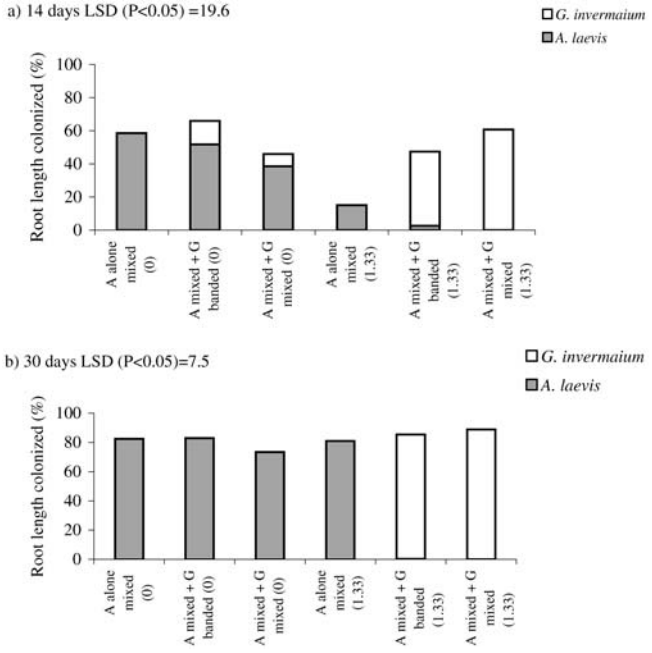

Fig. 1 Root length of subterranean clover colonized (%) by *Acaulospora laevis* (*A*) and *Glomus invermaium* (*G*) 14 and 30 days after sowing in limed (1.33 g $CaCO_3$ per kg soil) or unlimed soil (0 $CaCO_3$) (experiment 1). *A. laevis* inoculum was mixed throughout the soil in each pot; *G. invermaium* inoculum was either banded or mixed or was absent

Table 1 Shoot dry weight (mg per plant ±SEM) and root fresh weight (g per plant ±SEM) of subterranean clover 30 days of sowing, inoculated with *Acaulospora laevis* (*A*) alone mixed throughout the soil in each pot or with *Glomus invermaium* (*G*) either banded or mixed in limed ($CaCO_3$; g per kg soil) or unlimed soil (experiment1)

Inoculum	Shoot dry wt. $CaCO_3$		Root fresh wt. $CaCO_3$	
	0	1.33	0	1.33
A mixed +G mixed	140 (33)	146 (31)	1.24 (0.31)	1.20 (0.31)
A mixed + G banded	109 (21)	154 (26)	1.24 (0.31)	1.29 (0.23)
A alone mixed	116 (27)	78 (23)	1.15 (0.41)	0.69 (0.21)
LSD (P<0.05)	22		0.31	

Experiment 1

In soil with no added $CaCO_3$, *A. laevis* colonized most of the mycorrhizal root length at both 14 and 30 days after sowing (Fig. 1). The generally low percentage of roots colonized by *G. invermaium* at 14 days was higher (P<0.05) when the inoculum was placed in a band.

Addition of $CaCO_3$ reduced the growth rate of *A. laevis* mycorrhiza (Fig. 1). Mycorrhiza of *G. invermaium* predominated in soil containing *A. laevis* and $CaCO_3$, irrespective of inoculum placement (Fig. 1). Initially, *G. invermaium* colonized a higher percentage of mycorrhizal root length (P>0.05) when mixed throughout the soil than when banded (Fig. 1). However, this effect was only apparent at the 14 day harvest.

Shoot and root weights of plants inoculated with *G. invermaium* were not affected by application of $CaCO_3$ (Table 1). However, addition of $CaCO_3$ decreased dry weights of both shoots and roots of plants inoculated with *A. laevis* alone by more than 60%.

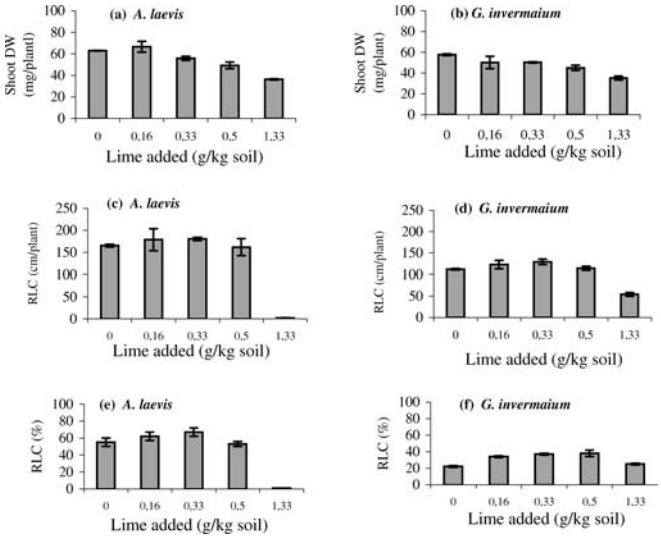

Fig. 2 Shoot dry weight, the rate and extent of root colonization by *A. laevis* and *G. invermaium* at different levels of limed soil 28 days of sowing (experiment 2) (*RLC* Root length colonized)

Experiment 2

Shoot dry weight gradually decreased with increasing application of lime irrespective of fungal species (Fig. 2a, b). Mycorrhiza formation by *A. laevis* was inhibited by the addition of 1.33 g $CaCO_3$ per kg soil but addition of 0.16 and 0.33 g $CaCO_3$ per kg increased the rate and extent of colonization by this fungus (Fig. 2c, e). By 28 days after sowing, plants at 0, 0.16, 0.33 and 0.5 g $CaCO_3$ per kg soil had similar lengths of root colonized by *A. laevis*. The responses of *G. invermaium* to addition of $CaCO_3$ were similar to those of *A. laevis*, but less severe (Fig. 2d, f). In the absence of $CaCO_3$, colonization by *G. invermaium* was delayed compared with the first three levels of $CaCO_3$ added. The effects of $CaCO_3$ on percentage root length colonized were similar to those for absolute root length colonized. Inoculum of *A. laevis* was contaminated with a fine endophyte (Ali 1969) in this experiment that randomly colonized up to 16% of root length by the harvest at 28 days, irrespective of the fungal inoculum added (data not presented).

Experiment 3

When *A. laevis* and *G. invermaium* were inoculated together, the $CaCO_3$ additions used in this experiment had little effect on total percentage of root length colonized

Table 2 Colonization by *A. laevis* (*A*) and *G. invermaium* (*G*) of subterranean clover grown at three levels of lime and three combinations of inoculum in experiment 3. Inoculum combinations (g per kg soil) were: A1G1 25:10, A1G2 25:25, A2G1 100:10). The doses of lime as $CaCO_3$ (g per kg soil) were: L1 0.16, L2 0.33, L3 0.50

Inoculum	Colonization by:	Percentage of root length colonized					
		14 days after sowing			28 days after sowing		
		L1	L2	L3	L1	L2	L3
A1G1	*A. laevis*	17	16	13	44	50	46
A1G2		11	11	8	20	17	17
A2G1		36	32	22	60	63	71
LSD (P<0.05)		10			12		
A1G1	*G. invermaium*	9	8	13	12	14	24
A1G2		19	20	27	49	59	61
A2G1		4	5	8	3	8	8
LSD (P<0.05)		10			9		
A1G1	Total	25	24	25	56	62	65
A1G2		30	33	34	66	72	74
A2G1		40	36	29	63	69	78
LSD (P<0.05)		10			12		

Table 3 Shoot dry weight (mg per plant), root fresh weight (mg per plant) and root length (cm per plant) of subterranean clover 28 days after sowing and inoculated with *A. laevis* and *G. invermaium* in different combinations (experiment 3). See Table 2 for treatment designations (*ns* not significant)

Inoculum	Shoot dry wt.			Root fresh wt.			Root length		
	L1	L2	L3	L1	L2	L3	L1	L2	L3
A1G1	61	60	60	494	508	483	284	239	246
A1G2	70	63	62	479	461	472	273	237	245
A2G1	73	65	58	503	462	500	228	205	212
LSD (P<0.05)	ns			ns			ns		

(Table 2). Increasing the inoculum level of either *A. laevis* or *G. invermaium* increased the percentage of roots colonized by each fungus by approximately 20% and 14% at 14 and 28 days after sowing, respectively (Table 2).

In general, addition of *G. invermaium* decreased mycorrhiza formation by *A. laevis*, but this effect was overcome by increasing the inoculum of *A. laevis* (Table 2). There was little effect of added $CaCO_3$ within the range used in this experiment on the percentage of mycorrhizal roots colonized by each species. For the 14 day harvest, addition of the lower inoculum of *G. invermaium* did not affect the percentage of roots colonized by *A. laevis* inoculated at the lower level, although by 28 days colonization by *A. laevis* was generally reduced (Table 2). This effect became more marked with an increase in the *G. invermaium* inoculum. There was little effect of $CaCO_3$ addition on this relationship, except for a decline in the percentage of roots colonized by *A. laevis* at the first harvest. Increasing the inoculum of *A. laevis* in the presence of the lower inoculum of *G. invermaium* increased root colonization by *A. laevis*, except for a temporary decline at 0.5 g $CaCO_3$ per kg soil (Table 2).

The percentage mycorrhizal root length formed by *G. invermaium* in the presence of increasing levels of *A. laevis* and with three levels of $CaCO_3$ followed the same pattern as for *A. laevis* in the presence of *G. invermaium* (Table 2). Thus, although the absolute lengths and proportions of mycorrhizal root differed for the two fungi, each fungus responded in the same way to the presence of the other fungus when $CaCO_3$ was applied to the soil.

At the three intermediate levels of $CaCO_3$, there was no difference in shoot growth in treatments with mixed communities of AM fungi (Table 3). Addition of 1.33 g $CaCO_3$ per kg soil decreased growth of shoots when plants were inoculated with either AM fungal species (Fig. 2). Addition of the higher inoculum of either *G. invermaium* or *A. laevis* increased shoot growth marginally, but not at the three intermediate levels of $CaCO_3$. There were no differences in root growth with the three intermediate applications of $CaCO_3$ or in relation to inoculation treatment (Table 3).

Discussion

The results of these experiments demonstrate that the innate competitiveness or aggressive characteristics of AM fungi may be overshadowed if the quantity of inoculum present or the soil conditions allow increased growth of hyphae in soil prior to root colonization. Inoculum quantity and $CaCO_3$ concentration both influenced the relative colonization by *G. invermaium* and *A. laevis*. *A. laevis* was not competitive in soil containing a high level of $CaCO_3$. At lower $CaCO_3$, mycorrhiza of each species predominated when its inoculum was higher. An increase in either the inoculum or $CaCO_3$ altered the relative amounts of infective hyphae of each fungus at the root surface. Therefore, these data support the hypothesis that factors decreasing the infectivity of hyphae of AM fungi near roots decrease their competitive ability in mycorrhiza formation (Abbott and Robson 1984). Although inoculum placement can influence the colonization of roots by AM fungi (Afek et al. 1990), varying the placement of inoculum of *G. invermaium* had little

effect on the relative competitive abilities of the two fungi used in this study.

A. laevis showed low competitiveness in the presence of *G. invermaium* in limed soil in the first experiment. In contrast, in the absence of *G. invermaium*, mycorrhiza formation was extensive but slower than in the unlimed soil. The lack of competitive ability and slower rate of *A. laevis* mycorrhiza formation was likely associated with slow germination of its spores at high pH (Hepper 1984) and limited growth of hyphae in soil at high pH (Porter et al. 1987b). Spores were the most likely form of propagule of *A. laevis* in this experiment as its hyphae cannot survive in dried root pieces (Tommerup and Abbott 1981) and they lose infectivity when disturbed by mixing in dry soil, as occurred during inoculum handling (Jasper et al. 1989).

The absence of an effect of $CaCO_3$ on relative levels of mycorrhiza formation by *A. laevis* and *G. invermaium* in the second experiment was unexpected. However, there was also no effect of $CaCO_3$ at the three intermediate levels applied when the fungi were introduced individually. The only exception was the initially slower rate of colonization by *A. laevis* at 0.5 g $CaCO_3$. This corresponded with a narrower soil pH range than in the first experiment. It was expected that *A. laevis* at 1.33 g $CaCO_3$ would have no competitive advantage in the presence of *G. invermaium* as occurred in the first experiment, but this level of $CaCO_3$ was not included in the third experiment. Despite the absence of an effect of $CaCO_3$ on interactions between the fungi at the levels applied, the data demonstrate reciprocal displacement of *A. laevis* and *G. invermaium* at higher inoculum levels. A similar effect was observed previously when soils containing *A. laevis* were watered prior to transplanting seedlings containing *G. invermaium* (Abbott and Robson 1984). The pretreatment allowed spores of *A. laevis* to germinate and effectively provided a higher inoculum level at the time the roots containing *G. invermaium* were introduced into the soil. Consequently, *G. invermaium* was out-competed by *A. laevis* in forming mycorrhizas on new roots.

The percentage of root length infected by *A. laevis* at 1.33 g $CaCO_3$ was close to 80% after 30 days in experiment 1 but almost zero after 28 days in experiment 2. A possible explanation for this marked difference at pH 7.3 is that the much higher (fourfold) inoculum of *A. laevis* used in experiment 1 was sufficient to overcome an effect of high soil pH on colonization by this fungus. Alternatively, there may have been a difference between the two isolates of *A. laevis* in response to soil pH.

In field soils, dominance by certain species of AM fungi could be expected where soil physical, chemical and biological characteristics favour the growth of their hyphae. There is little evidence for the two species used here that direct interaction between hyphae played a role in competitive colonization of subterranean clover. This contrasts with evidence that fine endophytes affect colonization by AM fungi with coarse hyphae independent of inoculum level (Wilson and Trinick 1983; Wilson 1984).

Effects of soil pH on joint colonization of roots by two *Glomus* spp. were apparent in the experiment of Koomen et al. (1987). In their case, the extent of sporulation by these two fungi was reversed when pH was altered. Similarly, major changes in the number of spores of four combinations of AM fungi species occurred with increasing addition of lime (Siqueira et al. 1990). Lime addition may alter the relationship between mycorrhiza formation and sporulation of AM fungi. Although the relative effect of lime on mycorrhiza formation and sporulation by each fungus was not determined, variation in relative number of propagules of these fungi with changes in soil pH will perpetuate shifts in the population of AM fungi in limed soils.

Adding $CaCO_3$ at a high level can decrease the availability of nutrients, especially phosphorus, to plants although this is not always the case. In addition, a decrease in plant growth may be caused by inhibition of the growth of mycorrhizal hyphae in alkaline soil and, thus, mycorrhizal root length (Abbott and Robson 1985). At the highest level of $CaCO_3$, the differences in plant growth may reflect the quantity of mycorrhizas formed at an earlier stage, as has been previously demonstrated (Carling et al. 1979; Smith et al. 1979). Differences between fungal species in forming mycorrhizas in limed soil could also be associated with differences in their response to the level of soil phosphate available for plant growth (Thomson et al. 1986) as well as to pH effects per se.

In this study, we quantified the mycorrhizal fungal community in roots according to their morphological features; this was possible because the fungi selected had distinctly different morphologies (Abbott 1982). AM fungi with less distinctive morphologies in roots can be quantified by PCR techniques (Jacquot et al. 2000).

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