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Interactions Between Mycorrhizal Colonisation, Nodulation and Growth of *Calliandra calothyrsus* Seedlings Supplied with Different Concentrations of Phosphorus Solution

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Abstract

Interactions between arbuscular mycorrhizal colonisation, nodulation and growth of *Calliandra calothyrsus* were investigated in an experiment in which mycorrhizal and non-mycorrhizal seedlings were grown in a glasshouse environment, in pots containing a sterilised substrate and supplied with four different concentrations of phosphorus solution (0, 7.5, 15 and 30 mg l^{-1} P). All seedlings were inoculated with *Rhizobium*. After eight weeks growth, assessments were made of stem, leaf and root growth, nodule dry mass and percentage mycorrhizal colonisation. Mycorrhizal colonisation was highest at 0 mg l^{-1} P and was reduced with increasing phosphorus application. Mycorrhizal inoculation increased seedling growth and nodulation most at lower concentrations (0 and 7.5 mg l^{-1} P) of phosphorus application. Phosphorus application improved growth and

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nodulation at 7.5 mg l^{-1} P compared with 0 mg l^{-1} P, but further increase in phosphorus application did not result in further growth benefits. Seedling growth was positively correlated with mycorrhizal colonisation and nodule dry mass with strongest relationships occurring for nodule dry mass. Growth response of seedlings to greater nodulation was maintained in mycorrhizal plants but not in non-mycorrhizal plants, even at high levels of P application when available P was not limiting. It is concluded that *C. calothyrsus* is dependent on mycorrhizal association in P-deficient soils, and that mycorrhizal inoculation has the potential to enhance its growth and nodulation under these conditions.

Keywords: Arbuscular mycorrhizas, nitrogen-fixing tree, growth responses

1. Introduction

Calliandra calothyrsus Meissner is a small, thornless, leguminous tree native to humid and sub-humid regions of Central America. In recent years its value as a multi-purpose tree for use in agroforestry has been recognised, both as a source of firewood and high quality fodder and as a hedgerow plant in alley cropping systems. In addition, the species grows well in acidic, infertile soils typical of many parts of the humid tropics. As a result, *C. calothyrsus* has been increasingly planted as an exotic in many tropical countries and evaluation of its potential benefits and optimisation of its growth has been undertaken (Lesueur et al., 1996; Mugendi et al., 1999a; 1999b; Ndufa et al., 1999).

Like many tree legumes, *C. calothyrsus* forms symbiotic relationships with both nitrogen-fixing bacteria and arbuscular mycorrhizal (AM) fungi. The main benefit of AM association to the plant is through enhanced phosphorus uptake and, because *C. calothyrsus* forms associations with AM fungi and nitrogen-fixing *Rhizobia*, it is able to sustain growth in both phosphorus (P) and nitrogen (N) deficient soils. Positive interactions between AM fungi and *Rhizobia* have also been demonstrated for many legumes: mycorrhizal colonisation has been shown to stimulate nodule formation and nitrogen-fixing activity in the host plant and, as effective nodulation depends on an adequate supply of P, it is thought that these benefits are largely P-mediated (Barea and Azcón-Aguilar, 1983).

To a large extent, plant growth response to AM colonisation is determined by soil fertility and in particular the availability of P. However, plant species which form AM differ in their susceptibility to root colonisation and in their dependence on AM formation to stimulate plant growth. Janos (1980) has shown that tropical trees exhibit a wide range of dependency on AM colonisation and more recent studies (Habte and Turk, 1991; Manjunath and Habte, 1992; Habte,

1995) on tree legumes used in agroforestry systems have also shown that some species show high dependency (e.g. *Leucaena leucocephala, Cassia siamea, Glyricidia sepium*) whereas others are less dependent (e.g. *Cassia reticulata, Sesbania pachycarpa*). As a result, dependent species are more likely to grow poorly when indigenous AM propagules present in field soils are deficient or ineffective.

Although extensive research programmes are in progress which evaluate the use of *C. calothyrsus* in agroforestry, comparatively few studies have examined its symbiotic relationships, which could be crucial in low-input farms and infertile soils. Reena and Bagyaraj (1990) and Ibrahim et al. (1996) have shown the potential for improving establishment and growth of introduced *C. calothyrsus* by mycorrhizal inoculation and P application, but do not relate mycorrhizal colonisation and plant growth to different concentrations of available P in the soil. Desmond (1995) found that increasing concentrations of available P improved plant growth and mycorrhizal colonisation of *C. calothyrsus*, but levels of colonisation observed were very low and may well have precluded any growth response due to mycorrhizal colonisation alone.

In this study, we aimed to examine the interactions between mycorrhizal formation, nodulation and plant growth and to evaluate the mycorrhizal dependency of *C. calothyrsus*. To do this, we compared the growth of mycorrhizal and non-mycorrhizal seedlings (both inoculated with *Rhizobium*) in soil containing increasing concentrations of available P. The research formed part of a wider study aimed at optimisation of growth and forage production by *C. calothyrsus* through evaluation and application of microsymbiont diversity (Lesueur, 2000).

2. Materials and Methods

Experimental design

A factorial experiment was used to test 2 mycorrhizal and 4 nutrient treatments. Treatments were laid out in 8 randomised blocks with each treatment represented once within each block.

Plant material

Seeds of *Calliandra calothyrsus* seedlot 12/91 ex. Honduras, supplied by the Centre for Natural Resources and Development, Oxford, UK (CNRD), were scarified by chipping off a small piece of the seedcoat and pre-germinated in petri-dishes on moistened, sterilised filter paper for 5 days.

Mycorrhizal inoculum

Pot cultures of *Glomus intraradices* Schenck & Smith (isolate UT 143-2, supplied by the International Culture Collection of Arbuscular and VA Mycorrhizal Fungi, West Virginia University, USA) were grown for 4 months in a sterilised loam/sand/grit mixture using cowpea (*Vigna unguiculata* L.) and millet (*Pennisetum typhoides* L.) as host plants.

Inoculation and set up of the experiment

The experiment was conducted in a glasshouse set to provide a day/night temperature regime of 28/20°C and relative humidity of 30–50%. Natural sunlight was supplemented with high-pressure mercury vapour lamps when necessary, to produce a day length of 14 h.

Free-draining, 1 litre pots were filled with a sterilised (autoclaved at 121°C and 1.03. 10^5 Nm^2 for 1 hour) mixture of horticultural coconut fibre and sand (1:1 by volume). Mycorrhizal pot cultures were thoroughly mixed and 14 g of inoculum (soil, spores and root fragments) was added to each pot at about 1 cm depth prior to planting. For control pots (i.e. non-mycorrhizal plants) the same amount of autoclaved inoculum was added. Three pre-germinated seedlings were then transplanted to each pot. At this point, the seedlings were inoculated with a *Rhizobium* suspension, consisting of isolate TAL 1455 (also supplied by CNRD, Oxford, UK) and crushed *C. calothyrsus* nodules, to ensure that all seedlings became nodulated. One week after planting, the seedlings were thinned to one per pot, retaining the seedling nearest to average height.

Phosphorus treatments and nutrient supply

Four phosphorus treatments were applied in the form of H_3PO_4 solution at three different concentrations (7.5, 15 and 30 mg l^{-1} P), and in the form of de-ionised water for 0 mg l^{-1} P. In addition to daily watering, all plants were supplied with Ingestad's solution (Ingestad, 1971) which had been modified to remove the phosphorus by replacing KH_2PO4 with KOH. Ingestad's and phosphorus solutions were applied to seedlings twice weekly, increasing the amount added from 10 cm^3 of each solution per pot per application in weeks one and two, to 15 cm^3 per pot per application in weeks three and four, and 20 cm^3 per pot per application in weeks five, six and seven.

Chemical analysis of the sterilised coconut fibre/sand mixture showed that it contained 345 mg K kg^{-1}, 9.4 mg NH_4^- N kg^{-1}, <0.5 mg NO_3^- N kg^{-1} and <0.2 mg P kg^{-1}. The mixture contained 4.05% organic matter and had a pH of 5.3.

Assessments

During the growth period, weekly measurements of plant height were made. Plants were harvested eight weeks after the experiment was set up. Shoots were removed and measurements were made of stem diameter, stem dry mass, leaf area, and leaf dry mass. After root washing, nodules were counted and removed for determination of dry mass. Mycorrhizal colonisation was assessed on a sub-sample of the roots so that root dry mass could be determined using the remaining roots. For sub-sampling, the root system was cut into root fragments about 1 cm long, thoroughly mixed and then 100 root fragments were removed at random. Fresh mass of the sub-sample and remaining roots was obtained and then the dry mass of the remaining roots was determined, so that the total root dry mass could be estimated by proportion. For assessment of mycorrhizal colonisation, root sub-samples were stained with trypan blue (Koske and Gemma, 1989) using a modified syringe method (Claasen and Zasoski, 1992) and percentage mycorrhizal colonisation was estimated using the gridline intersect method (Tennant, 1975).

Statistical analysis

Data were examined for normality and homogeneity of variances (Bartlett's test; Sokal and Rohlf, 1995), and transformed where necessary. Nodule dry mass data was normally distributed but showed heterogeneity of variance, so analysis was carried out on square root transformed data. One plant, which failed to nodulate and produced outliers in several data sets, was excluded from the analysis. Data were examined by two-way analysis of variance (ANOVA) using mycorrhizal inoculation and phosphorus application as treatment factors. Means were compared using Fisher's LSD test when the F-test from ANOVA was significant at $P<0.05$. Correlation coefficients and regression analysis were used to examine relationships between variates and P response curves were fitted when variates were significantly affected by P application.

3. Results

Weekly height measurements

After thinning to one seedling per pot, the initial height measurement showed that inoculated plants were significantly ($P=0.011$) taller than non-inoculated plants. For this reason ANOVA of weekly plant heights was adjusted using the initial height as a covariate. During the first 6 weeks of growth, plant height was not significantly affected by mycorrhizal inoculation

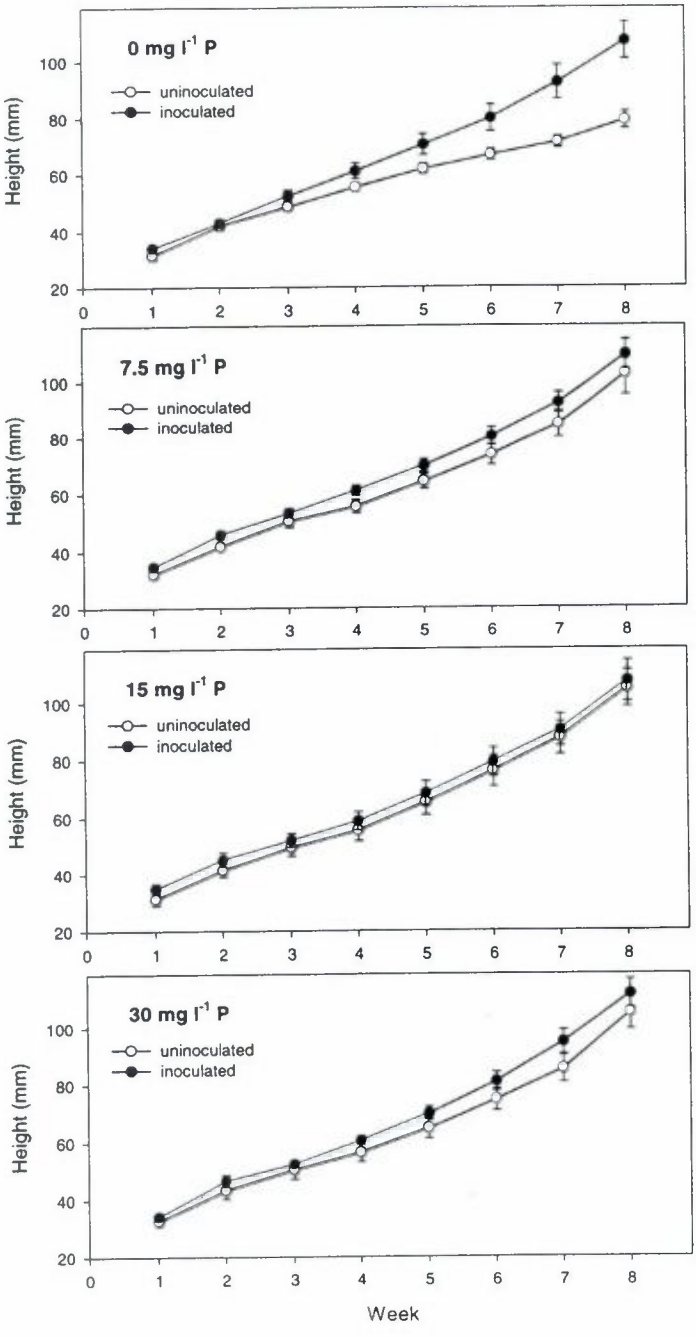

Figure 1. Weekly height growth of *Calliandra calothyrsus* seedlings in response to mycorrhizal inoculation and application of four different concentrations of phosphorus solutions. Error bars = ±SE.

Table 1. Effect of mycorrhizal inoculation on the growth of *Calliandra calothyrsus* seedlings after eight weeks

	Uninoculated	Inoculated	P value
Stem diameter (mm)	2.17 b[a]	2.37 a	0.004
Stem dry mass (mg)	99 b	127 a	<0.001
Leaf dry mass (mg)	430 b	567 a	<0.001
Leaf area (cm^2)	103 b	135 a	<0.001
Root dry mass (mg)	166 b	207 a	0.008
Nodule dry mass (mg)[b]	26.4 b	36.0 a	<0.001
Mycorrhizal colonisation (%)	0 b	29.1 a	<0.001

[a]Letters indicate significant differences within each row at $P<0.05$ as determined by ANOVA and Fisher's LSD test. [b]Square root transformations were performed on nodule dry mass data for analysis, significance is given against untransformed data.

Table 2. Effect of application of four different phosphorus solutions on the growth of *Calliandra calothyrsus* seedlings after eight weeks

	0 mg l^{-1} P	7.5 mg l^{-1} P	15 mg l^{-1} P	30 mg l^{-1} P	P value
Stem diameter (mm)	2.16	2.31	2.29	2.32	0.269
Stem dry mass (mg)	101	116	115	120	0.325
Leaf dry mass (mg)	416 b[a]	540 a	515 a	521 a	0.026
Leaf area (cm^2)	100 b	130 a	123 a	121 a	0.034
Root dry mass (mg)	179	199	178	189	0.732
Nodule dry mass (mg)[b]	17.2 b	34.3 a	34.8 a	38.6 a	<0.001
Mycorrhizal colonisation (%)[c]	44.7 a	31.5 b	23.9 c	16.1 d	<0.001

[a]Letters indicate significant differences within each row at $P<0.05$ as determined by ANOVA and Fisher's LSD test. [b]Square root transformations were performed on nodule dry mass data for analysis, significance is given against untransformed data. [c]Means given are for inoculated plants only.

or P application. After week seven however, inoculated plants were significantly ($P=0.030$) taller than uninoculated plants. Although no significant interactions were found, Fig. 1 shows that this effect was almost entirely due to uninoculated plants being smaller when no phosphorus was applied. At week eight, there was a significant ($P=0.037$) effect of P application on plant height, with seedlings given no phosphorus being smaller than those given 7.5, 15 and 30 mg l^{-1} P. As with week seven, no interactions between mycorrhizal inoculation and P application were found.

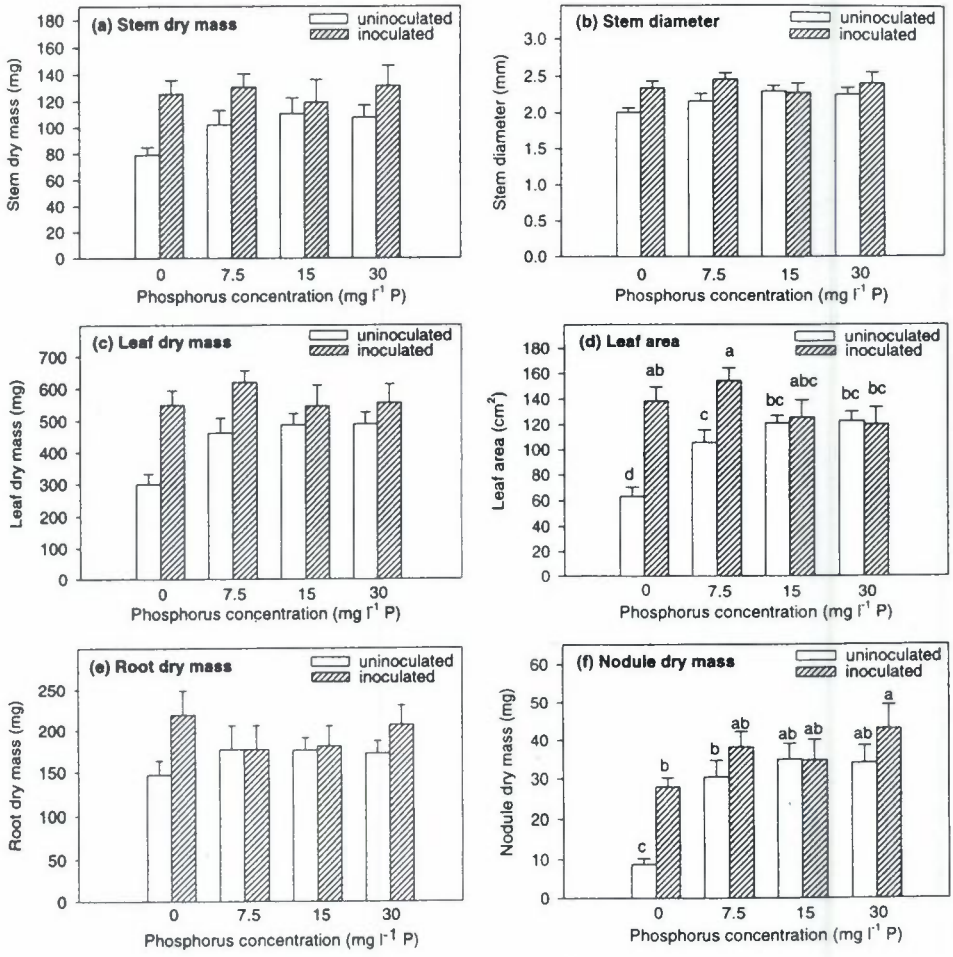

Figure 2. Effect of mycorrhizal inoculation and application of four different concentrations of phosphorus solutions on growth of *Calliandra calothyrsus* seedlings after eight weeks. Error bars = ±SE. Columns with different letters are significantly different at $P<0.05$ as determined by ANOVA and Fisher's LSD test.

Plant harvest

Main effects of mycorrhizal inoculation on seedling growth are summarised in Table 1. Mycorrhizal inoculation significantly increased stem dry mass, stem diameter, leaf dry mass, leaf area, root dry mass and nodule dry mass. All plants that received mycorrhizal inoculum became colonised by AM, whereas no colonisation was found in uninoculated control plants.

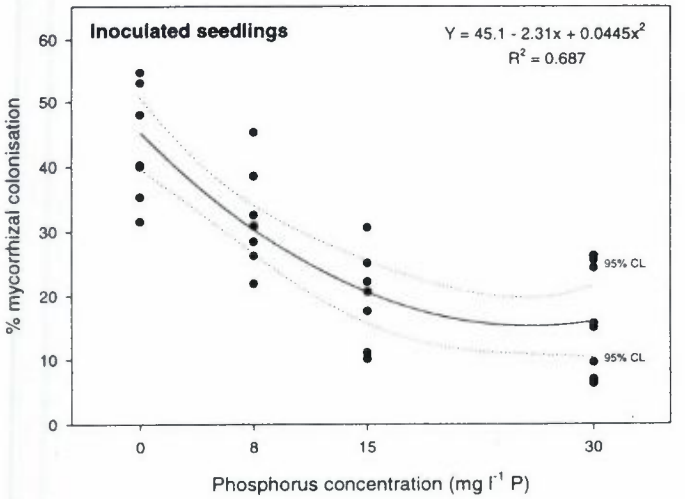

Figure 3. Fitted response curve of mycorrhizal colonisation of inoculated *Calliandra calothyrsus* seedlings to application of four different concentrations of phosphorus solution.

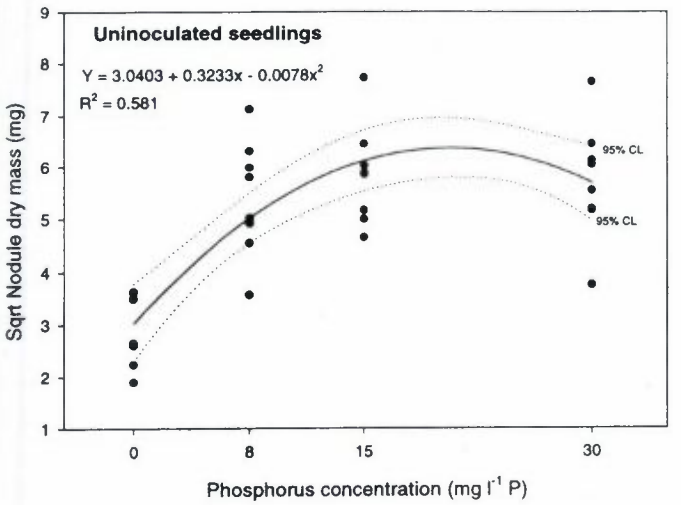

Figure 4. Fitted response curve of nodule dry mass of uninoculated *Calliandra calothyrsus* seedlings to application of four different concentrations of phosphorus solution.

Main effects of P application on seedling growth are summarised in Table 2. P application reduced percent mycorrhizal colonisation from 44.7% when 0 mg l^{-1} P was applied to 16.1% when 30 mg l^{-1} P was applied. Nodule dry mass was

also strongly affected by P application, with seedlings receiving 7.5, 15 and 30 mg l^{-1} P having twice the nodule dry mass of those receiving no phosphorus. Generally, P application had less impact on seedling growth than mycorrhizal inoculation: only significant differences in leaf dry mass and leaf area were noted, with seedlings receiving no phosphorus having smaller leaf area and leaf dry mass.

Significant interactions between mycorrhizal inoculation and P application were found for nodule dry mass ($P=0.001$) and leaf area ($P<0.001$). Nodule dry mass of non-mycorrhizal plants grown at 0 mg l^{-1} P was less than that of all other treatment combinations (Fig. 2f). Leaf area of non-mycorrhizal plants grown at 0 mg l^{-1} P was smaller than all other treatment combinations, while leaf area of non-mycorrhizal plants grown at 7.5 mg l^{-1} P was less than that of mycorrhizal plants grown at 0 mg l^{-1} P and 7.5 mg l^{-1} P (Fig. 2d). Similar differences were found for other growth parameters measured, with mycorrhizal inoculation increasing stem dry mass (Fig. 2a), stem diameter (Fig. 2b), leaf dry mass (Fig. 2c) and root dry mass (Fig. 2e) most at lower levels of P application.

Relationships between seedling growth, mycorrhizal colonisation, nodulation and phosphorus application

All parameters of seedling growth were positively correlated with both percent mycorrhizal colonisation and nodule dry mass, although correlation coefficients were much greater for nodule dry mass than for mycorrhizal colonisation (Table 3).

For mycorrhizal (inoculated) seedlings, percent mycorrhizal colonisation was negatively correlated with increasing P application. The P response curve (Fig. 3) showed that mycorrhizal colonisation decreased markedly from 0 mg l^{-1} P to 15 mg l^{-1} P, but that the rate of decrease slowed between 15 mg l^{-1} P and 30 mg l^{-1} P.

For non-mycorrhizal (uninoculated) seedlings, nodule dry mass was positively correlated to P application, whereas no relationship was found with mycorrhizal seedlings. The P response curve (Fig. 4) showed that nodulation of non-mycorrhizal seedlings increased up to P applications of 15 mg l^{-1} P, but did not increase at 30 mg l^{-1} P.

The strong positive relationship between seedling growth and nodulation was examined further by fitting separate nodule dry mass/shoot dry mass response curves for mycorrhizal and non-mycorrhizal seedlings (Fig. 5). ANOVA showed a significant difference ($P=0.004$) between these curves, which suggested that seedlings were more responsive to high levels of nodulation when they were mycorrhizal.

Table 3. Correlation coefficients (r) between growth, mycorrhizal colonisation and nodulation of *Calliandra calothyrsus* seedlings (n = 63)

	Mycorrhizal colonisation (%)	Nodule dry mass (mg)[a]
Stem diameter (mm)	0.323 *	0.745 ***
Stem dry mass (mg)	0.372 **	0.750 ***
Leaf dry mass (mg)	0.430 ***	0.811 ***
Leaf area (cm^2)	0.432 ***	0.739 ***
Shoot dry mass (mg)	0.424 ***	0.809 ***
Root dry mass (mg)	0.402 **	0.639 ***
Mycorrhizal colonisation vs. nodule dry mass	0.256*	

*, **, ***Significant at P<0.05, P<0.01 and P<0.001 respectively. [a]Square root transformations were performed on nodule dry mass data for analysis.

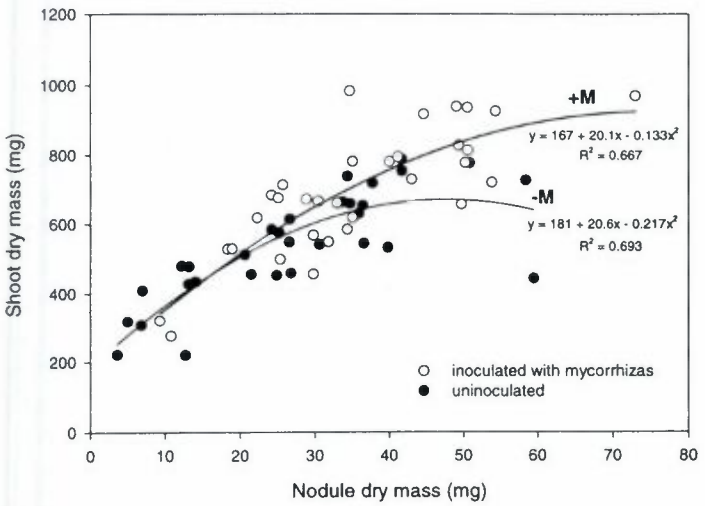

Figure 5. Fitted response curve of shoot dry mass of mycorrhizal (+M) and non-mycorrhizal (–M) *Calliandra calothyrsus* seedlings to nodulation (nodule dry mass).

4. Discussion

This study has shown that mycorrhizal inoculation benefits the growth of *Calliandra calothyrsus*, and supports the results of previous studies by Reena and Bagyaraj (1990), Desmond (1995) and Ibrahim et al. (1996). In our study

however, application of increasing levels of P reduced mycorrhizal colonisation and eliminated growth benefits attributable to mycorrhizal inoculation, whereas studies by Desmond (1995) and Ibrahim et al. (1996) showed that mycorrhizal inoculation only improved plant growth in P-deficient soils when P was applied. It is difficult to compare our study with that of Ibrahim et al. (1996) as mycorrhizal colonisation levels were not reported, while Desmond (1995) found much lower levels of mycorrhizal colonisation (1.5–18.4% after 13 weeks growth), which may have precluded any growth response to mycorrhizal inoculation alone. Our data would support this view and, had the 8-week growth period of our study been extended, colonisation levels and resulting growth benefits to plants growing at low P applications may have been much greater.

The results also show that the *C. calothyrsus* seedlings had a poor growth response to added P, which supports results obtained in field plots by Ndufa et al. (1999). This lack of response to added P and the concomitant low levels of AM colonisation suggests that, without adequate mycorrhizal colonisation, the *C. calothyrsus* seedlings were unable to take up sufficient P to maintain growth and therefore exhibited a high degree of mycorrhizal dependency.

The growth of mycorrhizal and non-mycorrhizal plants at 0 and 7.5 mg l^{-1} P indicates that, when available P was limiting plant growth, mycorrhizal plants were able to compensate for this. It is widely accepted that the main benefit plants receive from AM association is increased P uptake, and this suggests that the mycorrhizal *C. calothyrsus* seedlings were able to either prevent leaching of P from the pots or access normally unavailable P sources in the coconut fibre/sand substrate. At low P applications, mycorrhizal plants also increased leaf area and leaf dry mass more than stem or root growth. The enhanced leaf growth of mycorrhizal plants may have resulted in increased photosynthesis, carbon acquisition and greater allocation of carbon to the roots, which in turn may have stimulated AM colonisation and nodulation.

Even at high levels of P application (when available P was not limiting), mycorrhizal plants were able to maintain a positive growth response of seedlings to increasing nodulation whereas non-mycorrhizal plants were not. Kucey and Paul (1982) and Barea et al. (1987) found that N-fixation was greater in mycorrhizal than in non-mycorrhizal plants and it is possible that nodulation of the *C. calothyrsus* seedlings may have been generally ineffective to some degree and that N-fixation processes were stimulated by mycorrhizal plants. AM fungi are also known to significantly increase uptake of ammonium and trace elements such as copper and zinc (Smith and Read, 1997). Therefore at high P applications, mycorrhizal plants may have sustained plant growth by stimulating N-fixation and/or by directly supplementing N uptake.

This study has shown that mycorrhizal inoculation of *C. calothyrsus* increases plant growth in conditions of low P availability. The study also

shows that the species is largely dependent on AM fungi to sustain growth in soils of low fertility and will only respond to moderate applications of P. Inoculation with AM fungi will be effective and necessary if indigenous fungi in such soils are absent or ineffective. Current studies are examining the effectiveness of different AM fungi isolated from under *C. calothyrsus* growing in native and exotic locations. In due course effective isolates will be tested in combination with selected *Rhizobia* strains under nursery and field conditions (Lesueur, 2000).

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