LOCALIZED INCREASE IN NODULE ACTIVITY BUT NO COMPETITIVE INTERACTION OF COWPEA RHIZOBIA DUE TO PRE-ESTABLISHMENT OF VESICULAR–ARBUSCULAR MYCORRHIZA

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SUMMARY

Cowpea [*Vigna unguiculata* (L.) Walp.] plants were grown in a split-root system using a calcined montmorillonite clay (Turface) as the growth medium. At transplanting, either the vesicular–arbuscular mycorrhizal (VAM) fungus *Glomus macrocarpum* Tul. & Tul. and 100 mg hydroxyapatite (HAP) or 100, 200 or 400 mg HAP kg^{-1} without the VAM fungus were mixed into side 1 of the split-root system. Side 2 received only 100 mg HAP kg^{-1}. After 30 d, a combined inoculum of four *Rhizobium* isolates (TAL169, TAL173, TAL658 and IRC256) was applied to the surface of all pots. At harvest (60 d), root systems were separated from shoots, and nodule activity (acetylene reduction), plant dry weight, nitrogen (N) and phosphate (P) content and the percentage of VAM colonization were determined. There was no significant effect of treatment on either the N or P content of leaves or roots. The VAM fungus, however, significantly ($P < 0{\cdot}01$) increased shoot dry weight. Root dry weight and nodule activity were significantly increased only on the VAM side of the split-root system. Rhizobia from 30 nodules per half-root system were identified using fluorescent antibody techniques. Strain TAL173 was detected from 95% of the nodules and neither the VAM fungus nor P application affected competitive interaction by rhizobia for nodulation sites. Our work suggests the existence of a localized, non-systemic, non-P-mediated influence of a VAM fungus on root dry weight and nodule activity in cowpea.

Key words: *Vigna unguiculata*, vesicular–arbuscular mycorrhiza, *Rhizobium*, nodule activity, competitive interaction.

INTRODUCTION

The tripartite symbiosis between leguminous plants, *Rhizobium* spp. and vesicular–arbuscular mycorrhizal (VAM) fungi has been the subject of intensive research in recent years (Barea & Azcón-Aguilar, 1983). Generally, VAM fungi improve phosphate (P) nutrition of legumes which, in turn, enhances plant growth and N_2 fixation. The effects VAM fungi have on legumes can often be duplicated by increasing P availability to non-VAM plants (Carling *et al.*, 1978; Manjunath & Bagyaraj, 1984). However, physiological and morphological differences may occur betwen VAM and non-VAM plants even though their tissue P content is the same (Munns & Mosse, 1980; Kucey & Paul, 1982; Barea & Azcón-Aguilar, 1983; Pacovsky, Bethlenfalvay & Paul, 1986).

Most of the research directed toward legume–VAM–*Rhizobium* interactions has been concerned with development of the endophytes within roots and not the pre-infective interactions which may occur by direct or indirect means. It is known that a variety of factors influence the survival and nodulating abilities of rhizobia

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introduced into the soil (Freire, 1982; Eaglesham & Ayanaba, 1984). The ability of introduced rhizobia to compete for nodulation sites may be influenced by the growth rates of the bacteria (McLoughlin, Owens & Alt, 1985) but not necessarily by inoculum ratios (Franco & Vincent, 1976). Colonization of a root by a particular isolate of *Rhizobium* may also be affected by the activities of the resident microbial populations (Subba Rao, 1984). Nodulation and formation of mycorrhiza occur at the same time but the two endophytes probably do not compete for infection sites (Barea & Azcón-Aguilar, 1983). However, establishment of a VAM fungus within roots can alter microbial populations of the rhizosphere (Ames, Reid & Ingham, 1984; Meyer & Linderman, 1986) and this may affect distribution or development of nodules throughout the root system. If rhizosphere and rhizoplane populations of microbes are strongly affected by mycorrhizal root systems, then it is conceivable that the competitive interaction of introduced rhizobia for nodulation sites may also be affected. Little is known of mechanisms or techniques that can improve the ability of highly efficient strains of *Rhizobium* to compete with less efficient, but very competitive, indigenous populations. One such technique may be the use of VAM fungi to enhance the establishment of the desired rhizobia in roots and the soil.

Another aspect of legume–VAM–*Rhizobium* research which has not been addressed is whether the fungus can affect nodulation or N_2 fixation by *Rhizobium* only when the endophytes are in close proximity, or if a response occurs throughout the root system (systemic). This is important for understanding the distribution of rhizobia along the root system, and especially in regard to secondary (lateral root) nodulation. If we accept the theory that additional P can duplicate VAM-fungal effects, then a systemic response is probable because P is very mobile within plant tissues. Menge *et al.* (1978) demonstrated that, in a citrus split-root system, application of P to one side affected mycorrhiza formation on the opposite side. It is known that prior establishment of *Rhizobium* in one half of a soybean split-root system will inhibit nodulation on the other half (Kosslak & Bohlool, 1984) which further supports a systemic microbial response in plants. Bethlenfalvay, Brown & Stafford (1985) demonstrated a competitive interaction between a VAM fungus and *Rhizobium* when prior establishment of the fungus inhibited subsequent nodule development. In contrast to these studies, Schönbeck & Dehne (1979) proposed that increased resistance to root disease is localized at mycorrhizal sites with limited transmission of this effect within the root system and no transmission to the shoot. Also in a split-root system, the concentration of tobacco mosaic virus was found to be higher in VAM than non-VAM roots (Dehne, 1982). Therefore, there is evidence for either systemic or localized effects of VAM fungi on viruses or micro-organisms within roots. The localized or systemic response of rhizobia in legume root nodules to VAM fungal colonization has not been studied previously.

We conducted an experiment which addressed the following. (1) Can prior establishment of a VAM fungus influence the competitive interaction of rhizobia for nodulation sites? (2) Does pre-inoculation with the VAM fungus affect nodule activity? (3) Are responses of the introduced rhizobia to VAM formation localized or systemic? (4) Can VAM-mediated effects on nodulation be duplicated by increasing P availability to non-VAM plants?

Materials and Methods

Plants and growth conditions

Cowpea [*Vigna unguiculata* (L.) Walp., cv. California Blackeye no. 5222] seeds were purchased from Park Seed Co. (Greenwood, South Carolina, USA), surface-sterilized, and planted in a flat which contained a 1:1:1 mix of perlite: vermiculite:Turface. After 7 d, seedlings were removed, the tap root severed to induce lateral root formation, then replanted and grown for 2 more weeks. Growth chamber conditions were adjusted for a 14 h photoperiod (700 μmol m^{-2} s^{-1}, 30 °C, 50% RH) and 10 h night (22 °C, 70% RH). One-half-strength Ruakura nutrient solution (Smith, Johnston & Cornforth, 1983) was applied three times per week. After the initial 21 d growth period, seedlings were transplanted to plastic pots containing 1 kg of Turface (a granular, calcined montmorillonite clay). The Turface pH was adjusted to 6·8 with 3·4 g $CaCO_3$ kg^{-1}, and analysis revealed 6 mg NH_4-N kg^{-1}, 1 mg NO_3-N kg^{-1}, 0·1% OM and 34 mg P kg^{-1} (NH_4HCO_3-DTPA extracted). Phosphate was applied as hydroxyapatite (HAP) [$Ca_{10}(OH)_2(PO_4)_6$] at either 100, 200 or 400 mg kg^{-1}. The Turface, $CaCO_3$ and HAP were mixed together in a plastic bag prior to placement in each pot.

Each experimental plant was grown with its root system divided between two separate pots (side 1 and side 2). Side 1 consisted of one of four treatments: 100 mg HAP + VAM fungal inoculum (VAM + P_1) or 100, 200 or 400 mg HAP without the VAM fungus (P_1, P_2, P_4, respectively). The Turface in side 2 of all root systems received only HAP at the 100 mg kg^{-1} rate. There were six replications per treatment and plants were arranged in randomized complete blocks in the growth chamber. One-half-strength Ruakura nutrient solution minus P was applied (100 ml three times per week) to all pots. After the isolates of *Rhizobium* were applied (see below), N was reduced from 9·2 to 2·0 mM. Plants were grown for 60 d and watered as needed. Flower buds were removed as they developed since reproductive growth in legumes can result in an increase in nodule senescence and reduce N_2 fixation (Atkins, 1984).

Micro-organisms and inoculation

Pot culture inoculum of *Glomus macrocarpum* Tul. & Tul. was obtained from a potted strawberry [*Fragaria vesca* (L.) Duchesne cv. UC5] plant grown for 11 months. Thirty-nine cubic centimetres (52 g fresh weight) of inoculum, which contained 2540 spores, hyphae and root fragments, was mixed into the Turface of each pot of the VAM treatment at the time of transplanting. A microbial suspension was prepared by mixing 200 cm^3 of the VAM pot inoculum in 600 ml distilled water and passing the suspension through a 45 μm mesh screen twice. Ten millilitres were poured over the surface of all pots.

Isolates of *Rhizobium*, TAL169, TAL173, TAL658 and IRC256 were cultured on yeast-mannitol agar (YMA) or in yeast-mannitol broth (YMB), checked for purity and tested for their ability to nodulate cowpea grown in Turface in flasks plugged with cotton. Eight-day-old YMB cultures of all four rhizobia were sub-sampled to determine their populations by plate counting on YMA. A 200 ml sample was removed from each broth culture, combined in a sterile flask, and mixed on a magnetic stirrer for 30 min. Ten millilitres of the combined inocula of rhizobia were applied to the surface of each pot 30 d after transplanting to the split-root system. Cultures of *Rhizobium* TAL169, TAL173, TAL658 and IRC256 were determined to contain 9·7, 6·4, 11·3 and $6{\cdot}2 \times 10^8$ colony forming units per

millilitre, respectively, in the YMB cultures prior to mixing. Cowpea plants were not nodulated prior to inoculation with the rhizobia. This was determined by examining the root systems of extra plants and core samples removed from each pot.

Plant harvest, acetylene reduction and serology

Plant harvest was begun 60 d after transplanting. One or two complete blocks were harvested each day over a 4 d period. The root system of each pot was severed where the roots emerged from the stem. Shoot material (stem, petioles, leaflets) was oven-dried (70 °C, 48 h) and weighed. Root systems were shaken free of Turface without washing, sealed in 1 l jars, and nodule activity (acetylene reduction) determined according to standard methods (Bethlenfalvay *et al.*, 1985). Approximately 100 nodules were removed from the root system of each pot and stored at 5 °C in sealed vials containing a small amount of desiccant. A subsample of each root system was removed, stained (Phillips & Hayman, 1970) and assessed for the percentage of root length colonized by the VAM fungus (Marsh, 1971). The remaining nodules and root systems were oven-dried (70 °C, 48 h) and weighed.

Thirty randomly selected nodules from each pot (each half of all root systems) were assessed for the strains of *Rhizobium* they contained. The presence of IRC256 could be determined by the formation of very dark or piebald (part dark, part pink) nodules (Eaglesham *et al.*, 1982). All 30 nodules from each pot were checked serologically (Somasegaran & Hoben, 1985) for isolates of *Rhizobium* using antisera labelled with fluorescein isothiocyanate (FITC). Antisera were obtained from Mr H. Hoben, NifTAL Project, University of Hawaii. Strain IRC256 did not react with any of the antisera, nor were there cross-reactions between rhizobia and non-specific antisera. Background autofluorescence of nodule smears was minimal because of FITC-specific filtering of the Nikon epifluorescence system used.

Plant elemental analysis and statistics

Dried roots and leaves from each plant were ground in a Wiley mill to 60 mesh. Plant nitrogen (N) was determined by dry combustion on an ERBA model 1400 analyzer and P after the method of Allen (1940). An analysis of variance (ANOVA) was performed on the data to determine the significance of treatment effects. Paired *t*-tests were used to examine differences between the split-root systems within each treatment.

RESULTS

The VAM treatment had a significantly ($P < 0{\cdot}01$) greater shoot dry weight than did the other treatments (Table 1). None of the treatments differed significantly ($P > 0{\cdot}05$) in leaf N or P content. The VAM fungus significantly increased root dry weight and nodule activity on side 1 compared to side 1 of the non-VAM treatments (Table 2). There was no significant effect of treatment on root dry weight, N, P or nodule activity on side 2 (100 mg HAP only) comparisons of all treatments. Within the VAM-P_1 treatment, nodule activity was significantly ($P < 0{\cdot}01$) greater on side 1 (VAM) than side 2 (non-VAM). In the non-VAM treatments, P application to side 1 caused no differences between sides in any of the parameters measured. Due to the occurrence of numerous (> 300), small,

Table 1. *Shoot dry weight and leaf N and P content of cowpea* (Vigna unguiculata) *inoculated with* G. macrocarpum (*VAM*) *or grown at three rates of phosphate* (*hydroxyapatite, HAP*) *application*

Treatment	Shoot d. wt (g)	Leaf N (mg N g^{-1})	Leaf P (mg P g^{-1})
VAM-P_1*	22·07 a†	50·95 a	2·85 a
P_1	18·83 b	51·58 a	3·08 a
P_2	19·06 b	52·27 a	3·07 a
P_4	19·48 b	50·08 a	2·88 a

* P_1, P_2 and P_4 represent the addition of 100, 200 or 400 mg HAP kg^{-1} Turface, respectively, to side 1 of plants grown in a split-root system. Side 2 received 100 mg HAP kg^{-1} in all treatments. There were six split-rooted plants per treatment.

† Treatment means within columns not followed by the same letter are significantly ($P < 0·01$) different as determined by ANOVA.

Table 2. *Data obtained from cowpea* (Vigna unguiculata) *split-root systems treated with* G. macrocarpum (*VAM*) *or 100, 200 or 400 mg hydroxyapatite* (P_1, P_2, P_4, *respectively*) *per kilogram Turface* (*there were six replicates per treatment*)

Treatment*		Root d. wt (g)		Root N (mg N g^{-1})		Root P (mg P g^{-1})		C_2H_4 (μmol h^{-1} per pot)	
1	2	1	2	1	2	1	2	1	2
VAM-P_1	P_1	3·7 b	3·0 ab	31·1 a	31·0 a	2·9 a	2·7 a	38·0 x	17·7 y†
P_1	P_1	2·9 a	2·9 a	30·4 a	31·1 a	2·8 a	2·9 a	18·2 y	15·0 y
P_2	P_1	3·1 a	2·8 a	31·5 a	31·6 a	2·9 a	2·9 a	13·7 y	12·8 y
P_4	P_1	3·2 a	2·9 a	32·1 a	31·9 a	2·9 a	2·9 a	17·4 y	14·9 y

Treatment		Microbial abundance‡									
		VAM (%)		TAL169		TAL173		TAL658		IRC256	
1	2	1	2	1	2	1	2	1	2	1	2
VAM-P_1	P_1	33·2	0	0	0	29·7	30·0	0·5	0·7	0·3	0·0
P_1	P_1	0	0	0	0	29·8	29·3	0·0	0·0	1·3	1·8
P_2	P_1	0	0	0	0	29·3	30·0	0·8	0·5	1·0	1·0
P_4	P_1	0	0	0	0	29·0	28·8	0·8	1·0	1·2	1·3

* Treatments or data for side 1 (treated) or side 2 (100 mg hydroxyapatite only) of the cowpea split-root system.

† For each parameter within or across columns, treatment means not followed by the same letter are significantly (a, b, $P < 0·05$; x, y, $P < 0·01$) different.

‡ Incidence of isolates of *Rhizobium* (TAL169, TAL173, TAL658, IRC256) from 30 nodules examined per each half-root system.

closely packed nodules on each half-root system, we were unable to obtain accurate data on nodule mass or number.

VAM-fungal colonization of roots reached 33·2% on the VAM side of the VAM-P_1 treatment (Table 2) and no mycorrhizas were observed from non-inoculated pots. Nodulation by strain TAL173 dominated all root systems

regardless of fungal or P treatment. Strain TAL169 was not detected from nodules from any treatment. No significant ($P > 0{\cdot}05$) effect of the VAM fungus or P application was observed on the competitive interaction of rhizobia for nodulation within (side 1 *vs* side 2) or between treatments. Only 3·3 % of the 1440 nodules tested by the fluorescent antibody technique contained more than one strain of *Rhizobium*.

DISCUSSION

Preliminary tests showed that the isolates of *Rhizobium* used were effective in nodulating cowpea when inoculated individually into the Turface growth medium. Their effectiveness relative to each other was not determined. Testing of subsamples taken from the broth cultures of rhizobia before they were combined showed that the cultures were pure and serologically identical to the original stock cultures.

Neither *G. macrocarpum* nor P application produced a localized or systemic effect on the competitive interaction of rhizobia for nodulation. Strain TAL169 did not nodulate even though its population was higher than TAL173 and IRC256. However, Manjunath & Bagyaraj (1984) have used TAL169 successfully in a previous cowpea-VAM fungus study. In contrast, the inoculum population of TAL173 was nearly identical to IRC256 but lower than TAL169 and TAL658, yet it was identified from 95 % of the nodules examined. These results support the conclusion of Franco & Vincent (1976) that inoculum ratios alone are inadequate as a basis for predicting nodulation competitiveness by rhizobia.

Highly effective rhizobia may or may not be strongly competitive (Franco & Vincent, 1976). Although it would be worthwhile to study the influence of a VAM fungus on rhizobia of equal competitive abilities, it is important to know if even slight effects on nodule occupancy can occur with very aggressive rhizobia. Strain TAL173 was not altered in its competitive edge over the other rhizobia in response to *G. macrocarpum*, however, this result may change with a different host variety or soil type.

Turface can be used instead of soil as an excellent growth medium for potted plants (Plenchette, Furlan & Fortin, 1982). Since Turface does not strongly retain P or other nutrients, it is very useful for studies where frequent nutrient solution applications are desired (Plenchette, Furlan & Fortin, 1983a, b). Rather than preparing three different nutrient solutions, we applied HAP at three rates to the pots at planting. The HAP is not readily leached from the pot and provided a slow, soluble source of P to the plants. The amounts of HAP we applied were in excess of that required for optimum growth of soybean (*Glycine max* L.) (Bethlenfalvay, Bayne & Pacovsky, 1983). In the present study, the HAP provided enough P for good plant growth and excellent foliar and root P concentrations. In fact, the high plant P content was probably the cause of the low amount of mycorrhiza formation observed. Even though small, non-significant differences were found in foliar P concentrations, it should be pointed out that all the non-VAM treatments had higher levels of P in their leaves than the VAM plants. Since the non-VAM plant P concentrations were equal or slightly greater than the VAM plants, yet were much lower in their nodule activities (side 1 comparisons), this indicates to us that a non-P-mediated effect by the fungus on nodule activity occurred.

Cowpea has been shown to increase in N and P content and dry matter production after inoculation with VAM fungi (Sanni, 1976; Islam, Ayanaba & Sanders, 1980; Islam & Ayanaba, 1981a, b; Yost & Fox, 1982). It has been

proposed for cowpea (Manjunath & Bagyaraj, 1984) and other legumes (Carling *et al.*, 1978) that the effects of VAM fungi on increasing plant growth, nodulation and N_2 fixation are explained by enhanced P nutrition. Our study on cowpea does not support this explanation but is consistent with others who have suggested that VAM fungi have effects on legumes which are not duplicated by supplying additional P to controls (Munns & Mosse, 1980; Kucey & Paul, 1982; Barea & Azcón-Aguilar, 1983; Pacovsky *et al.*, 1986; Rajapakse & Miller, 1987).

Another important aspect of our study was the demonstration that *G. macrocarpum* had a localized, non-systemic effect on nodule activity. This is shown by the fact that side 2 (non-inoculated) of the VAM treatment had significantly less nodule activity than the VAM side. The proposal of Carling *et al.* (1978) that VAM fungi do not act directly with the bacteroids of *Rhizobium* may have to be modified to allow for a non-P-mediated interaction which occurs when nodules are in close proximity to areas of VAM colonization. Since we could not determine nodule number, size or weight (for reasons stated earlier), we cannot eliminate these as reasons for the higher nodule activity. Nitrogen fixation in cowpea is positively correlated with nodule fresh weight (Wadisirisuk & Weaver, 1985) and this, rather than nitrogenase efficiency in VAM plants, has been attributed to increases in N_2 fixation (Kucey & Paul, 1982). Since a complete nutrient solution was added three times each week, we do not feel that micro- or macro-elements were involved in the VAM fungal effects on nodule activity.

The slight but significant increase in root dry weight in the VAM root system may have resulted from altered source–sink relationships in addition to possible effects of nodule weight. Since nodulated-VAM root systems respire CO_2 at a greater rate than nodulated, non-VAM roots (Pang & Paul, 1980), more carbon may have been selectively shunted to the VAM half of the split-root system. Snellgrove *et al.* (1982) found that about 7% more carbon was translocated from shoots to mycorrhizal leek roots than from shoots to roots of non-mycorrhizal plants. Koch & Johnson (1984) obtained similar results in a citrus split-root system. This differential carbon movement may have supported the greater growth and nodule activity of the VAM cowpea root system, and this, in turn, may have stimulated the increased shoot growth in VAM plants.

Our work suggests that there is a much more complex and localized interaction between each component of the legume–VAM–*Rhizobium* tripartite system than previously known, and that this interaction cannot be attributed to plant P nutrition alone.

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