

Variation in the Response of Nodulating Pigeonpea (*Cajanus cajan*) to Different Isolates of Mycorrhizal Fungi

DAVID C. IANSON and R.G. LINDERMAN

*Oregon State University, Department of Botany and Plant Pathology, and
USDA-ARS, Horticultural Crops Research Laboratory, Corvallis, OR 97330, USA*
Tel. 503 750-8760, Fax 503 750-8764

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Abstract

Legumes form a tripartite symbiosis with diazotrophic bacteria and vesicular-arbuscular mycorrhizal (VAM) fungi. The interaction between these three symbionts is generally positive regarding growth and dinitrogen (N_2) fixation, but few studies have evaluated the interactions between a variety of the three microsymbionts. In this study, an effective N_2 fixing strain of the diazotrophic bacterium *Rhizobium* was evaluated alone or in combination with seven VAM fungi in terms of plant growth enhancement, nodulation and N_2 fixation on pigeonpea [*Cajanus cajan* (L.) Millsp. var. Corg-5]. Plants were fertilized weekly with a nutrient solution containing $40 \mu\text{g mL}^{-1}$ P and both VAM and non VAM plants had equal levels of tissue P. All plants were deprived of N fertilizer except the non VAM control which was fertilized weekly with a nutrient solution containing $220 \mu\text{g mL}^{-1}$ N. Measurements of plants harvested at 6 and 21 weeks yielded significant variation in VAM formation and in the effects of different VAM fungi on nodulation and N_2 fixation, but the level of VAM formation was not related to N_2 fixation. Some fungi formed extensive VAM, but did not affect nodulation; others formed low levels of VAM, yet greatly enhanced nodulation. These data suggest that a specific inter-endophyte interaction occurs between rhizobia and VAM fungi on pigeonpea that becomes apparent under N-limiting conditions, affects the process of nodulation, and is independent of P nutrition.

Keywords: *Cajanus*, N_2 fixation, nodulation, P nutrition, *Rhizobium*, vesicular-arbuscular mycorrhiza

1. Introduction

There are many reports on the interaction between vesicular-arbuscular mycorrhizal (VAM) fungi and *Rhizobium* (Cluett and Baucher, 1983; Barea and Azcón-Aguilar, 1985; Tilak, 1985; Linderman and Paulitz, 1989). The consensus is that VAM fungi influence the rhizobial symbiosis when plant-available soil P is limiting, resulting in enhanced nodulation (size and number) and N₂ fixation (Van Nuffelen and Schenck, 1984; Kawai and Yamamoto, 1986; Subba Rao et al., 1986; Ames and Bethlenfalvay, 1987). It has generally been assumed that the combination of VAM-fungal and rhizobial symbioses enhance plant growth more than with either symbiont alone. Many contend that growth enhancement when VAM are present is due to enhanced P or micronutrient uptake needed for N₂ fixation (Manjunath and Bagyaraj, 1984; Kawai and Yamamoto, 1986; Subba Rao et al., 1986). This is related to an increase in photosynthesis and photosynthate allocated to roots that have stronger C demand due to the presence of both symbionts (Kucey and Paul, 1982; Bayne et al., 1984; Brown and Bethlenfalvay, 1988; Brown et al., 1988). Others have observed that the tripartite interaction may not significantly increase photosynthesis and associated partitioning to the host roots (Brown and Bethlenfalvay, 1988).

Ames and Bethlenfalvay (1987) studied the tripartite interaction between the VAM fungus *Glomus macrocarpum* (Nicol. and Gerd.) and *Rhizobium* strains on cowpea [*Vigna unguiculata* (L.) Walp.] and demonstrated interactions that were not P-mediated. They used a split-root system to demonstrate a localized, nonsystemic increase in nodule activity in roots with both nodules and VAM. Although they used only one VAM fungal isolate and did not report nodule numbers or size (and thus cannot rule these out as affecting nodule activity), their work counters the view that improved P nutrition is the sole mechanism whereby VAM contribute to the enhanced N₂ fixation in the tripartite association.

Most research on *Rhizobium*-mycorrhiza interactions has been based upon a few VAM fungal isolates under differing environmental conditions and often with P-responsive hosts such as soybean [*Glycine max* (L.) Merr.] and cowpea (Hume et al., 1985; Skerman et al., 1988). However, different VAM fungal isolates have seldom been compared under uniform environmental conditions. Van Nuffelen and Schenck (1984) compared six isolates of VAM fungi on soybean, but they focused on fungal parameters (spore germination, penetration, and root colonization) rather than on effects of different VAM fungal isolates on nodulation and N₂ fixation. Inter-endophyte compatibility (Bayne et al., 1984), which may play an important role in the effectiveness of the overall

tripartite interaction, has received little attention. In an earlier experiment (Ianson and Linderman, unpublished data), we observed significant differences in the number and size of root nodules of plants inoculated with seven different VAM fungal isolates. Development of an effective N_2 -fixing association consists of a number of steps of which the induction of nodulation is only one (Vance, 1983). The VAM symbiosis could either directly or indirectly induce the expression of nodulation genes with no effect on N_2 -fixing capability of nodules, resulting in what Vance (1983) calls "ineffective nodules."

Our objectives were to see if VAM fungal isolates that increased nodule numbers in an earlier study also enhanced nodule activity and to compare VAM fungal isolates of different edaphic origin as to their ability to increase host growth and N_2 fixation.

2. Materials and Methods

Experimental design

Plants were completely randomized in accordance with a factorial design on greenhouse benches (22° C day, 18° C night) with 11 treatments: seven VAM fungal treatments [fertilized weekly with a modified N-free, low-P Hoagland's solution (130 mL)] and four non-VAM controls which were obtained by fertilizing non-VAM plants weekly (130 mL) with a modified Hoagland's solution (low-P) that varied in N content (100, 150, 200, or 300 μgmL^{-1} N as KNO_3). The series of non-VAM control treatments was used to screen for a single treatment similar in size and vigor to VAM treatments which would be used in further analysis. The decision to vary N in the non-VAM controls was made by observing in a previous experiment that varying P in non-VAM treatments did not result in plants equal in size to VAM plants and where non-VAM plants were observed to be chlorotic and lacking in N. Plants were maintained with supplemental lighting (high pressure sodium vapor lamps of PAR 250 $\mu\text{mol m}^2 \text{s}^{-1}$) for a 16 hr photoperiod. Plants for the first harvest were all inoculated with *Rhizobium*; plants for the second harvest were either inoculated or not with *Rhizobium*. Seedlings were transplanted into 84 m×84 mm×152 mm pots (volume 1080 cm^3). Plants for the first harvest were arranged in the greenhouse according to a completely randomized design and results were analyzed according to a One Way Analysis of Variance ($P > 0.05$, Ostle and Mensing, 1975). Where significance was detected, means were ranked and compared according to Fisher's Protected Least Significant Difference Test ($P < 0.05$, Ostle and Mensing, 1975). Plants for the second harvest were also completely randomized in the greenhouse and results

were analyzed according to a Multifactorial Analysis of Variance. Significant differences were compared as outlined above.

Microbial inocula

Seven VAM fungal isolates (6 morphospecies from 2 genera) were compared:

1. *Glomus etunicatum* (Becker & Gerd.) (*Get*) isolate – from Native Plants Inc., Salt Lake City, Utah. Origin – Florida.
2. *Glomus aggregatum/microcarpum* (Schenck & Smith)/Tul. & Tul.) (*Gmix*) isolates – from Lowell Young, USDA-ARS, Corvallis, Oregon. Origin – Oregon.
3. *Glomus deserticola* (Trappe, Bloss & Menge) [*Gd(C)*] isolate – from Lowell Young, USDA-ARS, Corvallis, Oregon. Origin – Southern California.
4. *Glomus mosseae* (Nicol. & Gerd.) (*Gm*) isolate – from Native Plants Inc., Salt Lake City, Utah. Origin – probably southern California.
5. *Gigaspora margarita* (Becker & Hall) (*Gmar*) isolate – from Native Plants Inc., Salt Lake City, Utah. Origin – probably Illinois.
6. *Glomus intraradix* (Schenck & Smith) (*Gi*) isolate – from Native Plants Inc., Salt Lake City, Utah. Origin – Florida.
7. *Glomus deserticola* (Trappe, Bloss & Menge) [*Gd(U)*] isolate – from Native Plants Inc., Salt Lake City, Utah. Origin – probably southern California.

Each VAM fungal inoculum consisted of spores, root pieces and hyphae in a sandy loam soil harvested from a previous experiment. Spore numbers were determined for each inoculum based on a saturation of infection level and each was diluted with sand to give 1500 spores per pot. The inoculum was localized in a column around the transplanted seedling and extending to the bottom of the pot. The bacterium used was a *Rhizobium* Cowpea strain (P132-1 Arhar) isolated from pigeonpea [*Cajanus cajan* (L.) Millsp. var. Corg-5] obtained from Dr. C.S. Singh (Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India). The bacteria were cultured at 30° C in Yeast Mannitol Broth (YMB) for 4 days before inoculation of plants.

Plant and soil preparation

Pigeonpeas were germinated in sterile distilled water at 27° C for 4 days and the germlings dipped into the YMB *Rhizobium* culture. The number of colony-forming bacteria per germling was estimated by dilution plating the bacteria washed from 5 inoculated germlings on Yeast Mannitol Agar (YMA) plates

incubated in the dark at 30° C for 5 days. The average number of bacteria was 2.547×10^7 germling⁻¹.

The soil used in this experiment was a 1:1 mixture of Willamette sandy loam (pH 6.) and river sand which contained: 0.02% total N, 12 mg kg⁻¹ P, 70 mg kg⁻¹ K and 8.5 me Ca per 100 g of soil. To reduce microfloral differences between non-VAM controls and VAM plants, the control inoculum was prepared from a *Gigaspora margarita* pigeonpea pot culture sand/soil medium that proved beneficial to rhizobial symbiosis in a previous pigeonpea experiment. The VAM fungal component of this inoculum was removed by air-steam pasteurization at 70° C for 30 min. The pasteurized medium was inoculated with microflora (other than VAM fungi) in a soil extract prepared by filtering nonpasteurized *Gigaspora* sand/soil medium (10% by volume of the total non-VAM control inoculum to be used) through Whatman #1 filter paper to retain VAM fungal propagules and yet let other rhizosphere microflora pass. The filtrate was mixed into the pasteurized medium and allowed to incubate, in order to increase populations of indigenous microorganisms, in the greenhouse for 10 days (Meyer and Linderman, 1986a). Plants were fertilized weekly with a N-free Hoagland's solution beginning at the first trifoliolate leaf stage. Iron was provided as Fe citrate (3.6 µg mL⁻¹ Fe).

First harvest

The first plants were harvested at 6 weeks. Roots and shoots were separated, and roots were reserved for assessment of N₂ fixation, VAM formation, nodulation, and dry weights. The root samples were divided into 2 groups of 10 replicates each. Nitrogen fixation assays, root dry weights, and nodule fresh weights were carried out on the first group; mycorrhizal formation was estimated on the second group. Plant shoots were dried at 60° C for 48 hr and weighed.

N₂ fixation and Rhizobium nodulation

Biological N₂ fixation (nodule activity) was estimated by the C₂H₂-reduction assay (ARA). At harvest, intact pigeonpea roots were blotted dry (to touch) and placed in 250 ml test tubes sealed with a rubber stopper. Ten percent of the tube gas volume was immediately removed by syringe and replaced with purified acetylene. After mixing (1 min), 1 cc of the gas was removed by syringe and sealed by sticking the needle into a rubber stopper. This first volume was used as a time-zero control. Tubes were incubated by placing them horizontally in an insulated box at room temperature. After 1 hr, a second 1 cc gas sample was withdrawn from the assay tube. Both samples

were analyzed for ethylene by injection into a Hewlett Packard HP5830 gas chromatograph. Quantification was by comparison with a known ethylene-in-air sample. Following the acetylene assay, root samples were spread over a grid and nodules ($> 500 \mu\text{m}$) were counted and fresh weights determined.

VAM fungal colonization

Whole fresh root samples (10 replicates) were blotted dry, weighed and cut into 0.5 to 1.0 cm segments. Segments were cleared overnight at 55°C in 10% KOH, stained in trypan blue (0.05%) in lactoglycerol (Phillips and Hayman, 1970), and assayed for mycorrhizal formation (Biermann and Linderman, 1981).

Second harvest

At 21 weeks, a second group of plants was harvested. Root and shoot dry weights, nodule weights, numbers, and nitrogenase activity, and VAM formation were assayed. Colonization was assayed on root subsamples from each plant. Shoot dry weights, nodule weights, numbers, and activity were assayed as previously outlined. P content of nodule tissue was determined colorimetrically according to Aziz and Habte (1987). Color was developed according to the molybdenum blue technique (Murphy and Riley, 1962).

3. Results

The influence of VAM fungal inoculation on root and shoot mass was similar for both harvests, in that inoculation by some VAM fungal isolates (i.e., *G. margarita* and *G. intraradix*) resulted in significantly larger shoots than inoculation by the other isolates (Figs. 1A,B). The difference in shoot weight was more pronounced at harvest, one with three distinct size groups (Fig. 1A). Differences in root weight were not as large as with shoots, except in the non-VAM control at 6 weeks (Fig. 1C). The group of non-VAM control plants fertilized with a solution containing $200 \mu\text{g mL}^{-1} \text{N}$ were the most uniform in size among the four control treatments and were the most similar in size to plants inoculated with VAM fungi. These were the plants chosen for further analysis. Adding $200 \mu\text{g ml N}$ to the non-VAM controls resulted in early root growth but may have delayed nodulation and N_2 fixation (Figs. 1C,2A). Nodules on plants in that treatment were consistently small, but by 21 weeks their ethylene production values were the highest of all treatments (Fig. 2B).

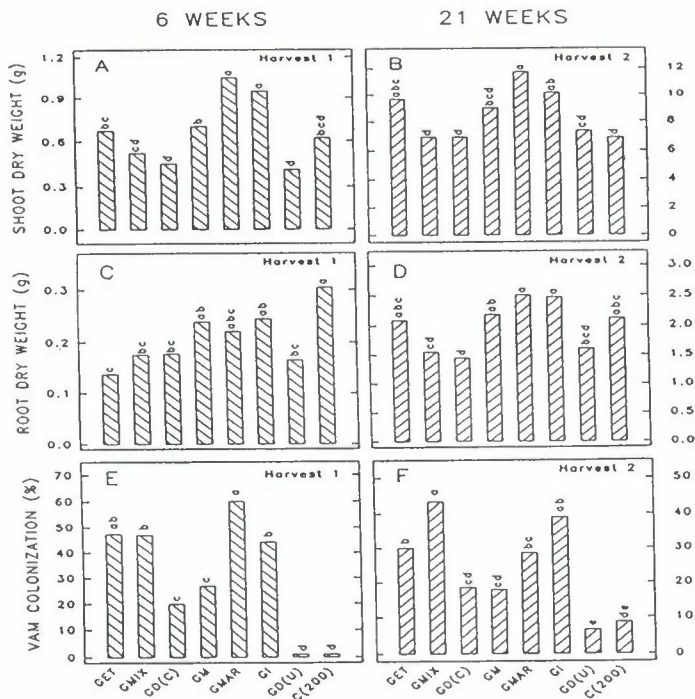


Figure 1. Shoot (A,B) and root (C,D) dry weights and VAM fungal colonization (E,F) for pi-gonpea as influenced by VAM fungal treatment. Means within a harvest and with common letters are not significantly different ($P < 0.05$). GET = *G. etunicatum*, GMIX = *G. microcarpum/aggregatum* mix, GD(C) = *G. deserticola* (California), GM = *G. mosseae*, GMAR = *G. margarita*, GI = *G. intraradix*, GD(U) = *G. deserticola* (NPI, Utah), and C(20) = non-inoculated control amended with 200 μg ml N as KNO_3 . The non-inoculated control was selected from four amended non-inoculated control treatments due to its uniformity in size with VAM treatments.

Number and ethylene production by nodules on plants inoculated with *G. intraradix* were high even through the second harvest, indicating that nodules remained relatively active regardless of their size (Figs. 2A-D, 3).

Inoculation of plants with the other VAM fungi resulted in the production of nodules of various sizes (Figs. 2C,D, 3), but by the second harvest, most of these, except for those inoculated with *G. deserticola* (U) were less active, regardless of mass (Fig. 2B). In general, nodule activity increased with increasing plant size at the first harvest (Figs. 1A-D, 2A,B) and with the exception of *Gi* acetylene reduction, did decrease with increases in shoot weight at the second harvest (Fig. 1B, 2B). Inoculation with either isolate of *G. deserticola* resulted in relatively small nodules, but their effects on nodule activity in the

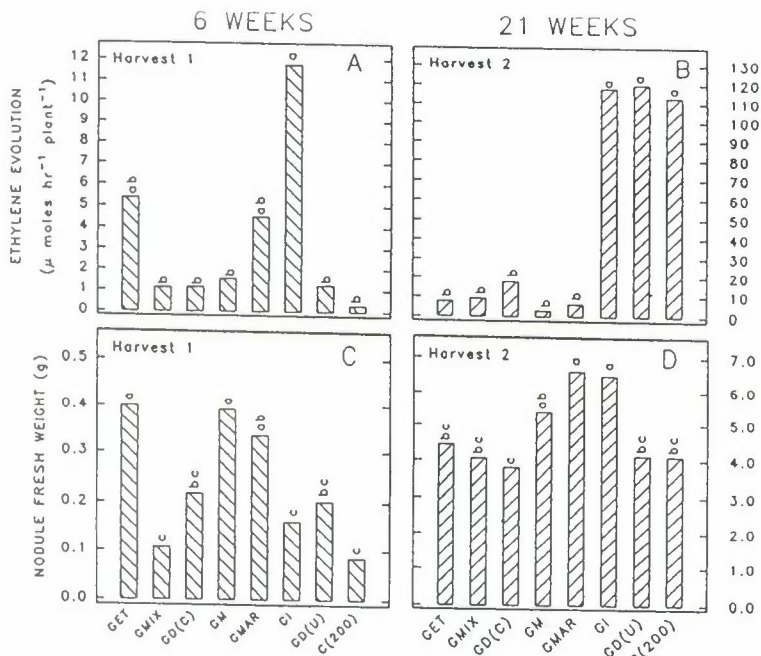


Figure 2. Mean ethylene (C_2H_4) evolution values (A,B) and nodule fresh weight (C,D) for pigeonpea as influenced by VAM fungal treatment. Means within a harvest and with common letters are not significantly different ($P < 0.05$).

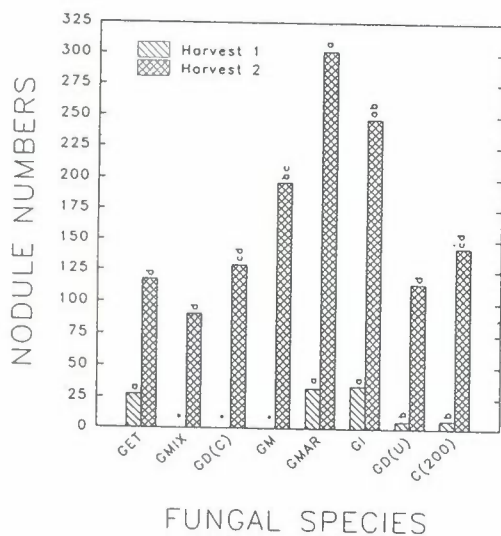


Figure 3. Mean nodule numbers for pigeonpea as influenced by VAM fungal treatment. Means with common letters are not significantly different ($P < 0.05$). * indicates missing values.

ARA were dissimilar. At the first harvest, *Gd(U)* and *Gd(C)* reduced similar amounts of acetylene, but *Gd(U)* nodule activity was strikingly increased by the second harvest, while that of *Gd(C)* was not (Table 1, Figs. 2A,B). Nodule activity also increased slightly with VAM fungal colonization early in the experiment (correlation coefficient (CC = 0.550, $R^2 = 0.30$) but decreased with increased colonization by the second harvest (CC = -0.37, $R^2 = 0.13$) (Figs. 1E,F, 2A,B). The appearance of some VAM in the non-inoculated plant roots was probably due to VAM fungal propagules passing through the filtering process. No significant differences were detected ($P < 0.05$) in the analysis of nodule P content on plants from the various VAM treatments.

4. Discussion

For legumes and rhizobia to form and maintain nodules capable of fixing N_2 , certain criteria must be met. Sprent (1989) listed a number of steps involved, including those external and internal to the host plant. Failure to meet these criteria whether due to genotypic incompatibility between rhizobia and the host or to nutritional stress on the symbiosis, results in decreased N_2 fixation and host growth. The tripartite interaction between VAM fungi, *Rhizobium*, and a host legume is more complex than the dual symbiosis between *Rhizobium* and host, and is less-well understood. Variations in VAM fungal isolate interactions with rhizobia on pigeonpea demonstrated in this study suggest even greater complexity. Some isolates supported the hypothesis that adding VAM fungi to the *Rhizobium*-legume symbiosis results in greater host growth, more and larger nodules, improved N_2 fixation, and increased VAM formation, while others did not. Others showed increased shoot dry weight by inoculating with isolates of *G. mosseae* (Smith and Daft, 1977; Asimi et al., 1980; Van Nuffelen and Schenck, 1984), while we did not. Van Nuffelen and Schenck (1984) observed that inoculating with *G. margarita* and *G. etunicatum* did not produce significant increases in shoot dry weight of soybean, and inoculating with *G. intraradix* increased shoot dry weight in one experiment and not in another. We, however, observed that inoculating with all three of these isolates significantly increased shoot dry weight of pigeonpea as compared to the non-inoculated control (Figs. 1A,B).

Compatibility within the tripartite symbiosis was also expressed in terms of *Rhizobium* colonization and nodule formation (numbers), growth, and function (nitrogenase activity). Some VAM fungal treatments significantly increased number and weight of nodules, and some decreased nodulation, compared to the non-VAM control (Figs. 2C,D, 3). Increased nodulation as affected by some VAM fungal isolates confirms many previous observations (Smith and

Table 1. Comparisons between two isolates of *Glomus deserticola*

	Shoot dry weight (g)	Sig. level	Root dry weight (g)	Sig. level	VAM%	Sig. level	N ₂ fixa- tion	Sig. level	Nodule fresh weight (g)	Sig. level	Nodule number	Sig. level
Harvest 1												
	Gd(U)	0.407	0.63	0.166	0.73	1.2	0.0009	1.22	0.87	0.81		
	Gd(C)	0.451	0.63	0.177	0.73	19.9	0.0009	1.14	0.87	0.81		
Harvest 2												
	Gd(U)	7.33	0.78	1.590	0.76	6.5	0.03	119.82	0.07	0.82	114.73	0.66
	Gd(C)	6.93	0.78	1.43	0.76	18.5	0.03	17.83	0.07	0.82	128.76	0.66

Daft, 1977; Smith et al., 1979; Varma, 1979). Decreased nodulation exhibited by some VAM fungal treatments, however, is not as commonly reported but does confirm observations by Bethlenfalvay et al. (1985).

These differential responses may be explained on the basis of mycorrhiza-induced physiological changes (or lack thereof) in the host, or in microbial composition and activity in the rhizosphere or rhizoplane. Linderman and Paulitz (1990) point out that VAM fungi and *Rhizobium* could directly interact in the rhizosphere in ways that may encourage *Rhizobium* to successfully infect root hairs. There is currently no experimental evidence to support this.

It is assumed that VAM fungi colonize roots before rhizobia and thereby alter the quantity and quality of root exudates available to *Rhizobium* in the rhizosphere. Among the constituents of these exudates are isoflavonoid compounds (Morandi et al., 1984), which have been reported to induce *nodD* gene expression that in turn regulates transcription of other nod genes (e.g. *nodA*, B, and C). Ultimately this would regulate the entire nodulation process (Phillips et al., 1988; Rolfe and Gresshoff, 1988; Sadowsky et al., 1988; Wijffelman et al., 1988). Sometimes these compounds chemotactically attract rhizobia (Caetano-Anollés et al., 1988). Alteration of root exudation by VAM could also favor certain groups of the rhizosphere microflora like pseudomonads that may interact with VAM fungi, rhizobia, and the host, allowing more initial rhizobial infections to proceed to nodulation. The presence of pseudomonads in the rhizosphere often increases nodulation (Grimes and Mount, 1984; Meyer and Linderman, 1986a), and VAM fungal colonization often selectively favors pseudomonad growth (Meyer and Linderman, 1986b). Certain VAM fungal isolates could alter root exudation patterns such that *nodD* gene expression is induced, possibly by enhancing the growth of "helper" microorganisms like pseudomonads, whereas others would not, or do so to a lesser degree. By these means, some VAM fungal isolates may exhibit more compatibility with the *Rhizobium*-legume system than others.

Colonization by some VAM fungi could delay a plant defense response and thus allow more rhizobial infections to proceed to nodule initiation. This could explain the significant differences in nodulation that we observed (Figs. 2C-D, 3). Researchers have demonstrated with split root systems that prior inoculation with one strain of *Rhizobium* results in a systemic plant response that inhibits subsequent nodulation by other *Rhizobium* (Singleton, 1983; Kosslak and Bohlool, 1984; Sargent et al., 1987) or in establishment of pathogens like *Fusarium* (Chakraborty and Chakraborty, 1989).

Once nodules begin to form within root tissues, their size and activity are probably closely related to host nutritional status. The balance between the

efficiency of nutrient uptake by VAM fungi and the cost to the host of maintaining a tripartite symbiosis could dictate the extent of VAM fungal colonization as well as the extent of nodule development and N_2 fixation. Differences in the efficiency of P uptake by certain VAM fungi would affect photosynthetic rates in the plant and ultimately the amount of photosynthate partitioned to the roots for the development and maintenance of *Rhizobium* nodules and mycorrhizal symbiosis. This might explain the variation in nodule weight, N_2 fixation and VAM fungal colonization when plants were inoculated with different VAM fungi (Figs. 1E,F, 2A-D, 3). Further evidence of the importance of balance between carbon cost and nutrient uptake as influenced by VAM fungi was seen in the drop from the first to second harvests in N_2 fixation and VAM formation in all fungal treatments except *G. deserticola* (U) and *G. intraradix* (Figs. 1E,F, 2A,B). At second harvest, plants were between flowering and seed set. The partitioning of C away from the root and both symbionts to the developing reproductive system may have deprived them, forcing nodule senescence and reducing N_2 fixation. In addition, it may have slowed VAM fungal spread in relation to root growth. This C drain could have been ameliorated more by compatible than incompatible VAM fungal isolates. This speculation is supported by the lack of a good relationship between nitrogenase activity and shoot and root dry weights (Figs. 1B,C, 2B).

In addition to C as a nutrient, the uptake of elements other than P, such as Cu and Zn, (essential to nodulation) could be preferentially affected by certain VAM fungal isolates. The uptake of these elements has been documented to increase with VAM formation (Pacovsky, 1986). The differences in nodule weight and activity observed in this study (Figs. 2A-D) with differing VAM fungal isolates may be due to the uptake of these essential elements.

Understanding that there are varying degrees of synergism involved in the interaction between VAM fungi, *Rhizobium*, host plant and the environment, and the role this plays in the rhizosphere is important and is the basis for much-needed further research. This variance may be important at both an inter- and intra-specific level. Although their effects on plant and nodule growth were the same, different isolates of *G. deserticola* affected nodule activity differently later in the experiment (Table 1, Fig. 2B). Plant growth and nodule activity, when viewed in light of the extent of VAM fungal colonization, suggest that one *G. deserticola* (U) isolate was more efficient than the other. This confirms the hypothesis of Stahl and Christensen (1990) and Morton (1990) that populations of a given VAM fungal species from dissimilar environments may be genetically different races or clones.

The results of this study suggest a need to consider this variability in re-evaluating our knowledge of VAM systems. From an applied aspect, combining

compatible symbionts with specific legume hosts for unique environmental conditions can enhance host establishment and survival under conditions of low or unavailable P and/or N. From the interest of basic research, our understanding of varying degrees of synergism involved in the VAM fungus-*Rhizobium*-host interaction provides a starting point for research on possible mechanisms of the interaction, exploiting the fact that two different VAM fungal isolates can colonize legumes, one enhancing N₂ fixation, the other not.

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