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David C. Ianson for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on December 21, 1990.

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Multifactorial experiments were conducted to document variation in plant growth and development as influenced by variation between VAM isolates and placement and density of inoculum in relation to host root development patterns. Experiments also were conducted to document variation in response of pigeon pea (Cajanus cajan) to dual inoculation with an effective nodulating Rhizobium and different VAM fungal isolates regarding enhanced N_2 fixation and drought tolerance.

Localizing and increasing inoculum density correlated well with enhanced VAM colonization and plant growth at early stages in the experiments. Degree of plant benefit, in most instances, varied with VAM fungal isolate as well as host species, and, in the case of pigeon pea, the addition of a compatible Rhizobium strain. In the N_2 fixation experiment, VAM colonization and effects on nodulation and N_2 fixation varied between fungal isolates, but level of colonization was not correlated with enhancement of N_2 fixation. Some VAM fungal isolates formed extensive VAM but effected nodulation little; others

colonized little yet dramatically enhanced nodulation. Generally where N_2 fixation was greatly enhanced, plant growth was also enhanced. Growth enhancement was largely independent of P fertilization. Non-VAM, Rhizobium-inoculated plants formed few nodules. In the drought study, plant growth varied in relation to enhanced nodulation and N_2 fixation under N limiting conditions. Induced drought tolerance varied between plants inoculated with different VAM fungal isolates but of equal size and tissue P. A correlation was observed between amount of external hyphae and the capacity of a VAM fungus to increase drought tolerance.

Localizing inoculum in relation to root morphology proved to establish VAM more rapidly with resultant plant growth responses. Localizing placement also allowed for the use of less inoculum. Additionally, legumes such as pigeon pea must be inoculated with Rhizobium and compatible VAM isolates to maximize nodulation and N_2 fixation. VAM isolates that enhance N_2 fixation, however, were not necessarily the best at increasing drought tolerance.

Variation in Plant Response to Inoculation with Different Isolates of Vesicular Arbuscular Mycorrhizal Fungi

by

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"OBESA DOMINA CANTAVIT"

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VARIATION IN PLANT RESPONSE TO INOCULATION WITH DIFFERENT ISOLATES OF VESICULAR ARBUSCULAR MYCORRHIZAL FUNGI

INTRODUCTION

Vesicular-arbuscular (VA) mycorrhizal fungi are indigenous to soils world-wide, and in fact, have been recently reported in the sub-antarctic (Smith and Newton, 1986). These fungi enhance the growth of many plants, largely through increased uptake of nutrients and water (Safir et al., 1971, 1972; Cooper and Tinker, 1978; Allen, 1982; Allen and Boosalis, 1983; Ames et al., 1983; Tinker and Gilden, 1983). In addition, mycorrhizae influence the quantity and quality of other rhizosphere organisms, some of which are known plant growth enhancers, and others are biocontrol agents (Meyer and Linderman, 1986a, 1986b).

Natural and man-made environmental disturbances reduce the number and efficacy of VA-mycorrhizal fungus propagules (Moorman and Reeves, 1979; Nemec, 1980; Allen and Allen, 1980; Gould and Liberta, 1981; Menge, 1982; Carpenter et al., 1982; Hayman et al., 1982; Allen et al., 1984). In addition, monoculture cropping systems common in major food producing nations often rapidly and dramatically alter soil microflora and disturb soil structure and thus soil water-holding capacity. These disturbances reduce plant survival and development in cultivated soils.

In "developed" countries we have ameliorated negative effects caused by disturbances and enhanced plant growth through the wide-spread use of nitrogen (N) and phosphorus (P) fertilizers, irrigation

and pesticides. The wide-spread, extravagant, and often inefficient use of N and P fertilizers is no longer economically or environmentally sound as both are based on non-renewable resources such as fossil fuels and rapidly vanishing P reserves (Cathcart, 1980; Newton and Burgess, 1983). The problem in "underdeveloped" countries is more basic in that many cannot afford the luxury of chemical amendments and must depend on nutritionally deficient soils to sustain plant growth (Mukerji and Kapoor, 1986). Under either agricultural system re-establishing VA-mycorrhizal colonization and enhancing the growth of associated organisms like free-living and symbiotic N_2 fixing bacteria could prove beneficial to host survival, growth and development. In addition it would lessen the need for N and P fertilizer input.

The time that may elapse for roots of annual plants to contact VAM propagules may delay development of significant levels of VAM and thus potential benefit from the symbiosis. Earlier establishment of mycorrhizae can be achieved by acknowledging and understanding isolate variation and by manipulation of inoculum in relation to the morphological root pattern. In legumes, which form a dual symbiosis with Rhizobium and VA-mycorrhizal fungi, isolate variation may also influence the establishment and function of Rhizobium and thus the amount of N fixed. A compatible combination of the two organisms would help plants tolerate environmental stresses including nutrient deficiency and drought. Accordingly, the objectives of these investigations were twofold: (1) to test the hypothesis that optimization of VA-mycorrhizal isolate, inoculum density and

placement in relation to root development patterns would increase colonization and the potential for host plant benefit; and (2) to document variation in response of a legume (pigeon pea) to dual inoculation with an effective Rhizobium and different VAM fungi regarding enhanced N_2 fixation and drought tolerance.

CHAPTER 1

INFLUENCE OF INOCULUM PLACEMENT AND DENSITY ON MYCORRHIZAL FUNGUS COLONIZATION AND GROWTH RESPONSE OF WINTER WHEAT (*Triticum aestivum*), GREEN PEPPER (*Capsicum annuum*), WESTERN RED CEDAR (*Thuja plicata*), AND PIGEON PEA (*Cajanus cajan*)

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SUMMARY

Compatibility of the host plant and VA-mycorrhizal fungus isolate and optimization of inoculum density and placement in relation to root development pattern were hypothesized to increase the potential for host plant growth. To test this hypothesis, inoculum densities (0, 100 and 200 spores ml^{-1}) of *G. intraradices* and *G. deserticola* were differentially placed (banded layer, a central column below the seed, or dispersed throughout the test soil) in containers seeded with winter wheat or green pepper. Pepper and wheat growth was greater when inoculum of *G. deserticola* but not *G. intraradices*, was localized in a column rather than dispersed. In another experiment, inoculum densities (0 or 100 spore ml^{-1}) of *G. intraradices*, *G. deserticola*, and two native inocula (one from a

forest nursery and one from a native old growth stand of western red cedar) were distributed in containers as in the first experiment. Into these pots were transplanted western red cedar seedlings. As in the first experiment, western red cedar was colonized and grew better in response to localized than to dispersed inocula, and grew better in the presence of either of the native inocula than G. intraradices or G. deserticola. In another experiment, inoculum densities (2.5, 5.0 or 10.0 spores ml^{-1}) of seven different VAM fungal isolates were placed in a central column beneath pigeon pea seedling transplants. In addition half of the plants were inoculated with Rhizobium compatible to pigeon pea. Initially, VAM fungus colonization and increased pigeon pea biomass were differentially affected by inoculum isolate and density, but later in the experiment the interaction between VAM fungus isolate and the presence of a compatible Rhizobium affected growth the most, with some VAM-Rhizobium combinations stimulating an increase in biomass more than others.

Thus selecting compatible VAM fungus isolates, choosing an appropriate inoculum density and localizing inoculum to optimize contact with roots results in earlier mycorrhization and more uniform growth enhancement.

INTRODUCTION

Vesicular-arbuscular mycorrhizal (VAM) symbiosis is essential in natural, undisturbed ecosystems where nutrient deficiency due to low soil nutrient content or nutrient unavailability would otherwise limit plant growth. This is especially true in soils of low or

unavailable phosphorus such as those found in the arid and semiarid tropics. In comparing natural-undisturbed to disturbed sage communities on Colorado oil shale lands, Reeves et al. (1979) found that 99 percent of the plants in the undisturbed ecosystem were VA-mycorrhizal fungi, whereas in nearby disturbed sage communities only 1 percent were mycorrhizal. Similar observations on the dominance of mycorrhizal plants in undisturbed communities have been made by Miller (1979) and Call and McKell (1982). In addition "about 95 percent of the world's present species of vascular plants belong to families that are characteristically mycorrhizal" (Trappe, 1987). The general hypothesis is that in natural undisturbed ecosystems mycorrhizal symbiosis is the rule, not the exception.

Whereas a primary value of mycorrhizal symbiosis in natural systems is enhanced nutrient uptake, its value in cultivated agricultural or horticultural systems may be in effects other than nutritional. The major benefits of VAM may be reduction of plant response to drought stress, transplant shock and other induced physiological responses that affect the overall success of plant establishment and vigor in a new environment. In addition the interaction of VAM with other potentially beneficial organisms such as Rhizobium may have a profound impact on agricultural systems, especially in countries where nitrogen fertilizer is unavailable or prohibitively expensive, and soils are low in available P.

In field soils, natural, evenly distributed populations of VAM fungi may be relatively low or concentrated in one area of the soil, such as a former root channel, and absent in the interspace. The

advantages of even distribution of VAM propagules to plant root growth have been documented. Abbott and Robson (1984) in studies conducted with Medicago truncatula observed a greater weight of mycorrhizal roots when Glomus sp. inoculum was evenly distributed in soil, but also observed that the initial rate of mycorrhiza formation and increased root weight were greatest when inoculum was localized in a band. These data demonstrate (when inoculum is dispersed in a container) that the chance of roots contacting an infective propagule increases as roots proliferate, but that localizing inoculum results in the earliest VAM establishment and plant growth benefit.

The time that may elapse for roots of annual plants to contact VAM propagules may delay the development of significant VAM and thus potential benefit from the symbiosis. The earlier establishment of mycorrhizae resulting from the placement of inoculum in relation to the morphological root pattern could result in increased potential benefits to plant growth and crop yield. The hypothesis that the optimization of host-compatible VAM fungi inoculum density and placement (as well as the use of a compatible isolate(s)) in relation to root development patterns would increase the potential for growth benefits was tested in three factorial experiments: the first with green pepper (Capsicum annuum (L.) var Early Bountiful) and winter wheat (Triticum aestivum (L.) var Tyee); the second with western red cedar (Thuja plicata (Donn.); and the third with pigeon pea (Cajanus cajan (L.) Millsp. var Corg-5). The pigeon pea experiment also evaluated the interaction between Rhizobium and VAM fungal isolates.

MATERIALS AND METHODS

EXPERIMENT ONE: VAM FUNGUS INOCULUM ISOLATE, PLACEMENT, AND DENSITY ON GREEN PEPPER AND WINTER WHEAT

Microbial Inoculum

Commercial inoculum of the mycorrhizal fungi Glomus deserticola Trappe, Bloss and Menge and G. intraradices Schenck and Smith were obtained from Native Plants Inc., Salt Lake City, UT and spores were separated (Allen et al., 1979) and counted. On the basis of the spore counts, the inocula were diluted with a 1:1 mixture of Willamette sandy loam to give three concentrations: non-VAM control (washings from the commercial inocula); 100 and 200 spores ml^{-1} of inoculum. To reduce microfloral differences between controls and VAM-inoculated plants, a control inoculum was prepared by removing the VAM component from the control soil medium by air-steam pasteurization at 70 C for 30 min. The pasteurized medium was inoculated with microflora (other than VAM) prepared by filtering nonpasteurized sand/soil medium of each VAM fungus isolate (10% by volume of the total control inoculum to be used) through Whatman #1 paper to retain VAM 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).

Plant and Soil Preparation

The soil medium used in this experiment was a 1:1 mixture of Willamette sandy loam (pH 6.0) and river sand which contained 0.02% total nitrogen, 10 mg kg^{-1} phosphorus, 74 mg kg^{-1} potassium, and 6.3 mequiv. calcium per 100 g of soil (Soil Testing Laboratory, Department of Soil Science, Oregon State University). Diluted inoculum was: layered or banded at 2.5 cm deep; placed in a central core below the seed, or dispersed throughout one liter pots (84 mm x 84 mm x 152 mm (volume 1080 cm^3)) (Fig. 1-1). Pots were seeded with winter wheat (Triticum aestivum (L.) var Tyee) or green pepper (Capsicum annuum (L.) var Early Bountiful).

Experimental Design

Pots were completely randomized on greenhouse benches with 18 fungal treatments (two VAM fungus isolates by three inoculum placements by three densities) per plant species. Ten replicates were planted for each fungal treatment.

Plant Nutrition

Beginning at two weeks after sowing (appearance of first true leaves on green pepper) plants were fertilized weekly with LANS (Long Ashton Nutrient Solution) modified by reduced phosphorus (P) (11 ppm P as NaH_2PO_4) to promote VA-mycorrhizal colonization. Iron was provided as Fe citrate at 2 ml l^{-1} fertilization volume (3.6 ppm Fe). Plants were grown under greenhouse conditions (21 C day, 20 C night)

and illuminated with supplemental light from high pressure sodium vapor lamps to ensure a 16 h photoperiod.

Harvests

Plants were harvested at three, six, and ten weeks after sowing. Roots and shoots were separated and shoots dried at 60 C for 48 h and weighed. VAM colonization was assayed by taking 1-cm subsamples from the center of the root system. The subsamples were weighed to estimate their proportion of the total root system weight and cleared overnight at 55 C in 10% potassium hydroxide and stained in trypan blue (0.05%) in lactoglycerol according to the methods of Phillips and Hayman (1970), and assayed for VAM colonization according to the method of Biermann and Linderman (1981). The remaining root system of each plant, minus the VAM subsample, was weighed fresh and oven dried at 60 C for 48 h and dry weight was determined. The ratio of dry weight to fresh weight was used to determine the expected dry weight of the root aliquot removed for VAM determinations (to be added to the whole root dry weight).

Statistical Analysis

Results of shoot and root dry weight and VAM colonization were analyzed according to a Multifactorial Analysis of Variance. 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).

EXPERIMENT TWO: VAM FUNGUS INOCULUM PLACEMENT, ISOLATE AND CONCENTRATION ON WESTERN RED CEDAR

VAM fungus inoculum

Commercial inoculum of the mycorrhizal fungi Glomus deserticola Trappe, Bloss and Menge and Glomus intraradices Schenck and Smith were obtained as previously described. In addition, native soil inoculum from two sources was obtained: One inoculum source was whole soil beneath a grove of second growth western red cedar located at the Phipps Nursery (Elkton, OR); the other was duff from beneath a stand of old growth western red cedar located west of Ozette Lake on the NW corner of the Olympic Peninsula, Washington, USA. VA-mycorrhizal fungus spores were separated and counted as previously described. In both native inocula the major mycorrhizal fungus component found was a fine endophyte and was assumed to be Glomus tenue (Green) Hall. On the basis of spore counts each inoculum was diluted (commercial inocula) or concentrated (native inocula) to give two densities: non-VAM control (washings from each inoculum respectively); or 100 spores ml^{-1} of inoculum. Native inocula, which had lower spore densities, were concentrated to a spore density of 100 ml^{-1} by sieving and concentrating spores and small root pieces (< 125 μm).

To reduce microfloral differences between controls and VAM-inoculated plants the control inoculum was prepared as in experiment one.

Plant and Soil Preparation

Seeds of western red cedar were sown in flats of 1:1:1 peat-sand-loam soil mix. The seeds were covered with vermiculite to hold moisture and the flats placed in a growth chamber for three weeks (20 C day/18 C night, 16 h photoperiod). Seedlings emerged in 10 days and at three weeks they were transplanted into 250 cm^3 tubes (Fig. 1-2).

Experimental Design

Pots were completely randomized on greenhouse benches with 24 fungal treatments (four VAM isolates or ecotypes by three inoculum placements by two densities, non-VAM control and 100 sp/ml (spores ml^{-1}) inoculum). Ten replicates were planted for each fungal treatment.

Plant Nutrition

Beginning at two weeks following transplanting, plants were fertilized weekly with LANS (Long Ashton Nutrient Solution) modified to deliver a low amount of phosphorus (P) (11 ppm P as NaH_2PO_4). The added P level was kept low to promote VA-mycorrhizal colonization. Iron was provided as Fe citrate at 2 ml l^{-1} fertilization volume (3.6 ppm Fe). Plants were grown under greenhouse conditions (21 C day, 20 C night) and illuminated with supplemental high pressure sodium vapor lamps to ensure a 16 h photoperiod.

Statistical Analysis

Results of shoot and root dry weight and VAM colonization were analyzed according to a Multifactorial Analysis of Variance. 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).

Harvests

Plants were harvested at five, eight, and ten weeks following transplant. Roots and shoots were separated and shoots dried at 60 C for 48 h and weighed. The root samples from ten replicate plants were divided into two groups of five. Root dry weights were carried out on the first group following oven drying at 60 C for 48 h; VAM colonization was estimated on the second group.

VAM Colonization

Whole fresh root samples (five replicates) were blotted dry (to touch), and cut into 0.5 to 1.0 cm segments. Segments were cleared overnight at 55 C in 10% potassium hydroxide and stained in trypan blue (0.05%) in lactoglycerol according to the methods of Phillips and Hayman (1970), and assayed for VAM colonization according to the method of Biermann and Linderman (1981).

EXPERIMENT THREE: VAM FUNGAL INOCULUM CONCENTRATION ON PIGEON PEA

Microbial Inoculum

VAM Fungi

Eight VAM fungal isolates or species were used in this experiment; species name and culture origin are listed in Table 1-1. The VAM fungus inocula used in this experiment were obtained from pot cultures of pigeon pea grown in a 1:1 mixture of Willamette sandy loam soil harvested from a previous experiment. Spore numbers were determined for these inocula and each was diluted with Willamette River sand to a spore density of 2.5, 5.0 or 10.0 spores ml^{-1} of inoculum. A 100 g aliquot of inoculum was placed in a column beneath each transplanted seedling (Fig. 1-1).

Rhizobium

The bacterium used was a Rhizobium Cowpea strain (P132-1 Arhar) isolated from Pigeon Pea and 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 four days before inoculation of plants.

Plant and Soil Preparation

Pigeon Pea seeds were germinated in sterile distilled water at 27 C for four days. The seeds were then dipped into the YMB Rhizobium culture. The number of colony forming bacteria per seed was estimated by dilution plating the bacteria washed from five inoculated seeds on Yeast Mannitol Agar (YMA) plates in the dark at

30 C for five days. Due to the size of the experiment, plants for two harvest times and two groups were planted one week apart. The bacterial counts from treated seed were: harvest 1-first group-0.83 x 10^6 CFU $seed^{-1}$; harvest 1-second group-1.47 x 10^6 CFU $seed^{-1}$; harvest 2-first group-83 x 10^6 CFU $seed^{-1}$; harvest 2-second group-16.6 x 10^6 CFU $seed^{-1}$.

The soil medium used in this experiment was a pasteurized 1:1 mixture of Willamette sandy loam (pH 6.0) and river sand which contained: 0.02% total nitrogen, 12 mg kg^{-1} phosphorus, 70 mg kg^{-1} potassium and 8.5 mequiv. calcium per 100 g of soil. To reduce microfloral differences between controls and VAM-inoculated plants, the Control inoculum was prepared from a Gigaspora margarita-pigeon pea pot culture sand/soil medium that proved beneficial to rhizobial symbiosis in a previous pigeon pea experiment. The VAM 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) prepared by filtering nonpasteurized Gigaspora sand/soil medium (10% by volume of the total control inoculum to be used) through Whatman #1 paper to retain VAM 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).

Experimental Design

Plants were completely randomized on greenhouse benches with seven fungal treatments. Plants for the first harvest all were inoculated with Rhizobium; plants for the second harvest were either inoculated or not with Rhizobium. Seedlings were transplanted into 84 mm x 84 mm x 152 mm pots (volume 1080 cm^3).

Plant Nutrition

Plants were fertilized weekly with a modified Hoagland's solution (Table 1-1) beginning at the first trifoliate leaf stage (three weeks following transplant). The macronutrients were diluted 1:1 with tap water before fertilization. Iron was provided as Fe citrate at 2 ml l^{-1} fertilization volume (3.6 ppm Fe).

First Harvest

At four weeks, the first plants were harvested, 20 replicates per treatment. Roots and shoots were separated, and roots were reserved for assessment of VAM colonization and dry weight. The root samples were subdivided into two groups of 10 replicates each. Root dry weights were determined on the first group following oven drying at 60 C for 48 h; VAM colonization was measured on the second group. Plant shoots were also dried at 60 C for 48 h and weighed.

VAM Colonization

Whole fresh root samples (ten plants) were blotted dry (to touch), weighed and cut into 0.5 to 1.0 cm segments. Segments were

cleared overnight at 55 C in 10% potassium hydroxide and stained in trypan blue (0.05%) in lactoglycerol according to the methods of Phillips and Hayman (1970), and assayed for VAM colonization according to the method of Biermann and Linderman (1981). Root subsamples were randomly collected from ten plant replicates and pooled into 6-8 samples per fungal treatment, and 25 segments per sample were examined for % root length with VAM colonization. The number of root segments examined per sample was determined statistically by examining 3 samples with 50 and 25 segment samples in each and comparing variability.

Second Harvest

At 12 weeks, a second group of plants was harvested. Root and shoot dry weights and VAM colonization were assayed. VAM colonization was assayed by taking 1-cm subsamples from the bottom of the root system and from 2.5 centimeters below the crown. The subsamples were weighed to estimate their proportion of the total root system weight. The remaining root system of each plant, minus the VAM subsample was weighed fresh and oven dried at 60 C for 48 hr and dry weight was determined. The ratio of dry weight to fresh weight was used to determine the expected dry weight of the root aliquot removed for VAM determinations (to be added to the whole root dry weight).

Statistical Analyses

Plants for the first and second harvest were sown according to a completely randomized design and results were analyzed according to a Multifactorial Analysis Of Variance. 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).

RESULTS

EXPERIMENT ONE

Wheat

Column placement of G. deserticola VA inoculum increased winter wheat root and shoot dry weight compared to dispersing or banding inoculum (Figs. 1-3 to 1-6; and Appendix Tables A-1 and A-2). Generally, increases in dry weight were less distinct in the roots than shoots, and not significant with G. intraradices. Increased root and shoot growth resulting from localizing G. deserticola inoculum was equal to increasing inoculum density (Figs. 1-3 and 1-4), but this did not occur with G. intraradices (Figs. 1-5 and 1-6).

In general, VAM colonization increased in response to increased inoculum density and to the localization of inoculum (Fig. 1-7), and this trend became significant at six and ten weeks. With respect to VAM fungal isolate, the general trend over time was for decreased VAM colonization with G. deserticola and increased colonization with G. intraradices (Fig. 1-7).

Green Pepper

Green pepper exhibited a significant positive shoot growth response to a moderate increase in, and column placement of, G. deserticola inoculum. This increase occurred as rapidly as three weeks and continued through the course of the experiment (Figs. 1-9 and 1-11; and Appendix Tables A-3, A-4, and A-6). Similar differences in root growth did not become significant until 10 weeks and then the difference between low and high inoculum density was not significant (Figs. 1-8 and 1-10; and Appendix Table A-5).

VAM colonization in green pepper appeared to be greatest when VAM fungal inoculum was localized (Fig. 1-12), and this trend continued through the course of the experiment with the exception that increasing inoculum density when the inoculum was dispersed throughout the bulk soil resulted in comparable VAM colonization values. As with winter wheat, there was a trend over time towards decreased colonization by G. deserticola and increased colonization by G. intraradices (Fig. 1-12).

EXPERIMENT TWO

Red Cedar

Inoculum from Phipps nursery induced a significant early shoot growth when compared to other inocula (Figs. 1-13 to 1-16). The effects on root growth of the interaction between all three main factors (VAM fungal isolate, inoculum placement and density) were significant as early as five weeks (Figs. 1-13 to 1-16 and Appendix Table A-7). Although a possible interaction between all three main

factors affected shoot growth by eight weeks, the two native inocula (Phipps Nursery and Olympic Peninsula) had the most significant effect. The significant early effects of the main factors on root growth had lessened by eight weeks (Figs. 1-13 to 1-16 and Appendix Table A-9). By ten weeks shoots were responding to all combinations of the three main factors except when the interaction between all three was analyzed (Figs. 1-13 to 1-16 and Appendix Tables A-11 to A-13). In general, shoot growth was greatest when all VAM fungal isolates except G. deserticola were localized (Figs. 1-13 to 1-16 and Appendix Tables A-11 to A-13). In addition, the dispersal of Olympic Peninsula control inoculum resulted in much more root and shoot growth than did dispersing the same inoculum with VAM fungal spores in it (Fig. 1-16 and Appendix Tables A-11 and A-12). Placing Olympic Peninsula duff in a band beneath the growing transplant resulted in the greatest shoot growth (Fig. 1-16). Although roots responded to all three main factors in combination, the greatest root growth values came from the Olympic Peninsula inoculum treatment (Fig. 1-16 and Appendix Table A-14).

G. deserticola and Phipps Nursery isolates resulted in the highest VAM colonization values early in the experiment, but by eight and 10 weeks the main factor, VAM fungal isolate, had less an influence alone than it did in combination with inoculum placement (Fig. 1-17 and Appendix Tables A-8 and A-10). At 10 weeks the localization of inoculum of G. deserticola, Phipps Nursery, and Olympic Peninsula inoculum around the root resulted in the greatest VAM colonization (Fig. 1-17 and Appendix Table A-10).

EXPERIMENT THREE

Pigeon Pea

Pigeon pea root growth was significantly increased early in the experiment by inoculation with some VAM fungal isolates, and shoot growth was significantly affected by the interaction between VAM fungal isolate and inoculum density (Fig. 1-18). Pigeon pea varied in response to inoculum density depending on which VAM fungal isolate was used, and this occurred in both roots and shoots (Fig. 1-18). Plants inoculated with isolates G. deserticola (C), Gig. margarita, and G. intraradices all increased root and shoot growth at four weeks in response to increasing inoculum density, but those inoculated with isolate G. deserticola (U) exhibited a decrease in growth (significantly so in the shoots) with increasing inoculum density. At 12 weeks the affects of the Rhizobium and VAM fungal isolate combination had a greater effect on root and shoot growth of pigeon pea than did inoculum density (Fig. 1-20 and 1-21 and Appendix Tables A-17 and A-18). A positive interaction occurred between Rhizobium and VAM fungal isolates G. etunicatum and G. intraradices, whereas combination with all other VAM fungal isolates resulted in a reduction in root and shoot growth (Fig. 1-20 and 1-21 and Appendix Tables A-17 and A-18). The main factor determining the amount of VAM colonization was the VAM fungal isolate used in the inoculum (Fig. 1-20 and Appendix Tables A-19 and A-20). Added Rhizobium and inoculum density also affected VAM colonization, but only in the interaction with VAM fungal isolate. Increasing inoculum density while adding another endophyte (Rhizobium) resulted in decreased VAM colonization

by isolates like G. deserticola (C) or G. deserticola (U), but increased colonization by the G. aggregatum/microcarpum mix (Fig. 1-22 and Appendix Tables A-17 and A-18).

DISCUSSION

The formation of vesicular-arbuscular mycorrhizae and the resulting plant growth responses depend in part on the VAM fungal isolate (Hall, 1976; Sanders et al., 1977; Abbott and Robson, 1981; Daniels et al., 1981; Jensen, 1984), inoculum placement and density (Carling et al., 1979; Sanders and Sheikh, 1983; Abbott and Robson, 1984; Walker and Smith, 1984; Frank et al., 1985; Haas and Krikun, 1985; Giovannetti and Avio, 1986; Sieverding, 1986), and plant root density (St John, 1980; Warner and Mosse, 1982; Bääth and Hayman, 1984). Models proposed for VAM formation and development are based on one or two of these factors while the others are often assumed constant. We observed the interaction of these factors and their effects on VAM colonization and plant growth in these three experiments.

Our hypothesis that rapid VAM colonization and earlier increased growth would result from localizing inoculum of a host-compatible VAM isolate around the developing root was confirmed with winter wheat and green pepper. Later, however, this advantage was lost as roots grew away from localized inoculum. We did not, however, predict the detailed nature of this process. We hypothesized that winter wheat would respond more rapidly to banding of inoculum than to a central column or dispersed inoculum. Earlier

colonization was expected because winter wheat has a fibrous root system with many shallow adventitious roots arising on the initial shoot. Fibrous roots would tend to come into contact with VAM fungal propagules more often if inoculum were banded (Abbott and Robson, 1984). However, although monocotyledons (like winter wheat) do form adventitious roots, they generally form a short-lived primary or seminal root, first (Esau, 1977; Russell, 1977). This seminal root would come into contact with inoculum more rapidly if it were localized in a central column below the seed. This was borne out by the fact that winter wheat initially responded more rapidly (VAM colonization and plant weight) to localizing inoculum in a central column extending from the soil surface below the seed. The rapidity of VAM colonization and enhanced growth are consistent with Frank et al. (1985) who observed greater plant growth and VAM colonization in Citation perennial ryegrass (Lolium perenne L.) when VAM inoculum was localized at the soil surface.

As with winter wheat, our hypothesis that rapid VAM formation and increased growth would result from localizing inoculum around a developing green pepper tap root was based on a perceived root growth pattern. We believed that green pepper would germinate and form a taproot system with less branching than winter wheat, and lateral branches would occur further down the main root (Esau, 1977). For this reason, we predicted that localizing inoculum in a central column would produce the most rapid growth benefits and colonization rates for green pepper.

Our results did not fully support this hypothesis. The effect of inoculum placement, when significant, was maximized by banding a higher density of inoculum. During harvests we observed that green pepper formed many branching lateral roots at the soil surface and these were colonized at the depth where banding inoculum occurred. These results were consistent with those of Abbott and Robson (1984) who noted heavy localized colonization of subterranean clover (Trifolium subterraeneum L.) and alfalfa (Medicago truncatula Gaertn.) occurring at the banding depth.

VAM colonization and plant growth response to localizing inoculum was slower in western red cedar than in winter wheat or green pepper. Western red cedar exhibited more rapid VAM colonization and plant growth when all inocula except G. deserticola, were localized around the growing tap root. Western red cedar responded more to differences in VAM fungal isolate than to inoculum placement.

VAM formation by the two native inocula (Phipps Nursery and Olympic Peninsula) resulted in significant root and shoot growth early in the experiment. These results suggest several hypotheses to explain the connection between VAM colonization and plant growth with these inocula on western red cedar. Since there was little direct correlation between VAM formation and early plant growth, the growth response might have been due to other edaphic (organic matter, soil clay content) or biotic (plant growth promoting rhizobacteria) factors in the inocula. On the other hand the VAM fungi in these inocula may have been especially efficient at nutrient uptake and

therefore little VAM colonization (as measured by internal morphology) was needed to enhance plant growth. The possibility of other organisms acting synergistically with VA-mycorrhizal fungi in the native inocula is supported by the fact that early root growth in the non-VAM control was similar if not greater than for VA-inoculated plants. It is therefore possible that in eliminating the VA-mycorrhizal fungus component, we stimulated other plant growth promoting organisms in the rhizosphere that no longer had to compete with VA-mycorrhizal fungi for plant root exudates, and/or the establishment and development of VAM was not a C drain on a young and developing western red cedar seedling. These results are consistent with observations of Parke et al. (1983) whose findings suggested that some biological factor other than VAM was responsible for stimulating the growth of western red cedar. The rapid response of western red cedar shoots to VA-mycorrhizal inoculation by the native inocula and the commercial G. intraradices inoculum is consistent with results observed by Kough et al. (1985), although, whereas they observed a decrease in growth enhancement from mycorrhizal colonization with seedling age, we did not. However, their experimental time frame was 320 days, and ours was 70 days. This difference in observation may have been attributable to a difference in root size in relation to pot volume and the difference in the length of experiment between our experiment and theirs. The lack of significant VAM colonization and plant growth when inoculated with G. deserticola might suggest an incompatibility between western red cedar and this VAM fungus under these experimental conditions.

The effect of VAM fungal isolate on colonization and growth of winter wheat and green pepper suggests that the extent of intraradical VAM colonization may be a poor indicator of plant growth response. With winter wheat the rapid colonization that occurred through six weeks with G. intraradices was not surprising. Glomus intraradices is known for producing large amounts of intraradical vesicles which, as Biermann and Linderman (1983) point out, could act as nourishment to colonizing hyphae via a hyphal network. In addition it produces chlamydospores inside the root, and it has been observed that VAM fungal isolates that produce these internal structures can more rapidly colonize roots and cause a greater host growth response than would occur when spores of the same species are used (Hall, 1976; Powell, 1976; Warner and Mosse, 1980). The lack of a positive growth increase by this fungal isolate in proportion to it's rapid colonization may suggest a proper balance between cost of VAM colonization to the host (in terms of carbon partitioned to the symbiont) and benefit of the symbiosis (increased nutrient uptake, stimulation of mycorrhizosphere organisms) was not achieved for winter wheat under these environmental conditions and the time frame of this experiment. That was not the situation, however, for G. deserticola on both winter wheat and green pepper. Glomus deserticola never colonized as extensively as G. intraradices but induced greater root and shoot growth (in both plants) than with G. intraradices. One possible explanation might be that G. deserticola produced more extraradical hyphae in these experiments and less intraradical fungal structures than did G. intraradices. If the total fungal biomass

(and hence the incurred C drain on the host) were the same for both fungi, the nutrient uptake through an increase in the volume of soil explored would have been greater with G. deserticola.

The effect of different VAM fungal isolate and inoculum density on VAM and plant growth response was most evident in experiment three with pigeon pea. Varying VAM isolate influenced the overall extent of VAM colonization and plant growth observed, but the rapidity with which these responses occurred was affected by both VAM isolate and inoculum density. The early interaction we observed between the two (i.e. isolate and density) confirms observations made by Walker and Smith (1984) on Trifolium subterraneum including the fact that the interaction is short-lived. The significant interaction between Rhizobium and VAM isolate was eventually more important than inoculum density in determining plant growth response. The overall significance of Rhizobium to the host-fungal association suggests the importance of N and N:P ratios, as has been observed by Pugh et al. (1981).

The eventual lack of a correlation between increased VAM colonization and stimulated plant growth with increasing inoculum density has also been observed by Owusu-Bennoah and Mosse (1979) and Smith (1981). The effect of increasing inoculum density of one VAM isolate (G. intraradices) influenced VAM formation and stimulated growth of winter wheat, yet the reverse was true with green pepper. With pigeon pea the effect changed with plant age and probable rooting density.

The observations made in these three experiments underscore the complexity of the VAM-host interaction. The objectives of inoculation with VAM symbionts is an increase in plant survival, growth and vigor, and beneficial modifications of the host rhizosphere, regardless of the extent of VAM colonization. These observations suggest the importance of understanding host-root morphology as well as experimental (and possibly field) conditions. In addition, we need to understand that compatible host-VAM combinations can achieve these objectives in less time. Proper placement of inoculum could insure early colonization by VAM fungi and potential benefits over a longer portion of the growing season.

Table 1-1. List of compounds used in a modified Hoagland's solution.

MACRONUTRIENTS (diluted 1:1 in final solution)	g $(100\ l)^{-1}$
K_2SO_4	27.5
$MgSO_4 \cdot 7H_2O$	49.0
KH_2PO_4	17.62
K_2HPO_4	22.4
$CaSO_4$	81.6
$CaCl_2 \cdot 2H_2O$	6.0
MICRONUTRIENTS (1 ml l^{-1} added to final solution)	**g l^{-1}**
H_3BO_3	0.23
$MnSO_4$	0.12
$ZnSO_4 \cdot 7H_2O$	0.22
$CuSO_4 \cdot 5H_2O$	0.08
$Na_2MnO_4 \cdot 2H_2O$	0.02
$CoCl_2 \cdot 6H_2O$	0.04
$NiCl_2 \cdot 6H_2O$	0.04

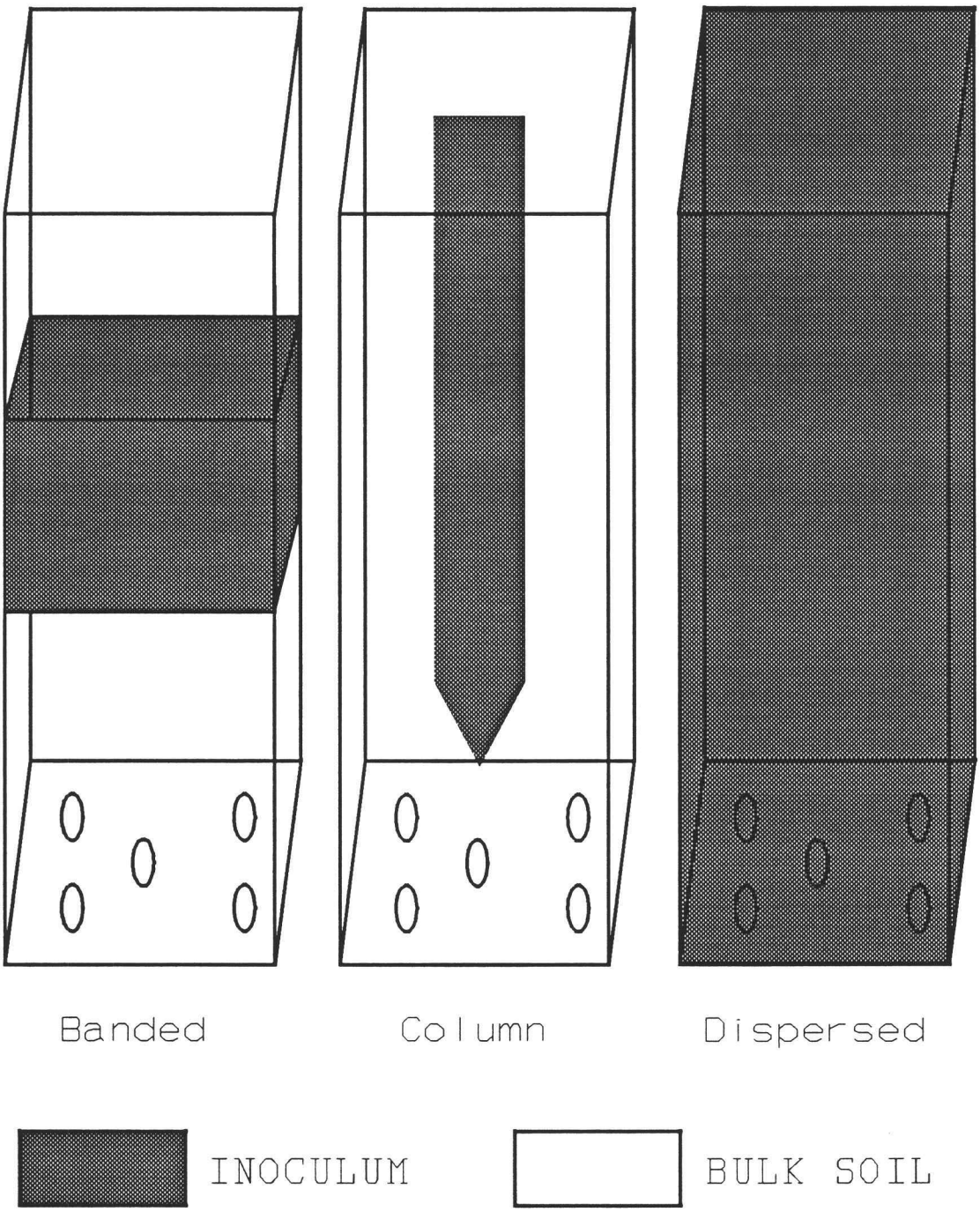

Figure 1-1. Inoculum distribution for experiment one with green pepper and winter wheat. Pot size was 84 mm x 84 mm x 152 mm (volume 1080 cm^3). Band was located 2.5 cm from soil surface.

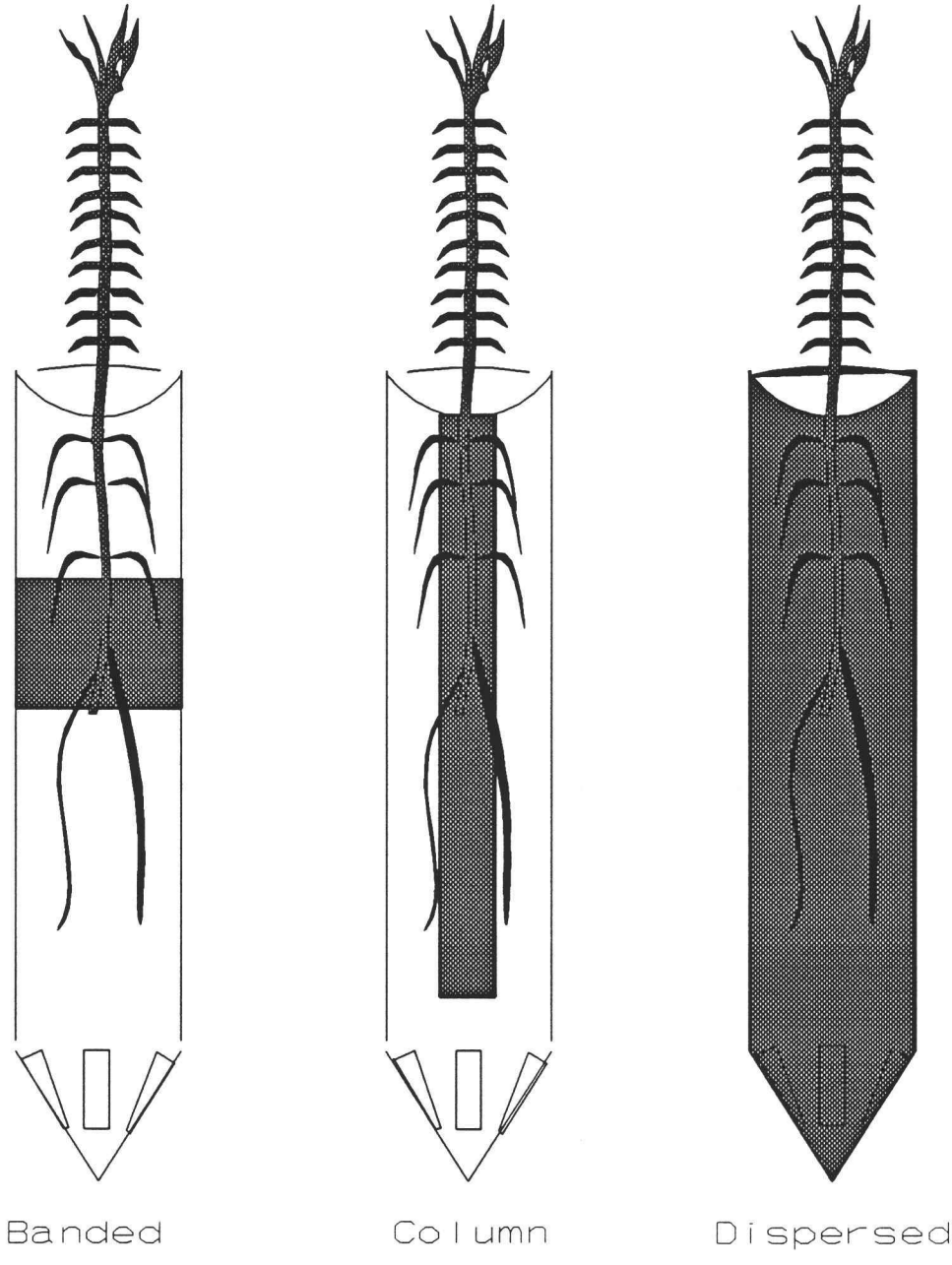

Figure 1-2. Inoculum placement for experiment two with western red cedar. Pot volume was 260 cm^3. Inoculum placed in shaded areas.

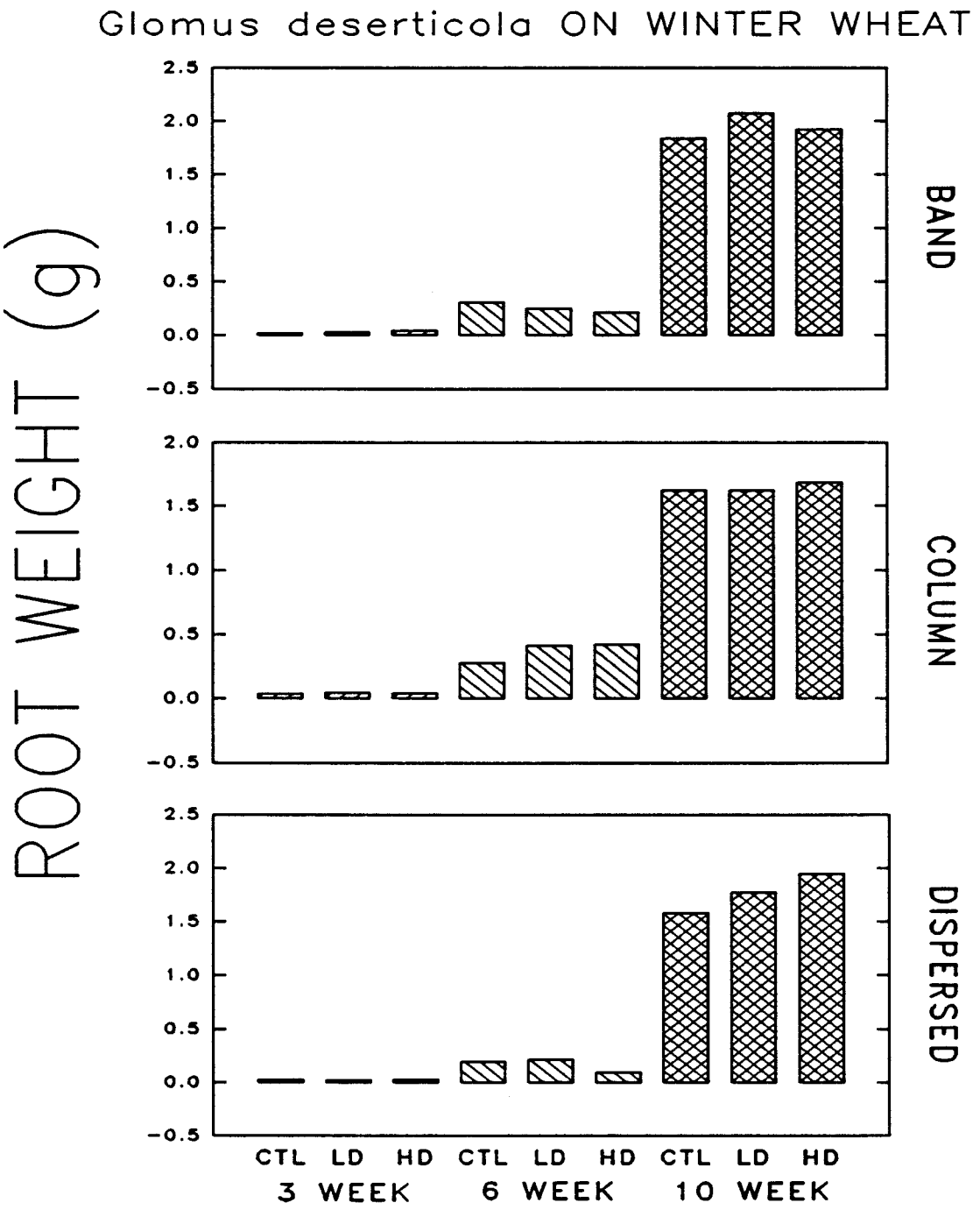

Figure 1-3. Winter wheat root dry weight at 3, 6, and 10 weeks as influenced by G. deserticola, inoculum placement, and density. CTL (non-VAM), LD-100 sp/ml, and HD-200 sp/ml.

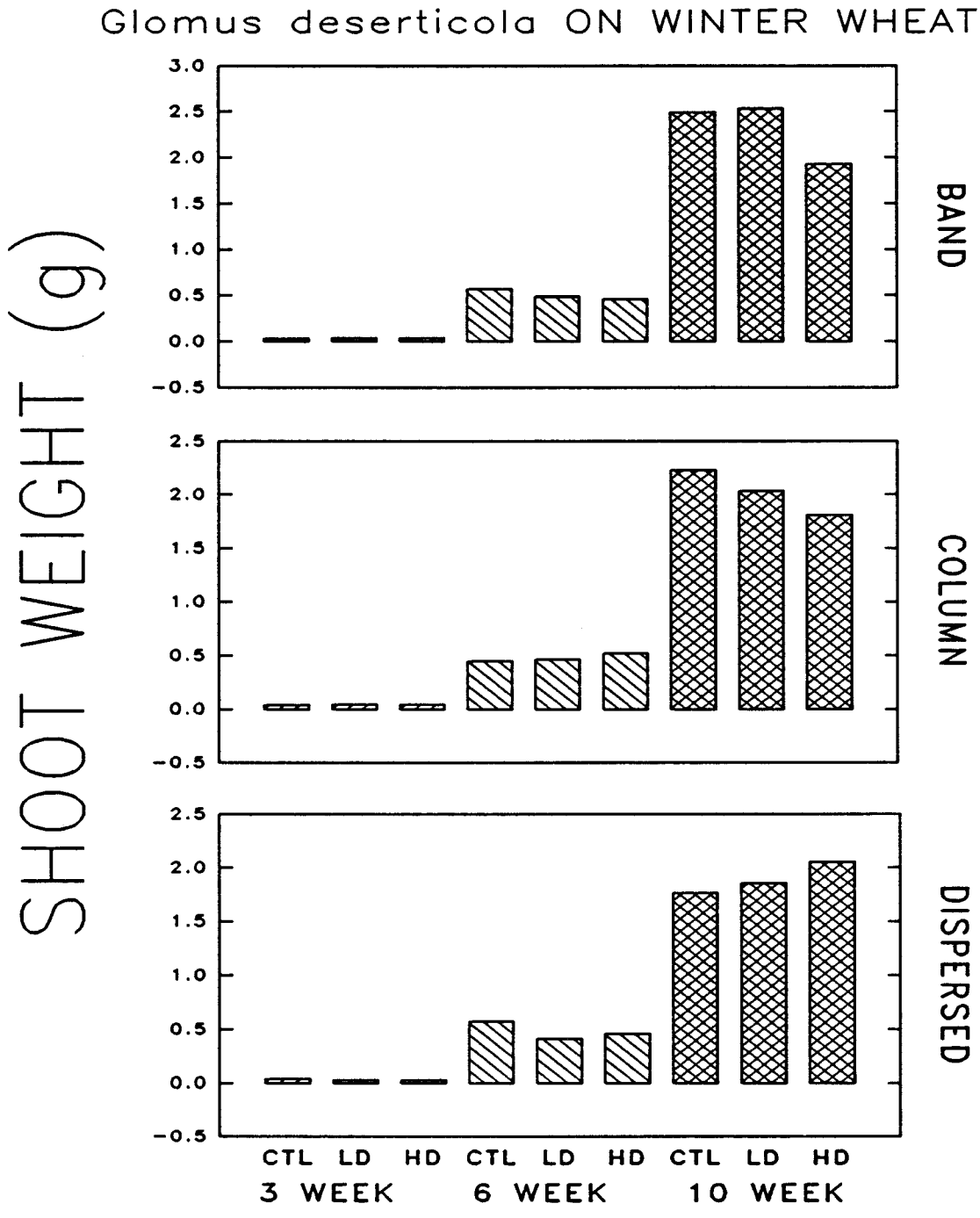

Figure 1-4. Winter wheat shoot dry weight at 3, 6, and 10 weeks as influenced by Glomus deserticola, inoculum placement, and density. CTL (non-VAM), LD-100 sp/ml, and HD-200 sp/ml.

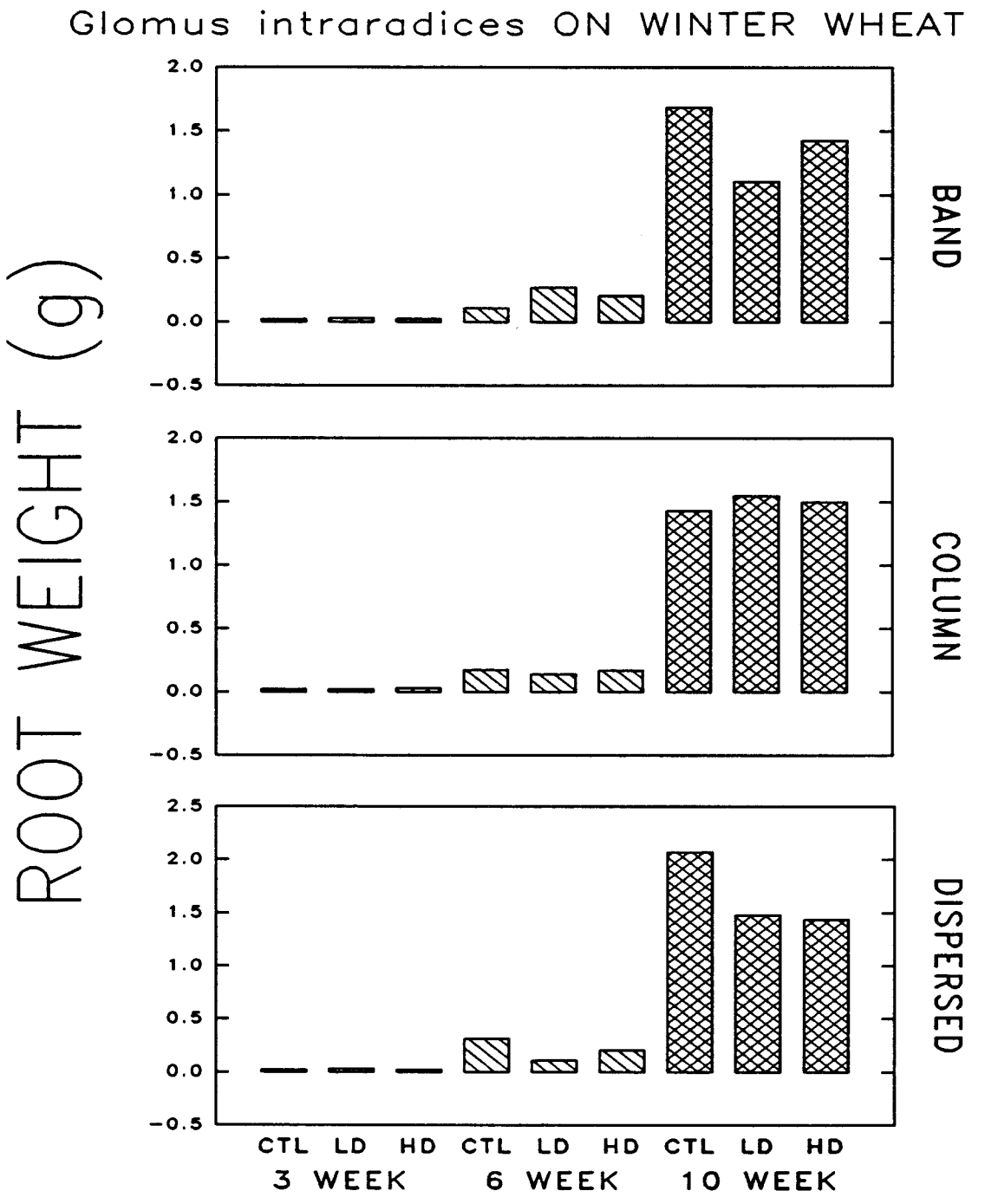

Figure 1-5. Winter wheat root dry weight at 3, 6, and 10 weeks as influenced by Glomus intraradices, inoculum placement, and density. CTL (non-VAM), LD-100 sp/ml, and HD-200 sp/ml.

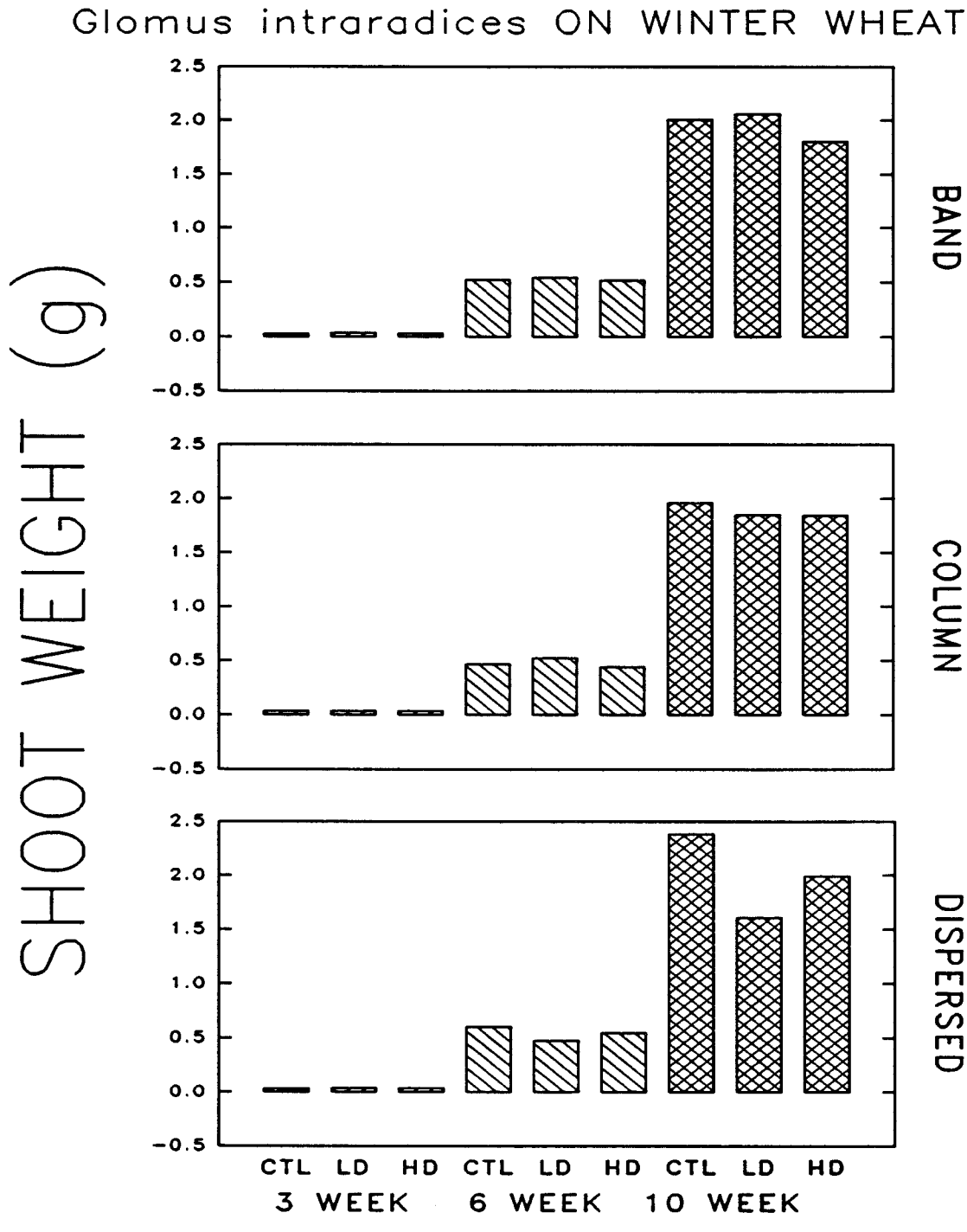

Figure 1-6. Winter wheat shoot dry weight at 3, 6, and 10 weeks as influenced by Glomus intraradices, inoculum placement, and density. CTL (non-VAM), LD-100 sp/ml, and HD-200 sp/ml.

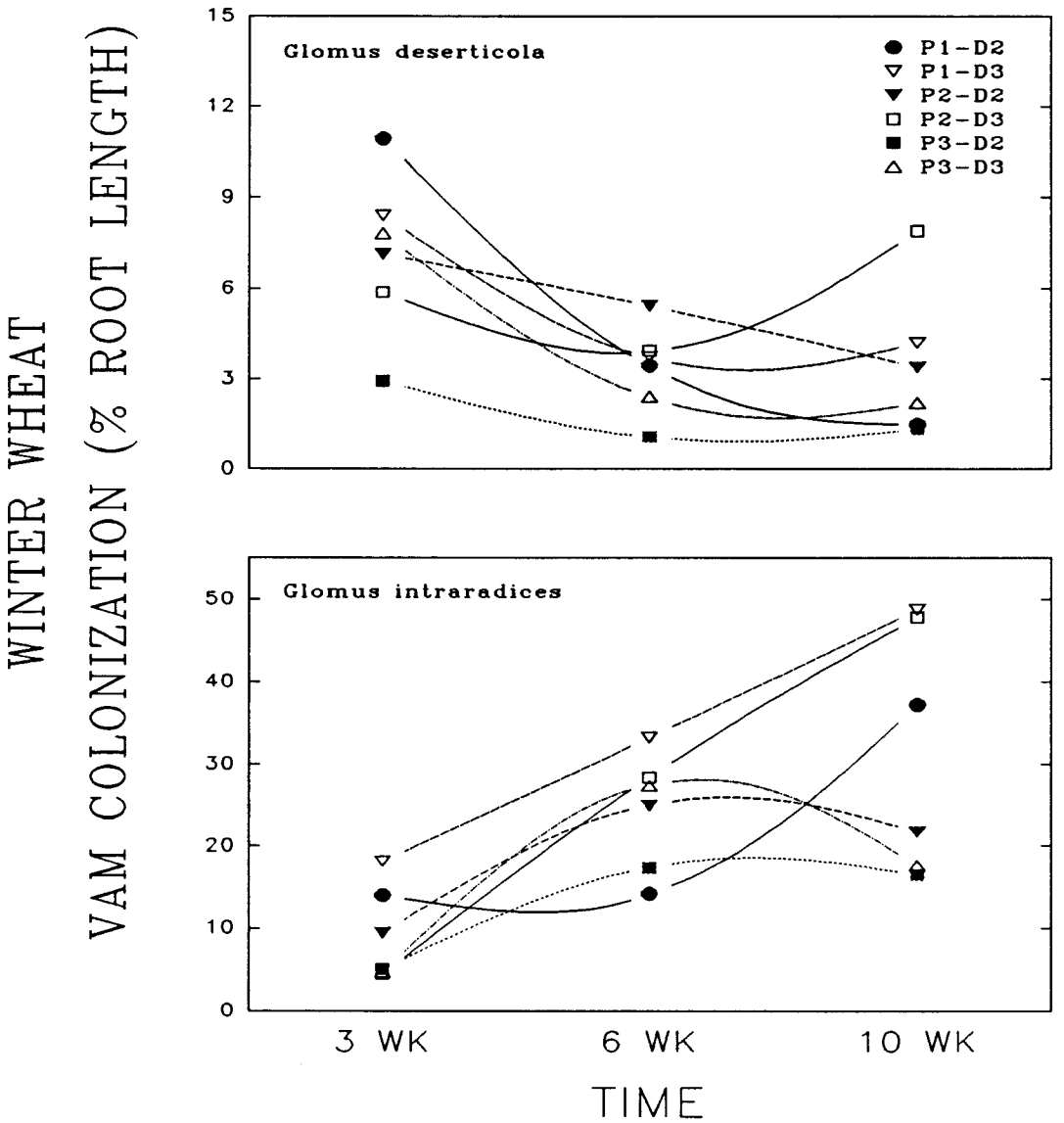

Figure 1-7. Winter wheat VAM colonization over time as influenced by VAM isolate, inoculum placement, and density. P1-band, P2-column, P3-dispersed, D2-100 sp/ml, and D3-200 sp/ml. Standard error values: (1) G. deserticola 3 WK-0.38, 6 WK-0.18, and 10 WK-0.39; (2) G. intraradices 3 WK-0.67, 6 WK-1.83, and 10 WK-1.20.

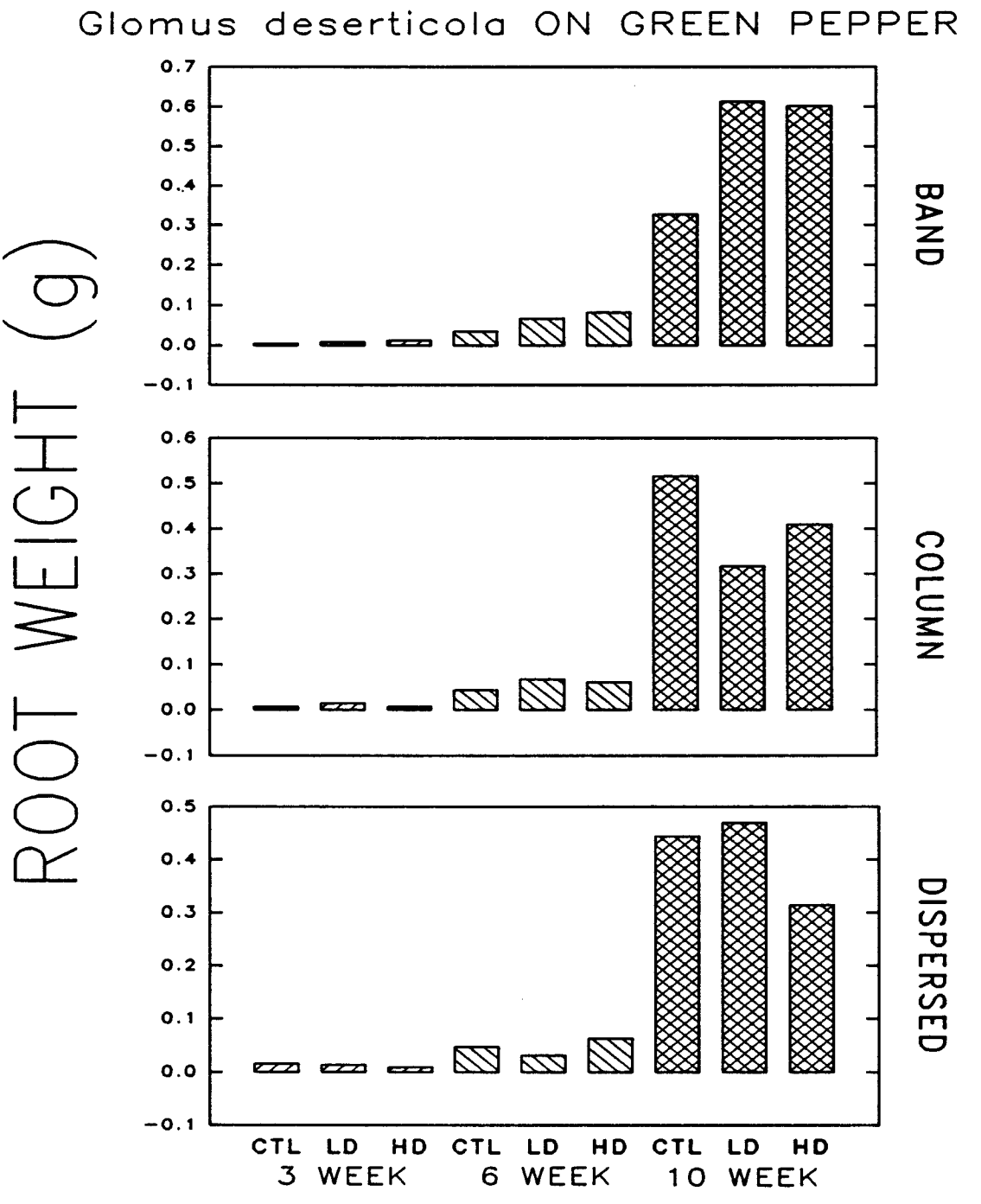

Figure 1-8. Green pepper root dry weight at 3, 6, and 10 weeks as influenced by Glomus deserticola, inoculum placement, and density. CTL (non-VAM), LD-100 sp/ml, and HD-200 sp/ml.

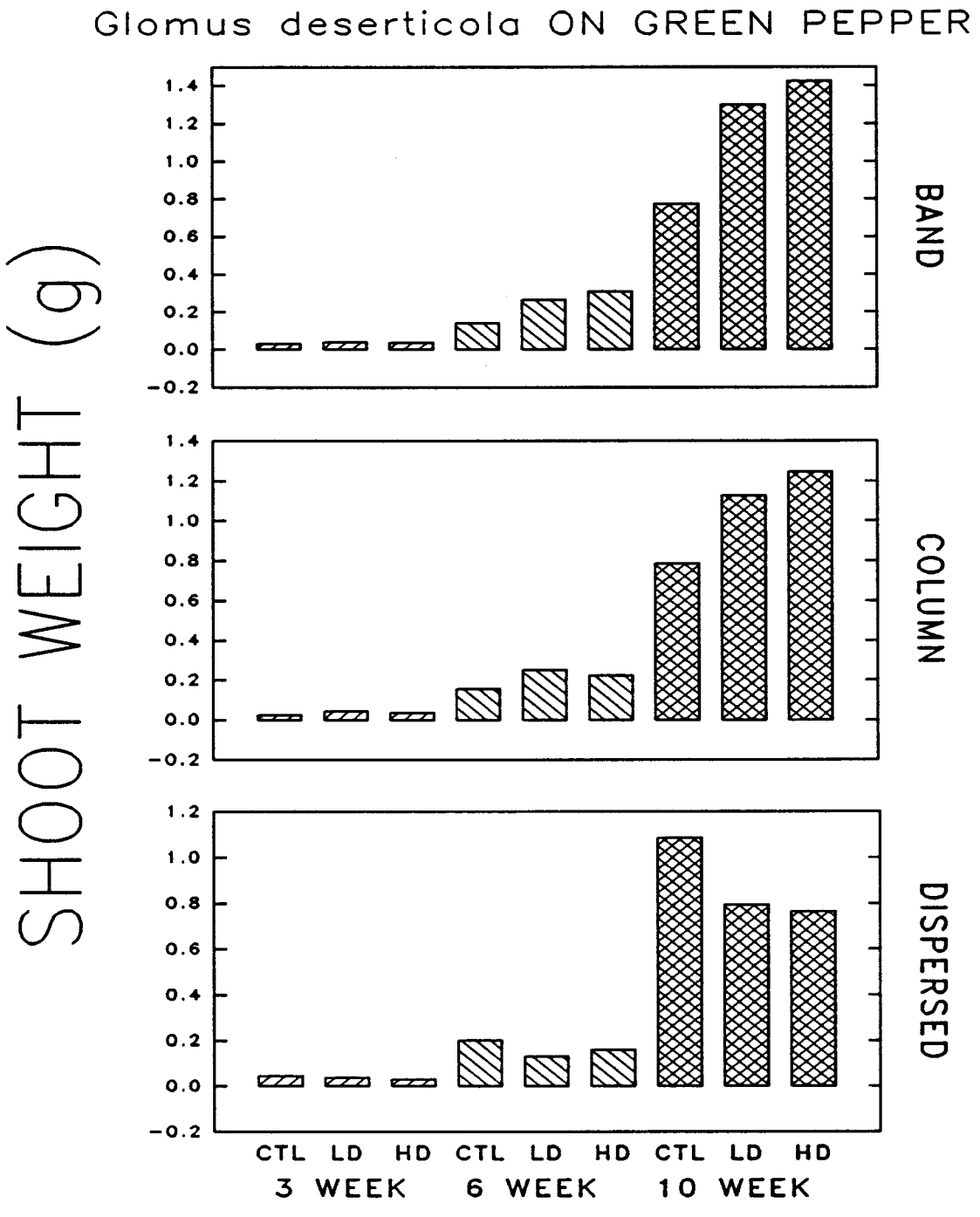

Figure 1-9. Green pepper shoot dry weight at 3, 6, and 10 weeks as influenced by Glomus deserticola, inoculum placement, and density. CTL (non-VAM), LD-100 sp/ml, and HD-200 sp/ml.

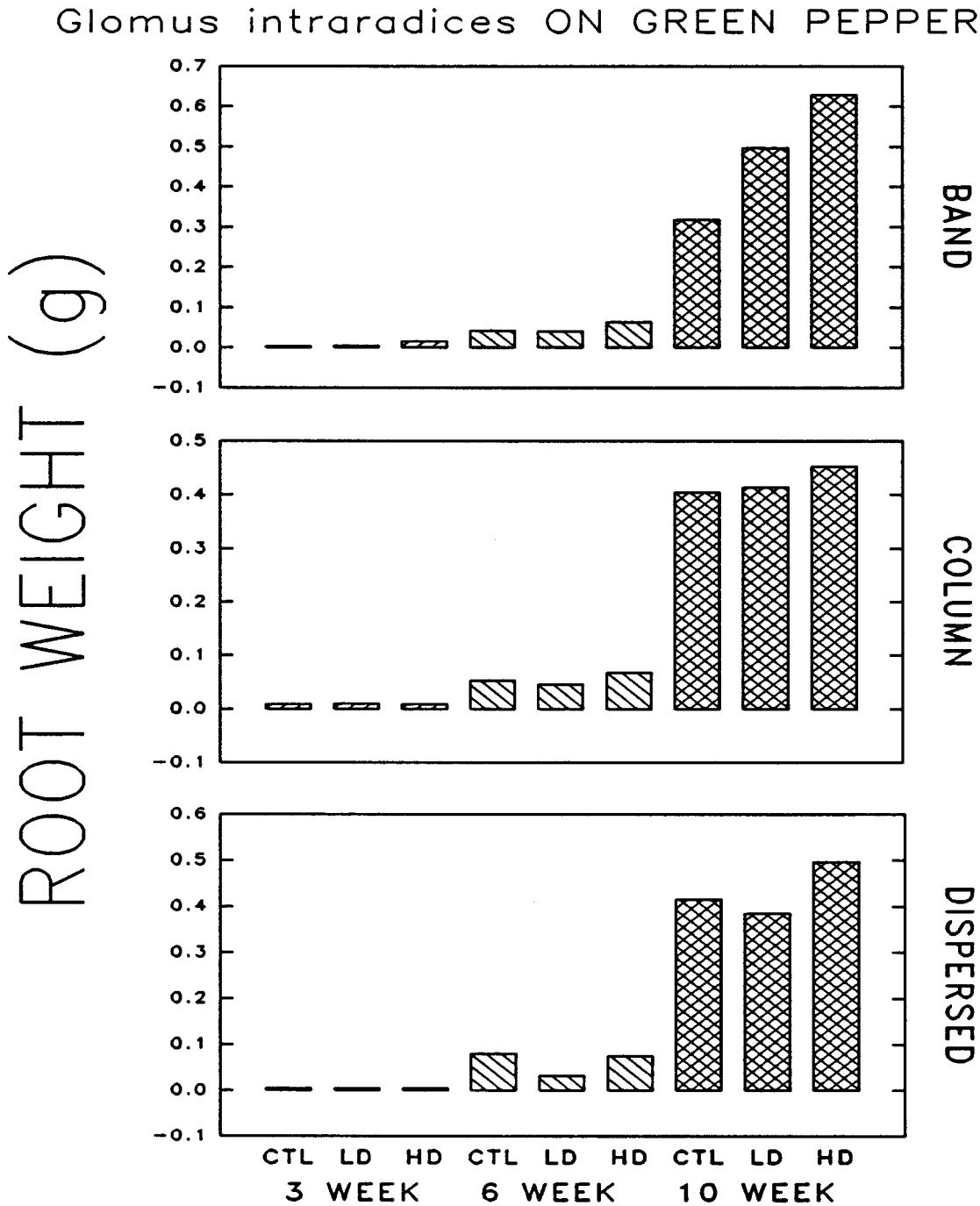

Figure 1-10. Green pepper root dry weight at 3, 6, and 10 weeks as influenced by Glomus intraradices, inoculum placement, and density. CTL (non-VAM), LD-100 sp/ml, and HD-200 sp/ml.

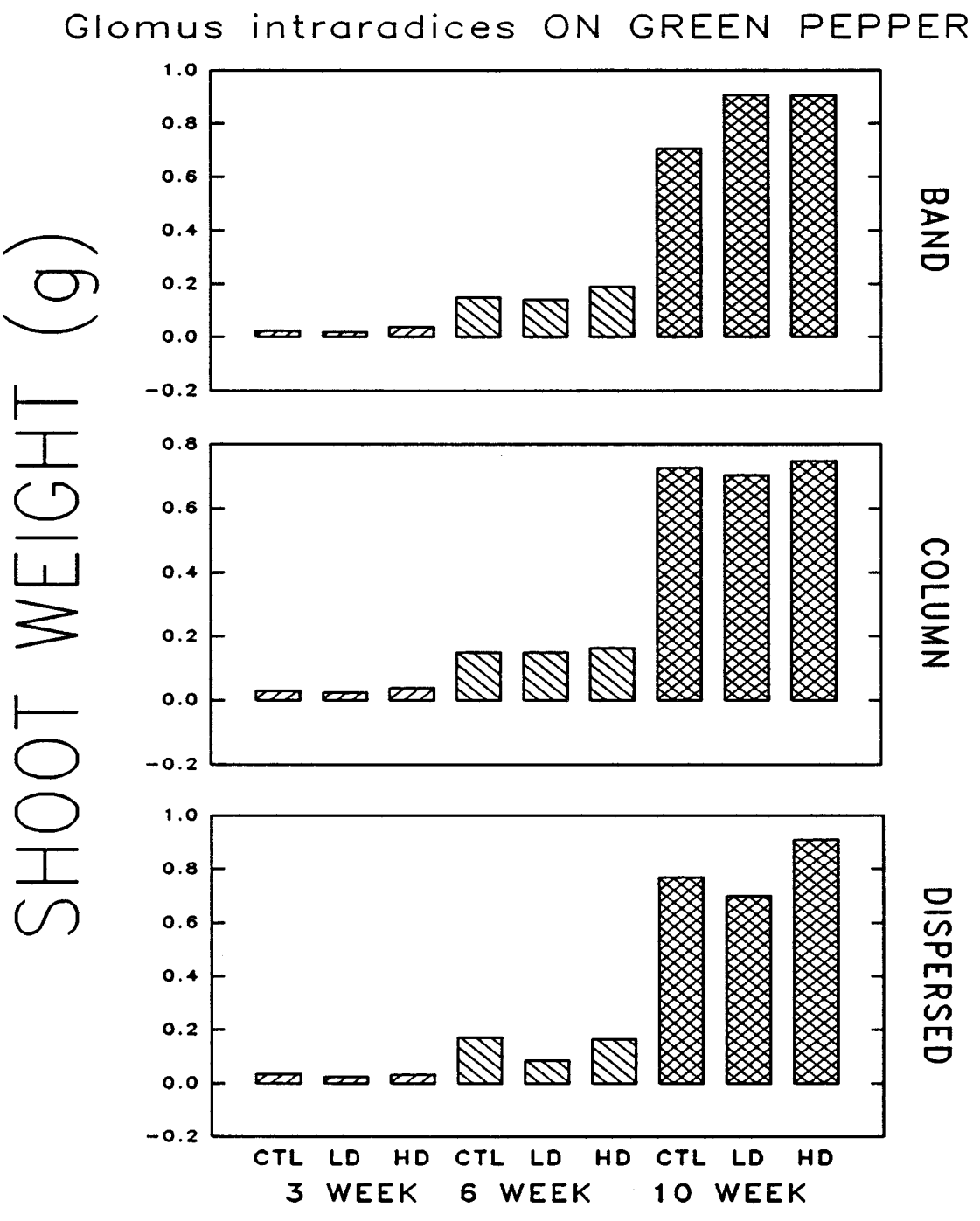

Figure 1-11. Green pepper shoot dry weight at 3, 6, and 10 weeks as influenced by Glomus intraradices, inoculum placement, and density. CTL (non-VAM), LD-100 sp/ml, and HD-200 sp/ml.

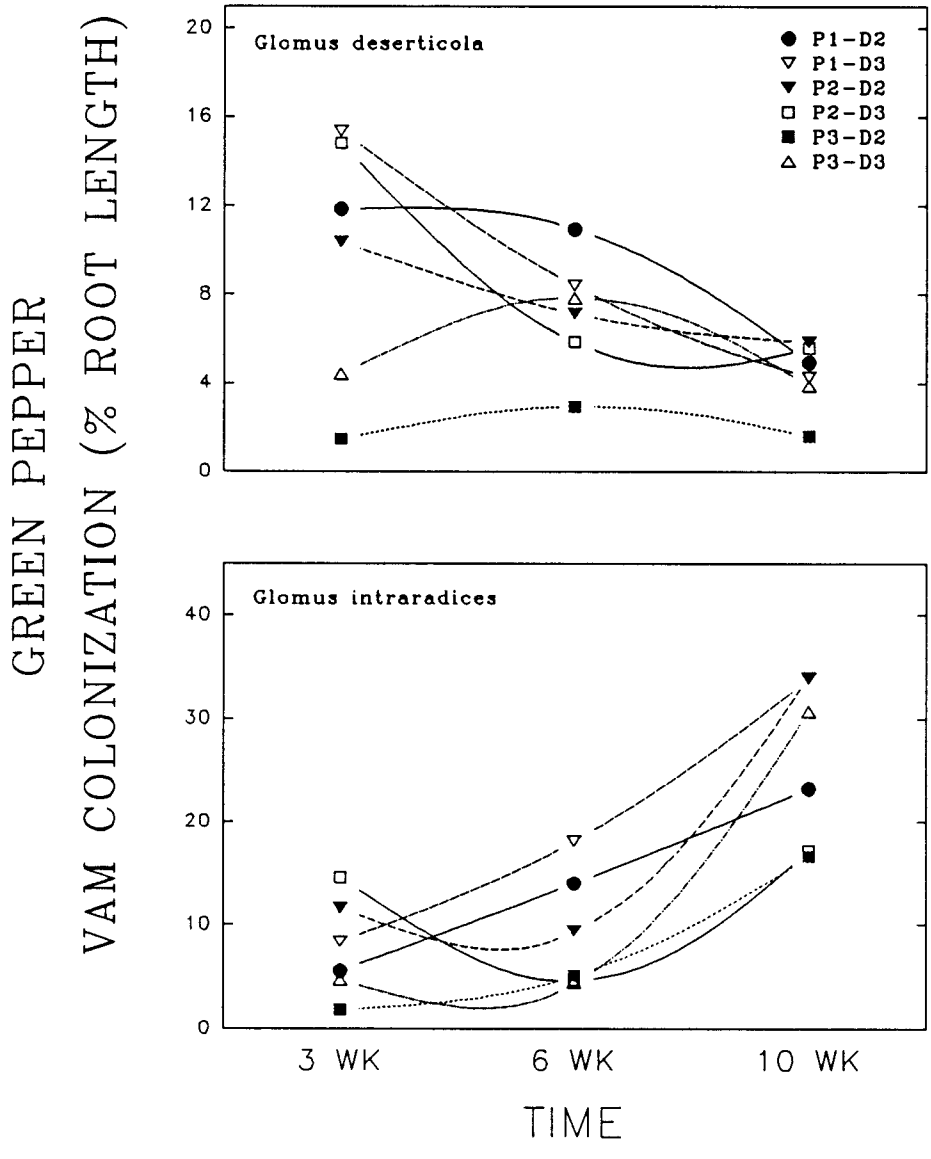

Figure 1-12. Green pepper VAM colonization over time as influenced by VAM isolate, inoculum placement and density. P1-band, P2-column, P3-dispersed, D2-100 sp/ml, and D3-200 sp/ml. Standard error values: (1) G. deserticola 3 WK-0.63, 6 WK-0.37, and 10 WK-0.167; (2) G. intraradices 3 WK-1.12, 6 WK-0.64, 10 WK-1.01.

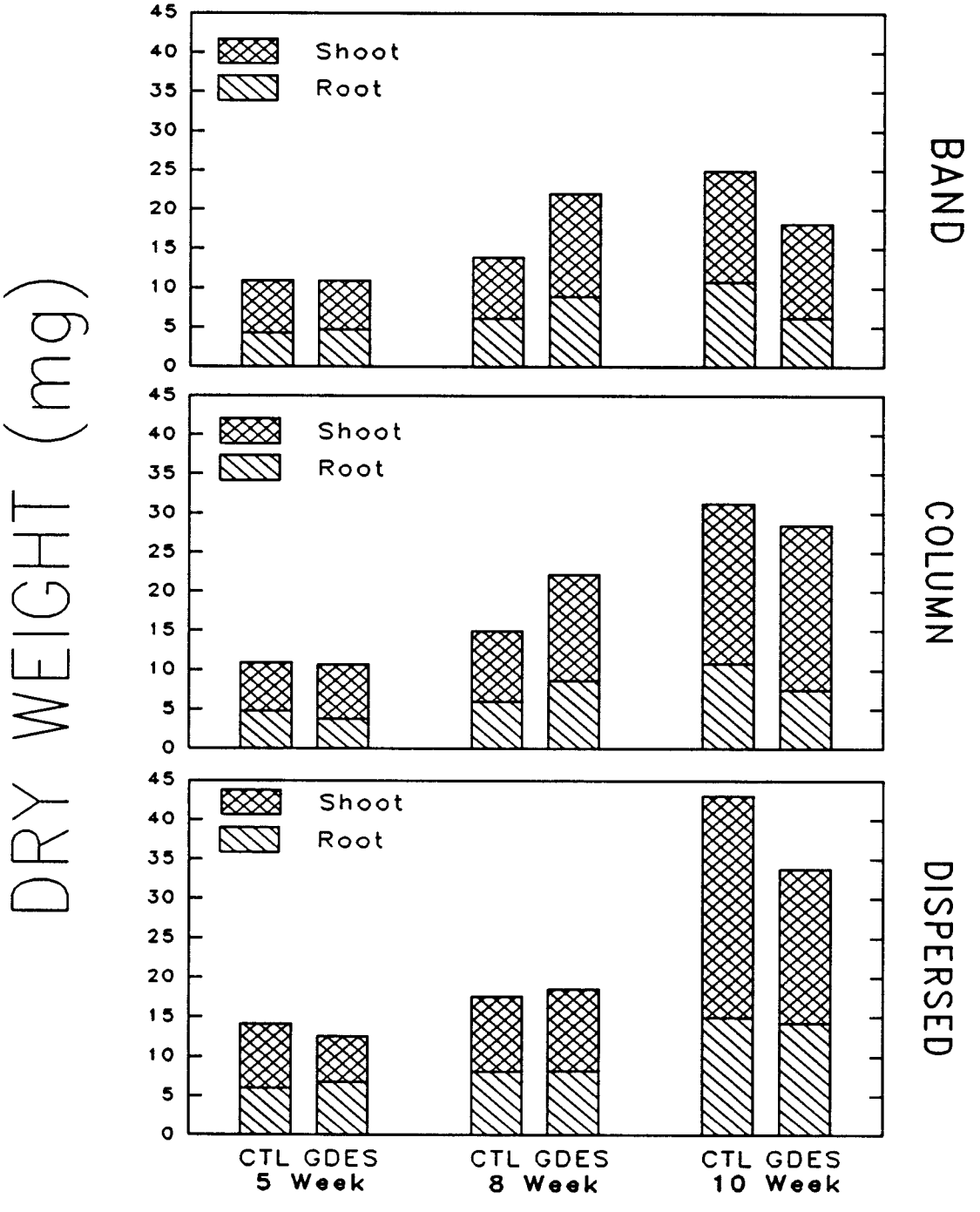

Figure 1-13. Western red cedar root and shoot dry weight at 5, 8, and 10 weeks as influenced by G. deserticola isolate, inoculum placement and density.

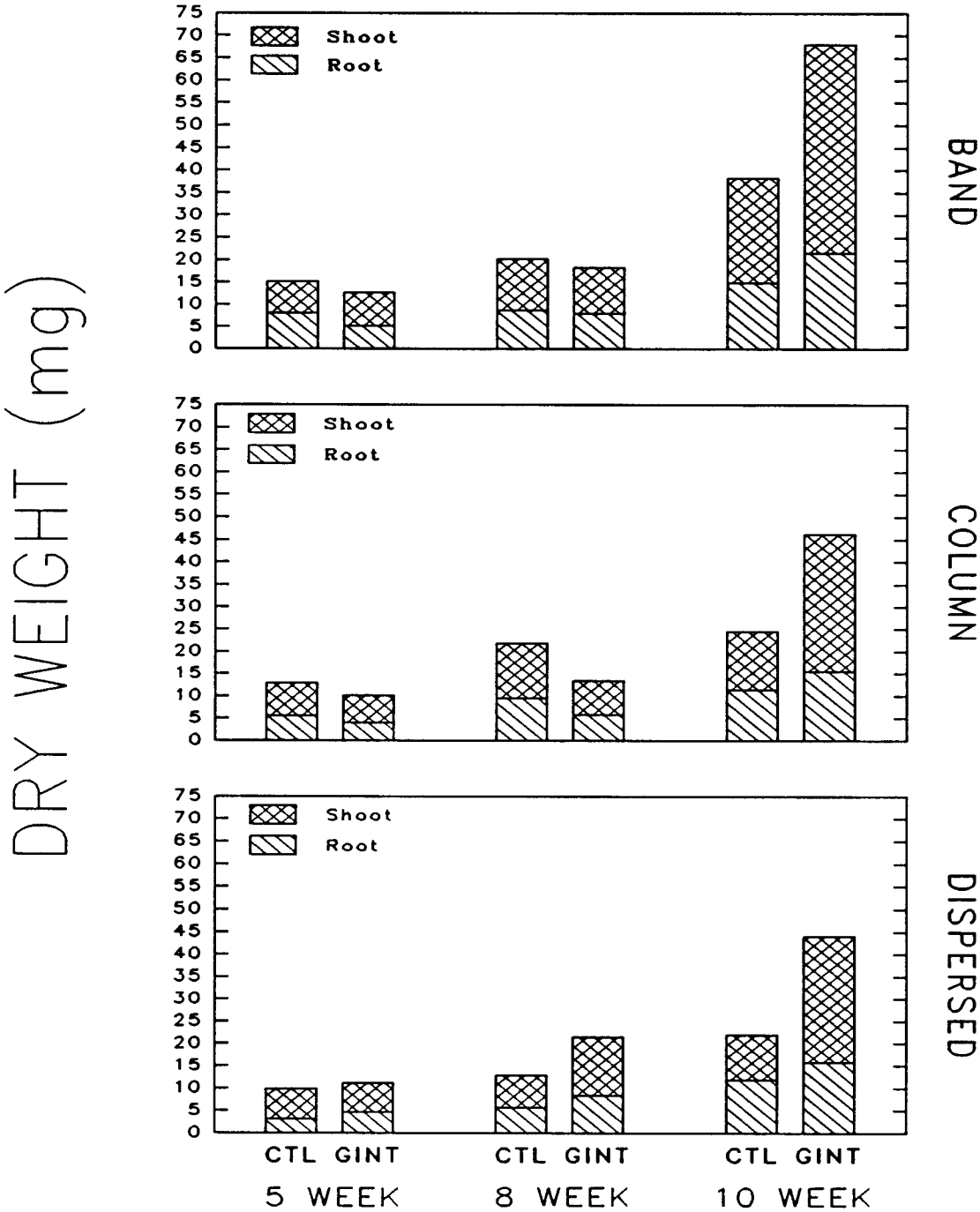

Figure 1-14. Western red cedar root and shoot dry weight at 5, 8, and 10 weeks as influenced by G. intraradices isolate, inoculum placement and density.

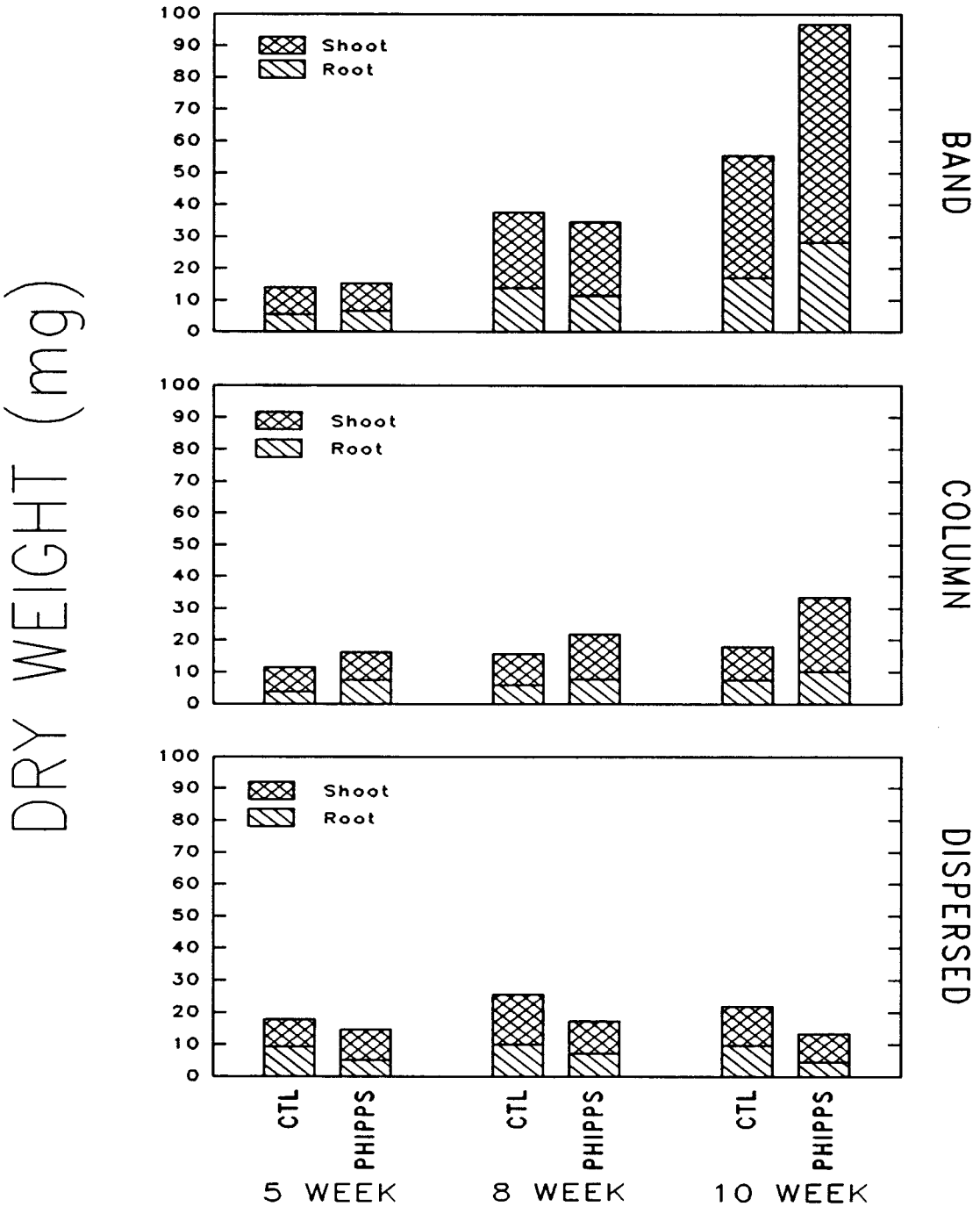

Figure 1-15. Western red cedar root and shoot dry weight at 5, 8, and 10 weeks as influenced by Phipps Nursery VAM fungal isolate, inoculum placement and density.

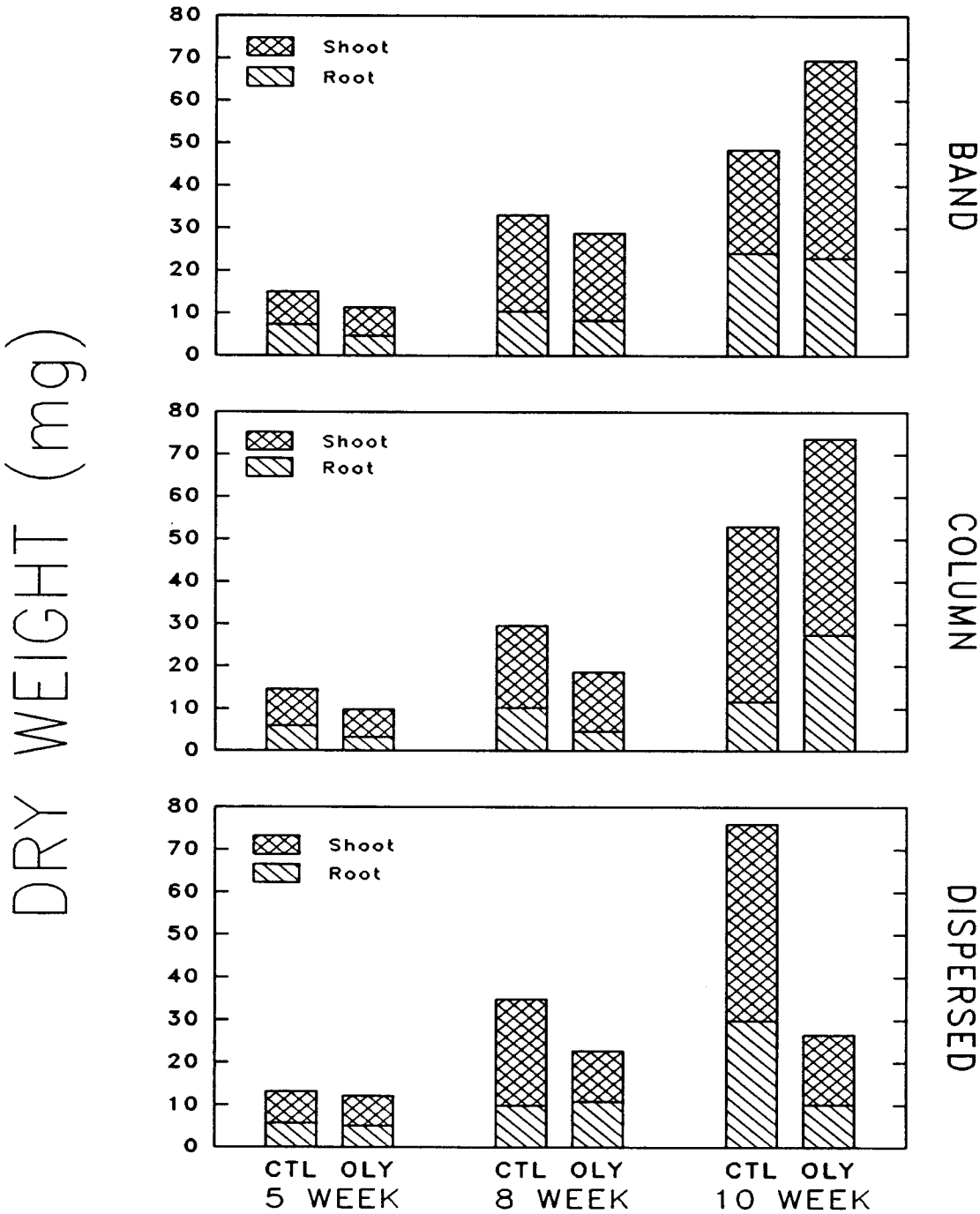

Figure 1-16. Western red cedar root and shoot dry weight at 5, 8, and 10 weeks as influenced by Olympic Peninsula VAM fungal isolate, inoculum placement and density.

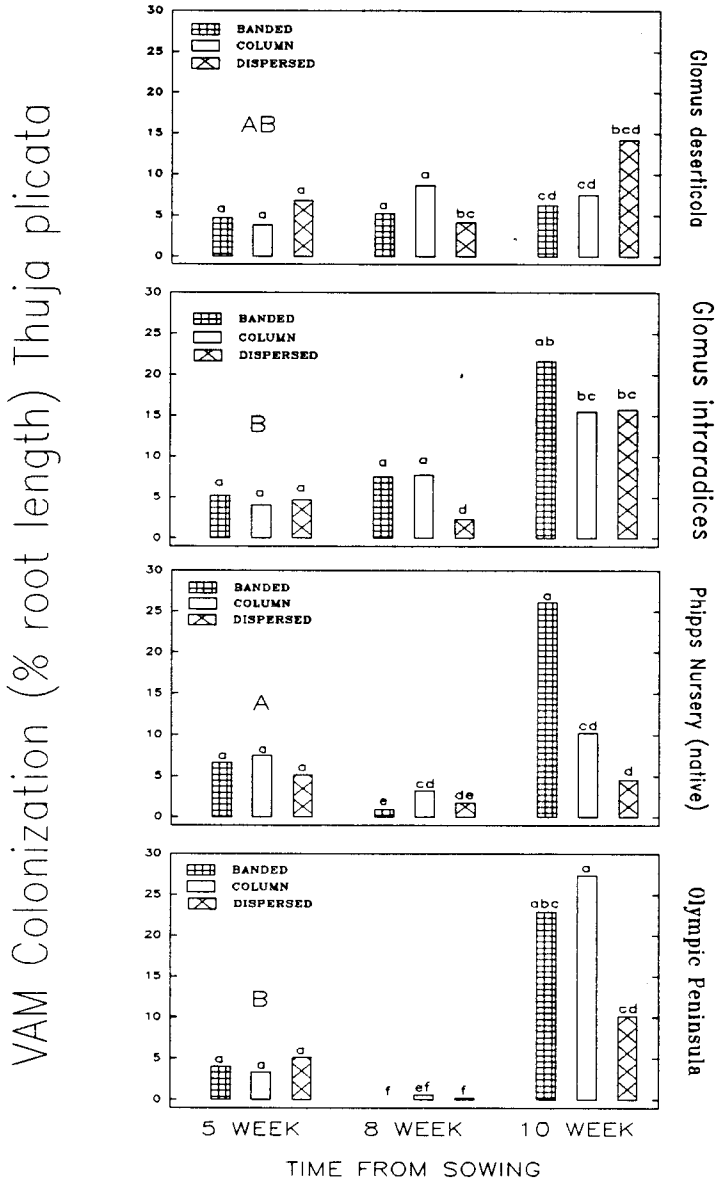

Figure 1-17. Western red cedar VAM colonization at 5, 8, and 10 weeks as influenced by VAM fungal isolate and inoculum placement. VAM fungal isolate influenced colonization at five weeks, the interaction between the two influenced results at eight and 10 weeks.

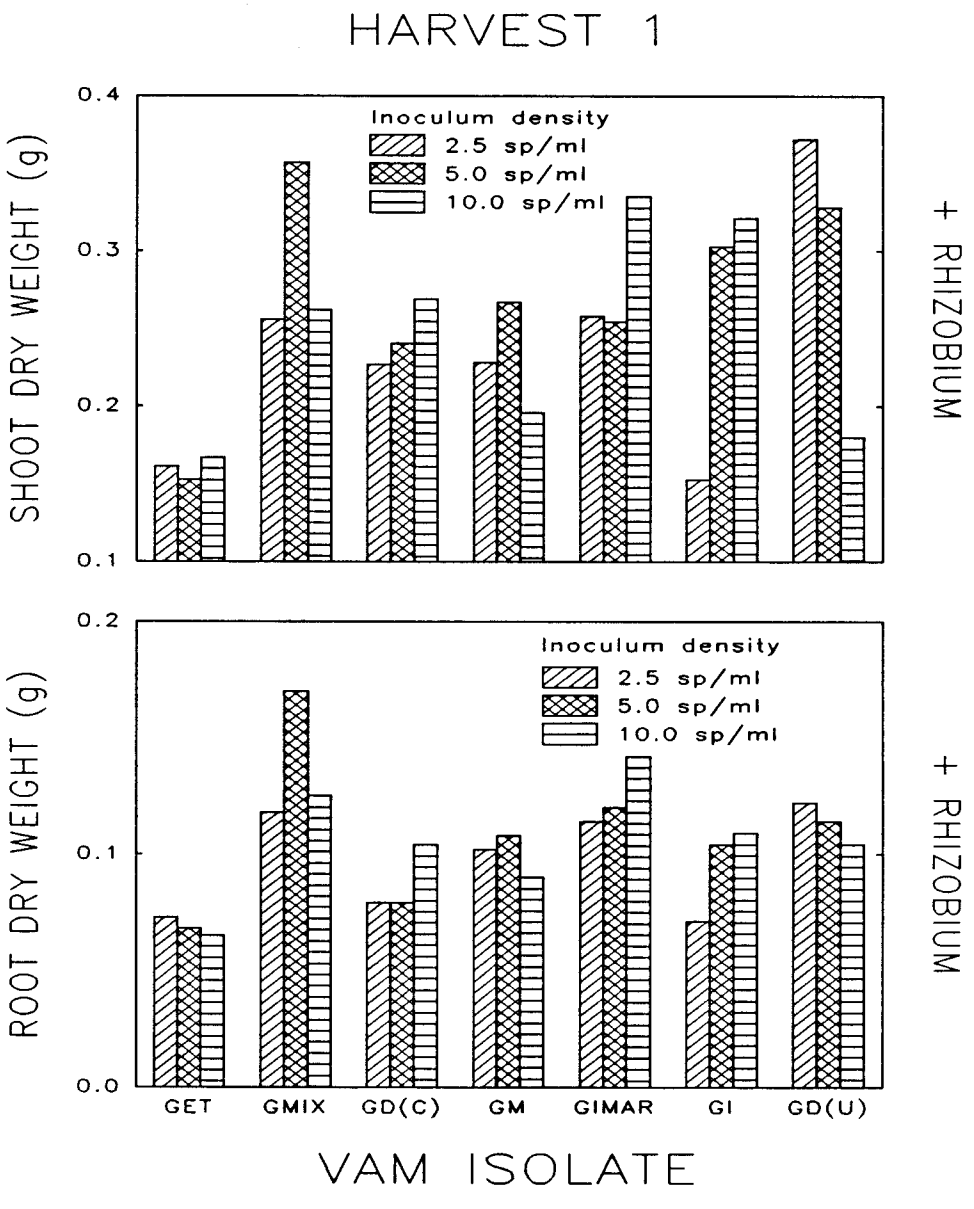

Figure 1-18. Pigeon pea root and shoot dry weight at four weeks as influenced by VAM fungal isolate and inoculum density. VAM fungal isolate significantly influenced root dry weight, and the interaction between the two influenced shoot growth.

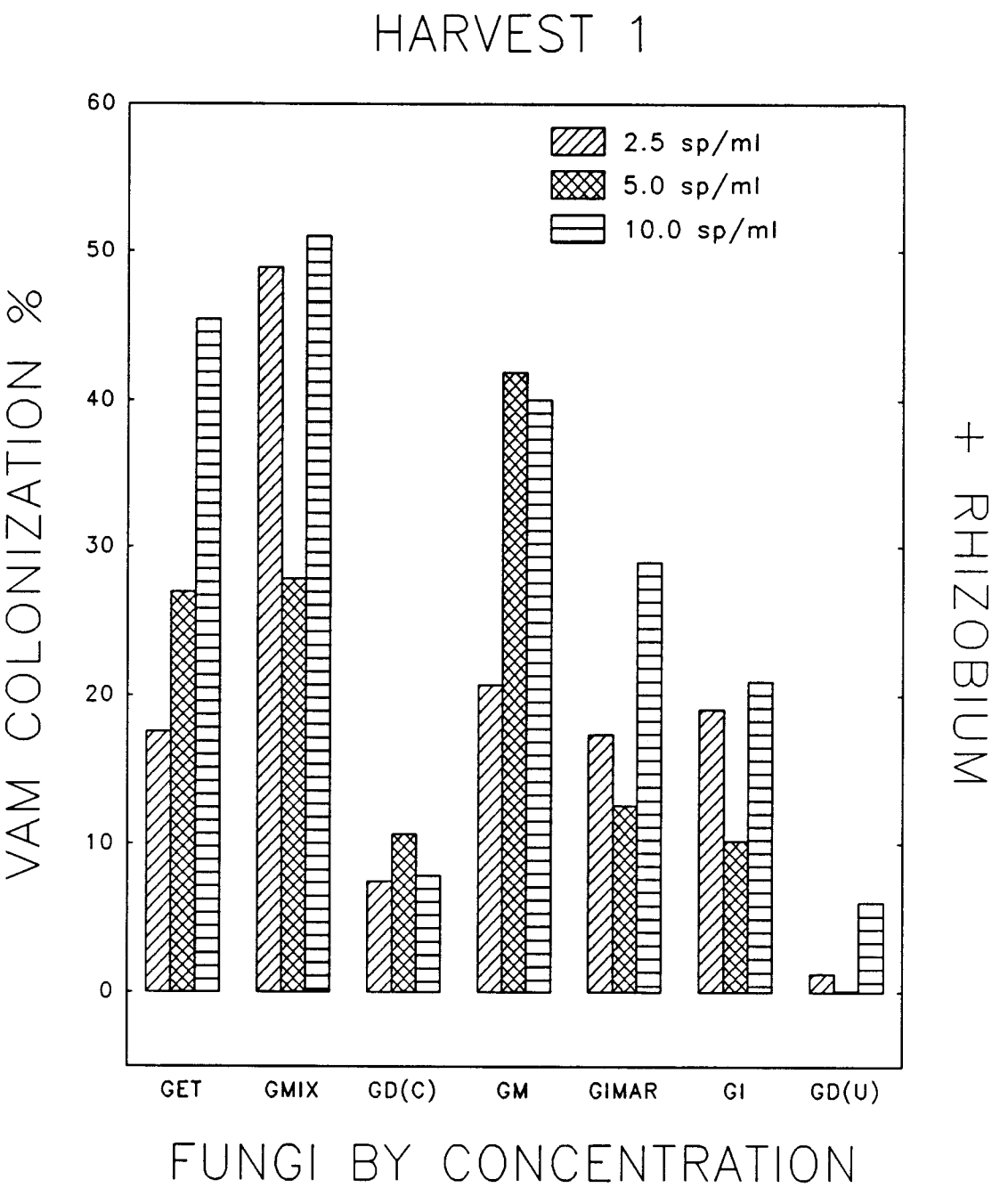

Figure 1-19. Pigeon pea VAM colonization at four weeks as influenced by VAM fungal isolate and inoculum density.

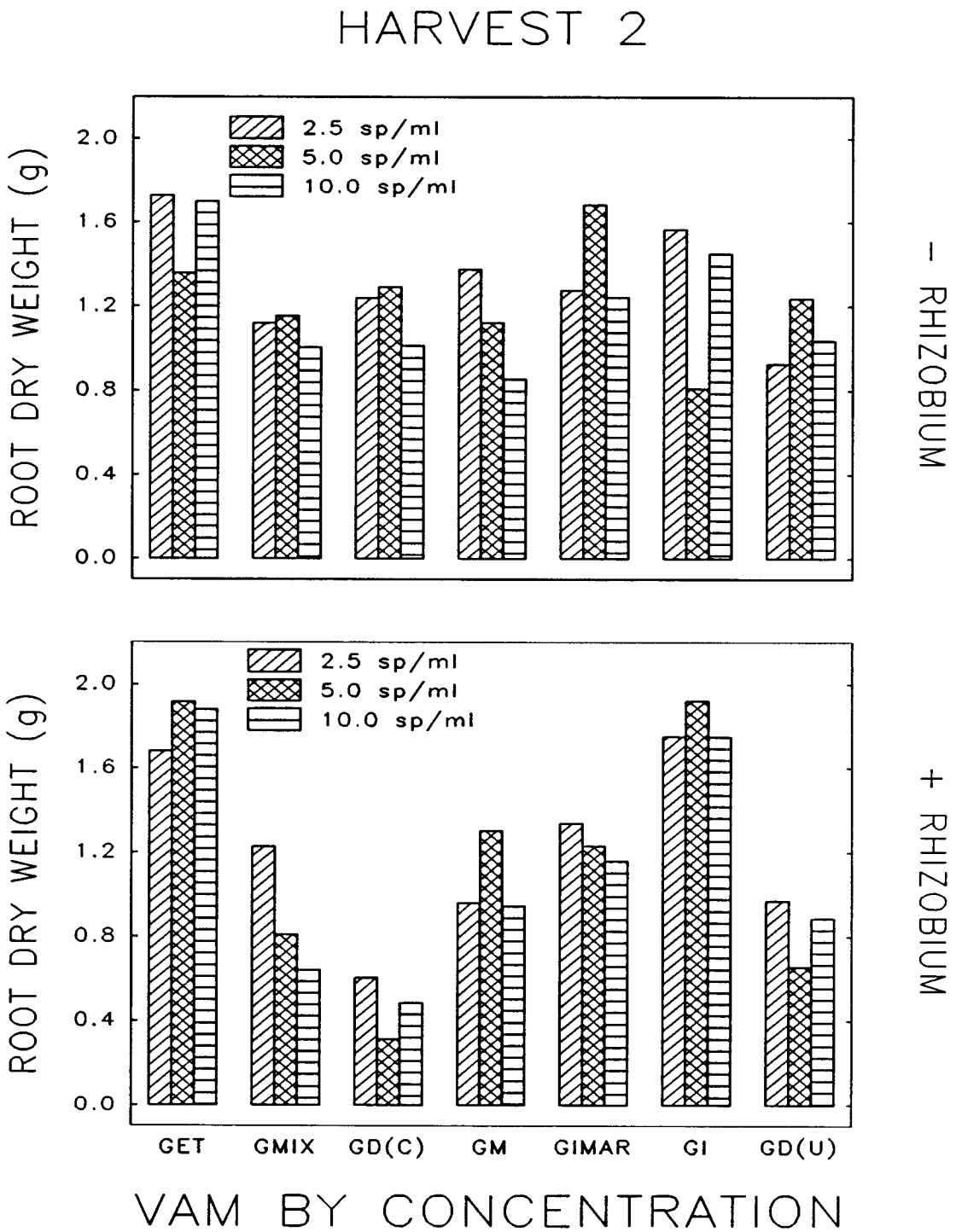

Figure 1-20. Pigeon pea root dry weight at 12 weeks as influenced by VAM fungal isolate, inoculum density, and added Rhizobium.

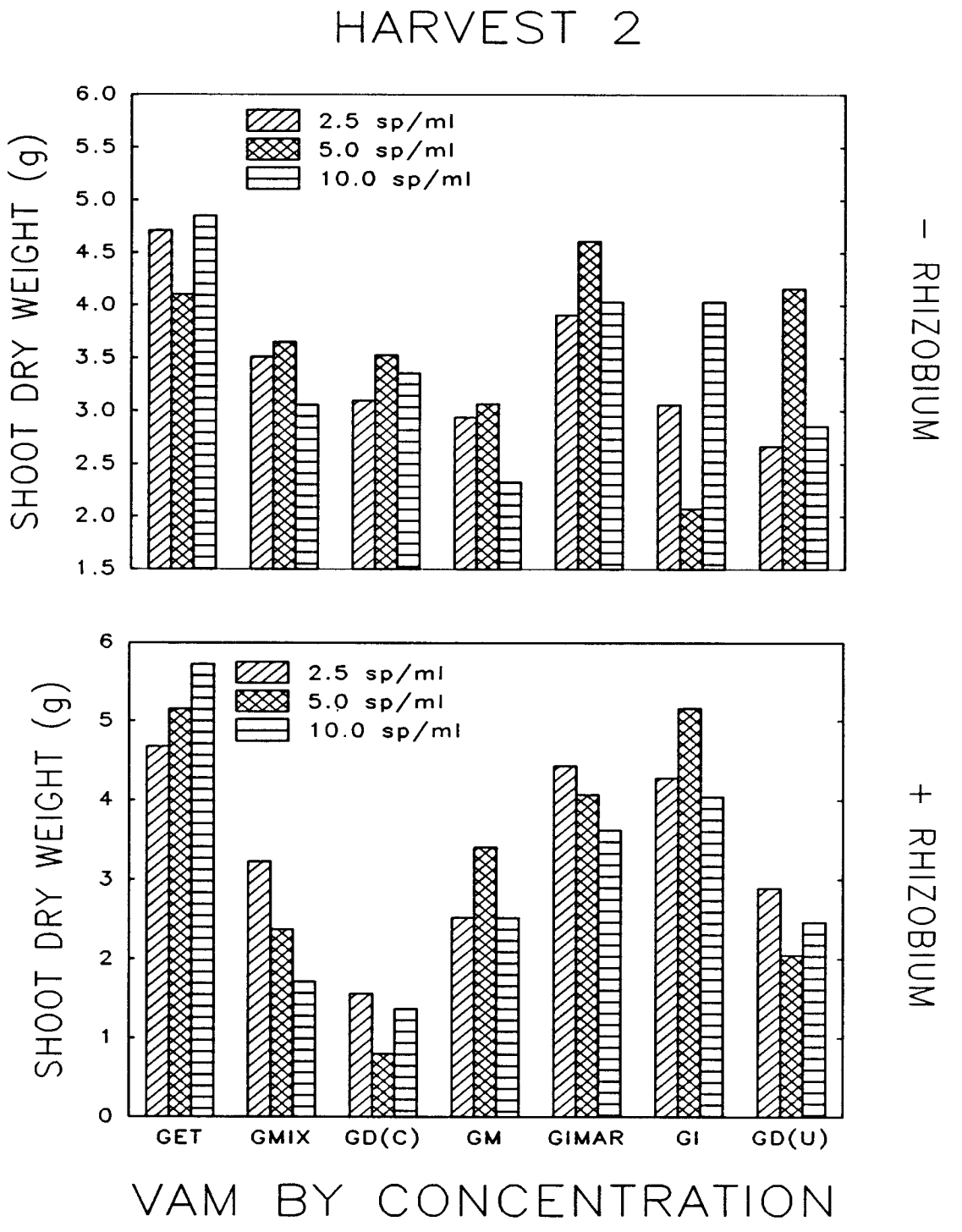

Figure 1-21. Pigeon pea shoot dry weight at 12 weeks as influenced by VAM fungal isolate, inoculum density, and added Rhizobium.

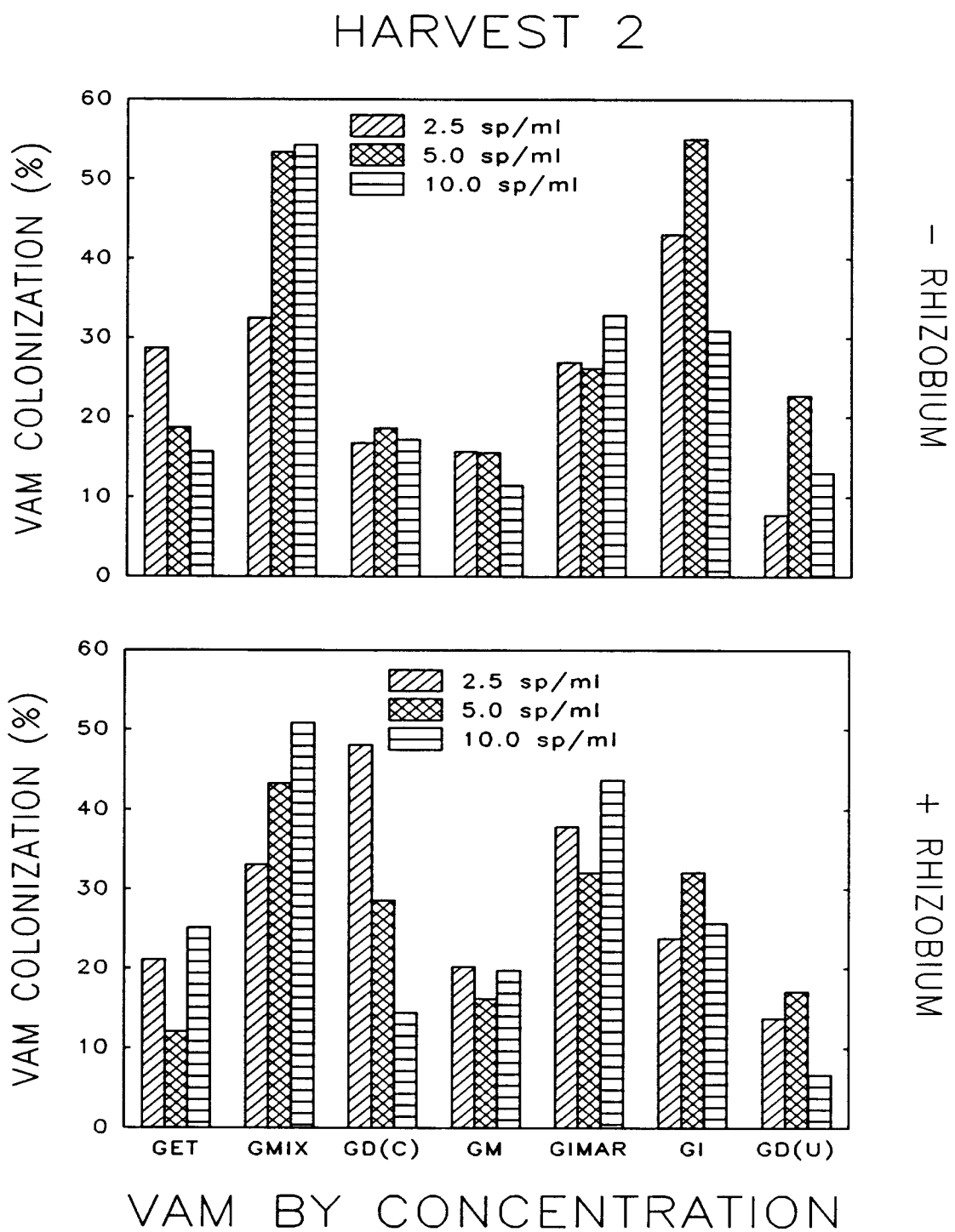

Figure 1-22. Pigeon pea VAM colonization at 12 weeks as influenced by VAM fungal isolate, inoculum density, and added Rhizobium.

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CHAPTER 2

VARIATION IN VA MYCORRHIZAL FUNGUS ISOLATE INTERACTIONS WITH RHIZOBIUM ON PIGEON PEA (CAJANUS CAJAN): I. NITROGEN FIXATION AND GROWTH OF PIGEON PEA

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SUMMARY

Legumes form a dual symbiosis with nodulating Rhizobium and Vesicular-arbuscular (VA) mycorrhizal fungi. Rhizobia in nodules fix N_2 and VA mycorrhizae help the plant tolerate environmental stresses and enhance nutrient uptake. Our objective was to document variation in response of pigeon pea to dual inoculation with an effective Rhizobium and different VAM fungi regarding enhanced plant growth, Rhizobium nodulation, N_2 fixation activity, and VA mycorrhizal colonization.

Seven VAM fungal isolates and a non-mycorrhizal control with or without added Rhizobium were compared. The controls were fertilized with 200 ppm N; all plants were fertilized with 40 ppm P, and N was withheld from VA inoculated plants. Plant harvests were at 6 and 21 weeks, at which time they were evaluated for extent of VAM

colonization, root and shoot weights, nodule number and weights, and nitrogenase activity.

Level of VAM colonization varied greatly between fungi, and the VAM effects on nodulation and N_2 fixation varied, but level of colonization was not correlated with enhancement of N_2 fixation. Some fungi formed extensive VAM, but had no effect on nodulation; others formed low levels of VAM, yet greatly enhanced nodulation.

These data support the contention that pigeon pea, and probably other legumes, must be inoculated with strains of Rhizobium and VA mycorrhizal fungi that are compatible with each other (interendophyte) in order to maximize nodulation, symbiotic N_2 fixation and thus growth enhancement under N-limiting conditions.

INTRODUCTION

Many researchers have investigated aspects of the interaction between VA mycorrhizal (VAM) fungi and Rhizobium (Cluett and Baucher, 1983; Barea and Azcón-Aguilar, 1985; Tilak, 1985; Linderman and Paulitz, 1989). The general consensus that has emerged is that VA mycorrhizae possibly influence the rhizobial symbiosis, resulting in enhanced nodulation (size and number) and nitrogen fixation (Van Nuffelen and Schenck, 1983; Kawai and Yamamoto, 1986; Subba Rao et al., 1986; Ames and Bethlenfalvay, 1987). It has generally been assumed that the combination of VAM and rhizobial symbioses would enhance plant growth more than with either symbiont alone. Many have supported the contention that growth enhancement when VAM are present is due to enhanced phosphorus (P) or micronutrient uptake (needed for

N_2 fixation) (Manjunath and Bagyaraj, 1983; Kawai and Yamamoto, 1986; Subba Rao et al., 1986), and to a general increase in photosynthesis and photosynthate partitioned to roots with a stronger C sink due to the presence of both symbionts (Pang and Paul, 1980; 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 result in significant increases in photosynthesis and associated partitioning to the host roots (Brown and Bethlenfalvay, 1987).

Ames and Bethlenfalvay (1987) studied the tripartite interaction between the VAM fungus Glomus macrocarpum and Rhizobium strains on Vigna unguiculata (cowpea) and demonstrated interactions that were not P-mediated. They used a split-root system to demonstrate a localized, non-systemic increase in nodule activity in roots with both nodules and VAM. Although they used only one VAM species and did not report nodule numbers or size (and thus cannot rule these out as affecting nitrogenase (nodule) activity), their work counters the broadly held view that improved P nutrition is the sole mechanism whereby VA mycorrhizae contribute to the enhanced N_2 fixation in the tripartite interaction.

Most of the research reported on Rhizobium-VAM interactions has been based upon a few mycorrhizal fungus species under differing environmental conditions and often with P-responsive hosts (eg. Glycine max and Vigna unguiculata (Hume et al., 1985; Skerman et al., 1988). However, little work has been done comparing different VAM fungi under uniform environmental conditions. Exception to that, however, was the work of Van Nuffelen and Schenck (1983) who compared

six species of mycorrhizal fungi on soybean (Glycine max), but their main interest was on fungal parameters (spore germination, penetration, and root colonization) and not on the effects of different fungal species on Rhizobium nodulation and N_2 fixation. The subject of inter-endophyte compatibility (Bayne and Bethlenfalvay, 1987), which may play an important role in the effectiveness of the overall VAM-Rhizobium-host interaction, has received little attention. In an earlier experiment (Ianson and Linderman, unpublished results), we observed significant differences in number and size of rhizobial nodules on roots of plants inoculated with eight different VAM fungi. Development of effective N_2-fixing associations consists of a number of steps of which the induction of nodulation is only one (Vance, 1983). VA mycorrhizae 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 those VAM fungal species that induced an increase in numbers of nodules in an earlier study also enhanced N_2-fixing activity of the nodules, and to compare VAM mycorrhizal fungi of different ecodaphic origin to identify the most compatible (in terms of increasing host growth and N_2-fixation) interendophyte combination.

MATERIALS AND METHODS

Microbial Inoculum

VAM Fungi

Seven VAM fungal isolates or species were used in this experiment. Species name and culture origin are listed in Table 2-1. The VAM inocula used in this experiment were obtained from pot cultures of pigeon pea grown in a 1:1 mixture of Willamette sandy loam soil harvested from a previous experiment. Spore numbers were determined for these inocula and each was diluted with Willamette River sand to a spore density of 15 spores ml^{-1} of inoculum. A 100 g aliquot of inoculum was placed in a column beneath each transplanted seedling (Fig. 2-1).

Rhizobium

The bacterium used was a Rhizobium Cowpea strain (P132-1 Arhar) isolated from Pigeon Pea (Cajanus cajan (L.) Millsp. var Corg-5) and 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 four days before inoculation of plants.

Plant and Soil Preparation

Pigeon Pea seeds were pregerminated in sterile distilled water at 27 C for four days and dipped into the YMB Rhizobium culture. The number of colony forming bacteria per seed was estimated by dilution plating the bacteria washed from five inoculated seeds on Yeast

Mannitol Agar (YMA) plates in the dark at 30 C for five days. Due to the size of the experiment, plants for two harvest times and two groups were planted one week apart. The bacterial counts from treated seed were: harvest 1-first group-0.8334 x 10^6 seed^{-1}; harvest 1-second group-1.47 x 10^6 seed^{-1}; harvest 2-first group-83 x 10^6 seed^{-1}; harvest 2-second group-16.6 x 10^6 seed^{-1}.

The soil medium used in this experiment was a 1:1 mixture of Willamette sandy loam (Ph 6.0) and river sand which contained: 0.02% total nitrogen, 12 mg kg^{-1} phosphorus, 70 mg kg^{-1} potassium and 8.5 mequiv. calcium per 100 g of soil. To reduce microfloral differences between controls and VAM-inoculated plants, the Control inoculum was prepared from a Gigaspora margarita-pigeon pea pot culture sand/soil medium that proved beneficial to rhizobial symbiosis in a previous pigeon pea experiment. The VAM 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) in a soil extract prepared by filtering nonpasteurized Gigaspora sand/soil medium (10% by volume of the total control inoculum to be used) through Whatman #1 paper to retain VAM 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).

Experimental Design

Plants were completely randomized on greenhouse benches with 11 fungal treatments (seven fungi and four non-VAM controls). Plants for the first harvest all were inoculated with Rhizobium; plants for the second harvest were either inoculated or not with Rhizobium. Seedlings were transplanted into 84 mm x 84 mm x 152 mm pots (volume 1080 cm^3).

Plant Nutrition

Plants were fertilized weekly with a modified Hoagland's solution (Table 2-2) beginning at the first trifoliate leaf stage (three weeks following transplant). The macronutrients were diluted 1:1 with tap water before fertilization. Iron was provided as Fe citrate at 2 ml l^{-1} fertilization volume (3.6 ppm Fe). The non-VAM controls were fertilized with the modified Hoagland's, but with varying amounts of KNO_3. Final solutions for these controls contained 100, 150, 200, or 300 ppm nitrogen as KNO_3.

First Harvest

At six weeks, the first plants were harvested, 20 replicates per treatment. Roots and shoots were separated, and roots were reserved for assessment of nitrogen fixation, VAM colonization, nodulation and dry weights. The root samples were subdivided into two groups of 10 replicates each. Nitrogen fixation assays, root dry weights, and nodule fresh weights were carried out on the first

group, VAM colonization was estimated on the second group. Plant shoots were dried at 60 C for 48 h and weighed.

Nitrogen Fixation and Rhizobium Nodulation

To estimate bacterial nitrogen fixation (the reduction of atmospheric dinitrogen to ammonia as catalyzed by the nitrogenase enzyme), the indirect method of measuring the capacity of the bacterial enzyme to reduce acetylene (C_2H_2) to ethylene (C_2H_4) was used. At harvest intact Pigeon Pea roots were blotted dry (to touch) and placed in 250 ml test tubes sealed with a special rubber stopper (Fig. 2-2). Ten percent of the tube gas volume was immediately removed by syringe and replaced with purified acetylene. After time for mixing, a 1 cc volume of the gas phase 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 one hour, 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. Each control C_2H_4 value (first sample) was subtracted from its corresponding second sample. Gas chromatographic parameters were: oven temperature-isothermal run at 55 C, injection port temperature-150 C, Flame Ionization Detector-temperature-250 C, attenuation-2 ⇑ 2, chart speed 0.2 cm min^{-1}. Following the acetylene assay, root samples were spread

over a grid and nodules (> 500 μm) were counted, removed and fresh weights determined.

VAM Colonization

Whole fresh root samples (ten replicates) were blotted dry (to touch), weighed and cut into 0.5 to 1.0 cm segments. Segments were cleared overnight at 55 C in 10% potassium hydroxide and stained in trypan blue (0.05%) in lactoglycerol according to the methods of Phillips and Hayman (1970), and assayed for VAM colonization according to the method of Biermann and Linderman (1981). Root subsamples from ten plant replicates were randomly pooled into 6-8 samples per fungal treatment and 25 segments per sample were examined for % root length with VAM colonization. The number of root segments examined per sample was determined statistically by examining 3 samples with 50 and 25 segment samples in each and comparing variability.

Second Harvest

At 21 weeks, a second group of plants was harvested. Root and shoot dry weights, nodule weights, numbers, and N_2-ase activity and VAM colonization were assayed. VAM colonization was assayed by taking 1-cm subsamples from the bottom of the root system and from 2.5 cm below the crown. The subsamples were weighed to estimate their proportion of the total root system weight. The remaining root system of each plant, minus the VAM subsample was weighed fresh and oven dried at 60 C for 48 h and dry weight was determined. The ratio

of dry weight to fresh weight was used to determine the expected dry weight of the root aliquot removed for VAM determinations (to be added to the whole root dry weight). Shoot dry weights, nodule weights, numbers, and activity were assayed as previously outlined. Phosphorus content of nodule tissue was determined colorimetrically according to Aziz and Habte (1987). Nodule tissue from each treatment was randomly pooled to give three samples per treatment. These (oven-dried) nodules were ground in a Wiley mill to pass through a number 60 sieve. One hundred mg of tissue was dry ashed at 500 C for 3 h. The ash was digested in 1 N HCl and diluted with 20 ml distilled water. Color was developed according to the molybdenum blue technique (Murphy and Riley, 1962).

Statistical Analyses

Plants for the first harvest were sown 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 and results were analyzed according to a Multifactorial Analysis Of Variance. Significant differences were compared as outlined above.

RESULTS

The influence of VAM isolate on plant root and shoot mass was similar for both harvests. Inoculation by some VAM fungi (i.e. Gig. margarita and G. intraradices) resulted in earlier VAM establishment and significantly larger roots and shoots (Figs. 2-3, 2-4, 2-5) than with the other VAM fungi. This trend continued throughout the time course of the experiment. Plants inoculated with G. etunicatum established VAM early, but root and shoot size increases were only apparent by the second harvest. Plants inoculated with G. mosseae had less VAM colonization than with Gig. margarita, G. intraradices, or G. etunicatum, but had comparable increased root growth as did the non-VAM control (Figs. 2-3, 2-4, 2-5). Plants inoculated with either isolate of G. deserticola were consistently among the smallest (Figs. 2-3, 2-4). The difference between root and shoot mass in control plants fertilized with different levels of N was striking (Figs. 2-3, 2-4). Adding 200 ppm nitrogen to the controls resulted in relatively large roots, but did not appear to affect the relative acetylene reducing efficiency of the nodules. Nodules on plants in that treatment were consistently small, but their ethylene production values were among the highest of all treatments (Figs. 2-6, 2-7, 2-8). Number, fresh weight, and ethylene production by nodules on plants inoculated with G. intraradices were high even through the second harvest, indicating that nodules remained relatively efficient regardless of their size (Figs. 2-6, 2-7).

Inoculation of plants with Gig. margarita resulted in the production of more and larger nodules than any other treatment (Figs.

2-7, 2-8), but by the second harvest those nodules were less efficient in the acetylene reduction assay (ARA) (Fig. 2-6). Inoculation with either isolate of G. deserticola similarly resulted in relatively small and few nodules, but their effects on nodule efficiency in the acetylene reduction assay were dissimilar. At the first harvest, Gd(U) and Gd(C) reduced similar amounts of acetylene per unit mass of nodules, but Gd(U) nodule activity was strikingly increased by the second harvest, but that of Gd(C) was not (Figs. 2-6, 2-8). The acetylene reducing efficiency of nodules on all VAM inoculated roots, except Gd(U), decreased from the first harvest to the second harvest, whereas nodule numbers increased (Figs. 2-6, 2-8). Plotting fungal treatment means for nodule fresh weight versus plant root and shoot weights revealed that nodule weight increased with shoot weight at both harvests (Figs. 2-9); however, establishing nodules by the young seedlings at harvest 1 resulted in a decrease in root weight (Fig. 2-10). Establishment and maintenance of Rhizobium nodules varied with inoculation by different VAM, especially early in the experiment. As a result it was difficult to establish any correlation between nodule fresh weight and root and shoot weights (Figs. 2-9, 2-10). By the second harvest, however, a correlation was evident, and the data suggested that nodule fresh weight increased with increasing root and shoot weights (Figs. 2-9, 2-10).

Variations in nodule activity in the ARA versus shoot and root weight occurred between plants inoculated with different VAM isolates or species. The great variation within treatments by the second harvest date, however, obscured most trends (Figs. 2-11, 2-12). In

general, nodule activity increased with increasing plant shoot and root weight at the first harvest (Figs. 2-11, 2-12). Despite variability within fungal species treatments, acetylene reduction did decrease with increases in shoot weight at the second harvest (Fig. 2-11).

All VAM-inoculated plants, except those inoculated with Gd(U) and the non-inoculated control, exhibited increased growth from harvest 1 to harvest 2 that were not proportional to rate and extent of VAM colonization (Fig. 2-6). The appearance of some VAM fungal structures in the non-inoculated plant roots was probably due to VAM propagules passing through the filtering solution process. No significant differences were detected ($P<0.05$) in the analysis of nodule phosphorus content on plants from the various VAM fungus treatments.

DISCUSSION

In order for legumes and symbiotic rhizobia to form and maintain functional nodules, capable of fixing atmospheric nitrogen, 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 nitrogen fixation and host yield. The tripartite interaction between VAM, Rhizobium, and a host legume is more complex than the dual symbiosis between Rhizobium and host, and is less well understood. Furthermore, variations in

VAM fungal isolate interactions with rhizobia on Pigeon Pea demonstrated in this study indicate even greater complexity. Some isolates supported the accepted hypothesis that the addition of VA mycorrhizae to the Rhizobium-legume symbiosis results in greater host growth, more and larger nodules, improved nitrogen fixation and increased VAM colonization, but some isolates did not. Previous workers showed increases in shoot dry weight from inoculation with the VAM isolate G. mosseae (Smith and Daft, 1977; Assimi et al., 1980; Van Nuffelen and Schenck, 1984), but we did not. Van Nuffelen and Schenck (1984) observed that inoculation with Gig. margarita and G. etunicatum did not produce significant increases in shoot dry weight of soybean when compared to a non-inoculated control, and that plants inoculated with G. intraradices had increased shoot dry weight in one experiment and not in another. We, however, observed that inoculation with all three of these isolates significantly increased shoot dry weight of pigeon pea as compared to the non-inoculated control (Fig. 2-3).

Compatibility within the tripartite symbiosis was also expressed in terms of Rhizobium colonization and nodule formation (numbers), growth, and function (nitrogenase activity). Some VAM treatments significantly increased number and weight of nodules, and some decreased nodulation compared to the control (Figs. 2-7, 2-8). The observed increased nodulation as affected by some VAM fungi concurs with numerous previous observations (Smith and Daft, 1977; Smith et al., 1979; Varma, 1979). The decreased nodulation exhibited

by some VAM treatments, however, is not as commonly reported, but does concur with the report by Bethlenfalvay et al. (1985).

These differential responses may be explained on the basis of VAM-induced changes (or lack thereof) in the host plant physiology, or changes in the microbial composition and activities in the rhizosphere or on the rhizoplane. A number of interactions between VAM, Rhizobium, plant host and other rhizosphere microflora have been summarized by Linderman and Paulitz (1989). They point out that VA mycorrhizal fungi and Rhizobium could directly interact in the rhizosphere in some way that encourages more Rhizobium to successfully infect host root hairs. There is currently no experimental evidence to support this idea.

It is generally assumed that VA mycorrhizal fungi colonize roots before rhizobia and thereby alter the quantity and quality of root exudates available to the Rhizobium in the rhizosphere. Among the constituents of these exudates are isoflavonoid compounds (Morandi and Bailey, 1984), some of which have been reported to induce nod D gene expression which in turn regulates transcription of other nod genes (eg. nod A, B, and C) and thus ultimately the nodulation process (Peters et al., 1986; Redmond et al., 1986; Djordjevic et al., 1987; Györgypal et al., 1988; Phillips et al., 1988; Rolfe and Gresshoff, 1988; Sadowsky et al., 1988; Wijffelman et al., 1988). In some cases these compounds chemotactically attract rhizobia (Caetano-Anallés et al., 1988). Alteration of plant root exudation by VAM could also favor certain groups of rhizosphere microflora like pseudomonads that would interact with VAM, rhizobia,

and the host, allowing more initial rhizobial infections to proceed to nodulation. The presence of pseudomonads in the rhizosphere has been observed to increase nodulation (Azcón-Aguilar and Barea, 1978; Grimes and Mount, 1984; Meyer and Linderman, 1986a), and colonization by VA mycorrhizal fungi have been shown to selectively favor the growth of pseudomonads (Meyer and Linderman, 1986b). Perhaps certain VAM alter root exudation patterns such that nod D gene expression is induced, possibly by enhancing the growth of "helper" microorganisms like pseudomonads, whereas other VAM would not (or to a lesser degree). By these means, some VAM isolates would be compatible in the Rhizobium-legume system, others not.

Sargent et al. (1987) point out that fewer than 10% of initial Rhizobium infections ever proceed to nodule formation. The rest are aborted, perhaps due to a host defensive response. Differences in nodulation may also be due to a delayed plant defense response (i.e. phytoalexin production) as affected by certain VAM. Rhizobium nodulation takes place via bacterial adhesion to root hairs, followed by invasion by bacterial infection threads and nodule initiation. Phytoalexins could interrupt this process. 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 disease organisms like Fusarium (Chakraborty and Chakraborty, 1988). Colonization by some VA mycorrhizal fungi could delay this plant defense response and thus allow more rhizobial infections to

proceed to nodule initiation. This could explain the significant differences in nodulation we observed (Fig. 2-7).

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 and the cost to the host of maintaining a tripartite symbiosis could dictate the extent of VAM colonization as well as the extent of nodule development and N_2 fixation. Differences in the efficiency of P uptake by certain VAM 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 colonization when plants were inoculated with different VA mycorrhizal fungi (Figs. 2-5, 2-6, and 2-7). Further evidence of the importance of balance between carbon cost and nutritional uptake as influenced by VAM was seen in the sharp drop from the first to second harvests in N_2 fixation and VAM colonization in all fungal treatments except G. deserticola (U) (Figs. 2-5 and 2-6). The second harvest occurred when plants were at the stage between flowering and seed set. The partitioning of C away from the root and both symbionts to the developing reproductive system may have deprived the symbionts, forcing nodule senescence and reducing N_2 fixation as well as slowing VAM spread in relation to root growth. We speculate that this C drain could have somehow been ameliorated more by compatible than incompatible VAM isolates. This speculation

is supported by the lack of a good correlation between nitrogenase activity and shoot and root dry weights (Figs. 2-11, 2-12).

The uptake of elements other than P, such as Cu and Zn, (essential to the Rhizobium nodulation) could be preferentially affected by certain VAM isolates. The uptake of these elements have been documented to increase with mycorrhizal colonization (Pacovsky, 1986). The differences in nodule weight and activity observed in this study (Figs. 2-6, 2-7) with differing VAM isolates may be due to the uptake of these essential elements.

Understanding that there is a "compatibility factor" involved in the interaction between VAM-Rhizobium-host-environment and the role this plays in the rhizosphere is important and is the basis for needed further research. From the applied aspect, "teaming up" compatible symbionts with specific legume hosts for specific environmental needs can enhance host establishment and survival under conditions of low or unavailable P and/or N. From the interest of basic research, our understanding that there is a compatibility phenomenon involved in the VAM-Rhizobium-host interaction provides a starting point for research on the possible mechanisms of the interaction, exploiting the fact that two different VAM isolates can colonize a legume but one enhances N_2 fixation, the other not.

Table 2-1. List of VAM fungal isolates and their origin.

VAM SPECIES	CULTURE ORIGIN	ABBREVIATION
Glomus etunicatum	Native Plants Inc.	Get
Glomus aggregatum/microcarpum	Lowell Young (ARS)	Gmix
Glomus deserticola	California via Lowell Young (ARS)	Gd(C)
Glomus mosseae	Native Plants Inc.	Gm
Gigaspora margarita	Native Plants Inc.	Gimar
Glomus intraradices	Native Plants Inc.	Gi
Glomus deserticola	Native Plants Inc.	Gd(U)

Table 2-2. List of compounds used in a modified Hoagland's solution.

MACRONUTRIENTS (diluted 1:1 in final solution)	g $(100\ l)^{-1}$
K_2SO_4	27.5
$MgSO_4 \cdot 7H_2O$	49.0
KH_2PO_4	17.62
K_2HPO_4	22.4
$CaSO_4$	81.6
$CaCl_2 \cdot 2H_2O$	6.0
MICRONUTRIENTS (1 ml l^{-1} added to final solution)	**g l^{-1}**
H_3BO_3	0.23
$MnSO_4$	0.12
$ZnSO_4 \cdot 7H_2O$	0.22
$CuSO_4 \cdot 5H_2O$	0.08
$Na_2MnO_4 \cdot 2H_2O$	0.02
$CoCl_2 \cdot 6H_2O$	0.04
$NiCl_2 \cdot 6H_2O$	0.04

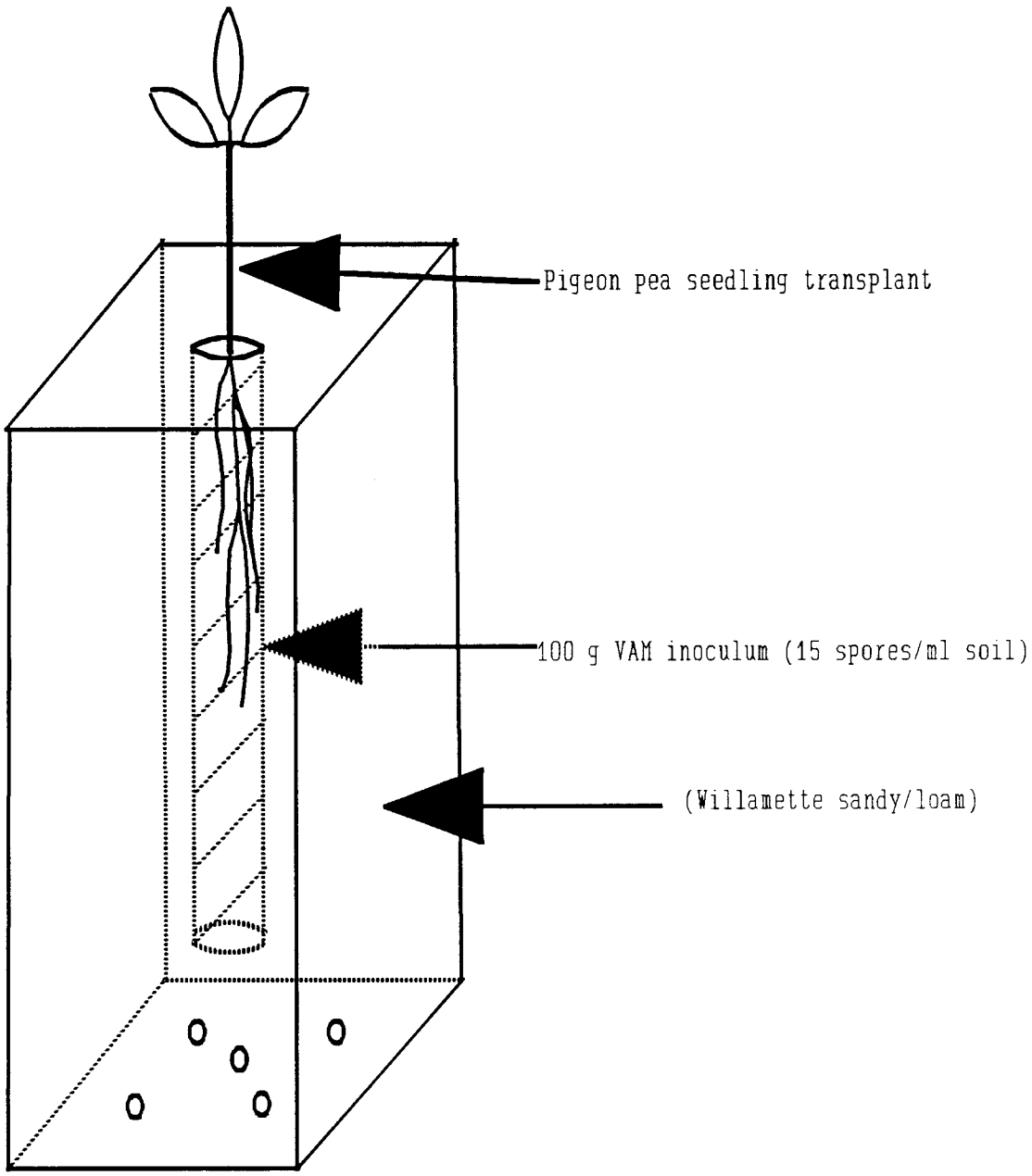

Figure 2-1. Design of VAM inoculation procedure using a central cylinder of inoculum.

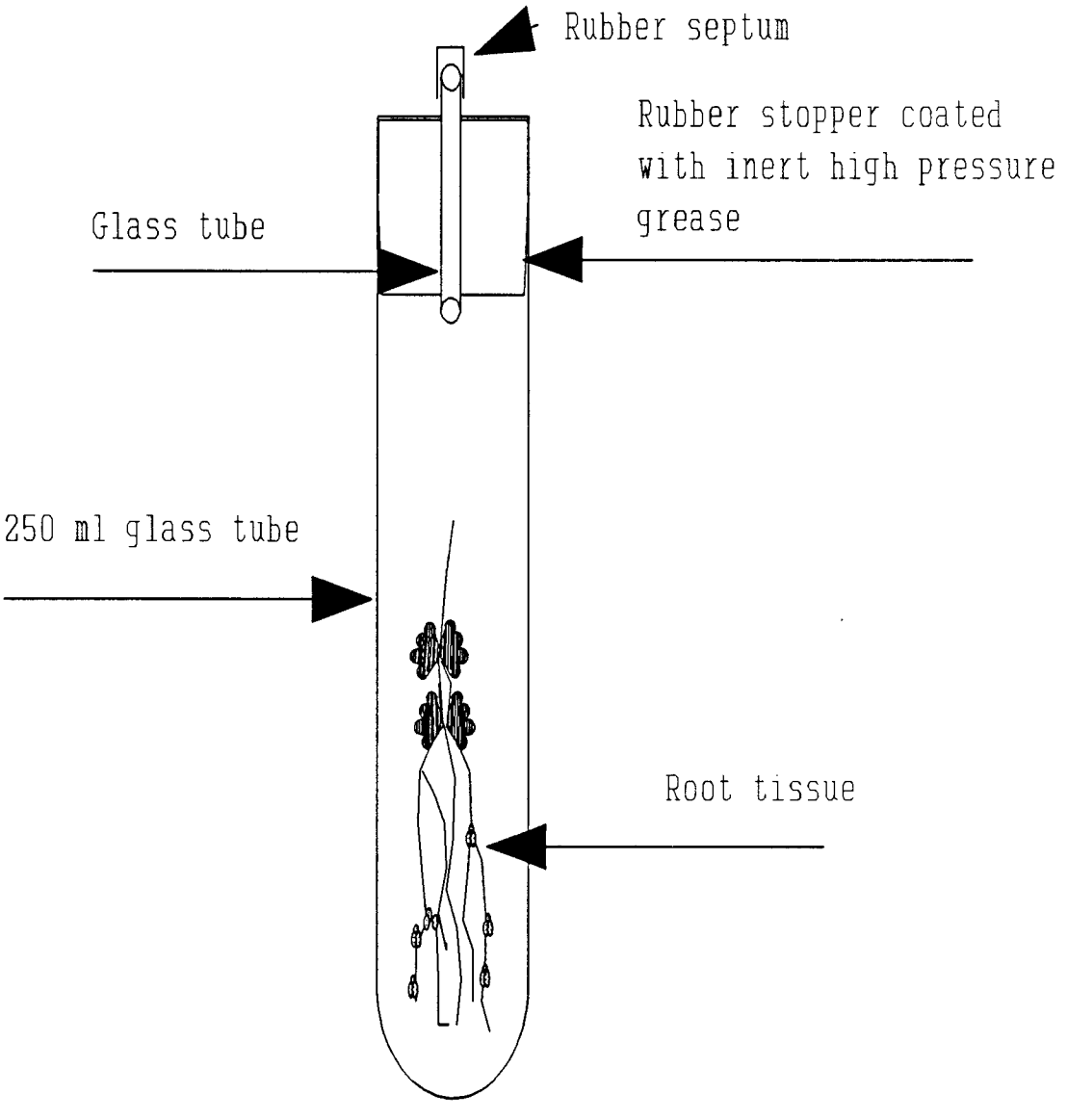

Figure 2-2. Incubation apparatus for acetylene reduction assay.

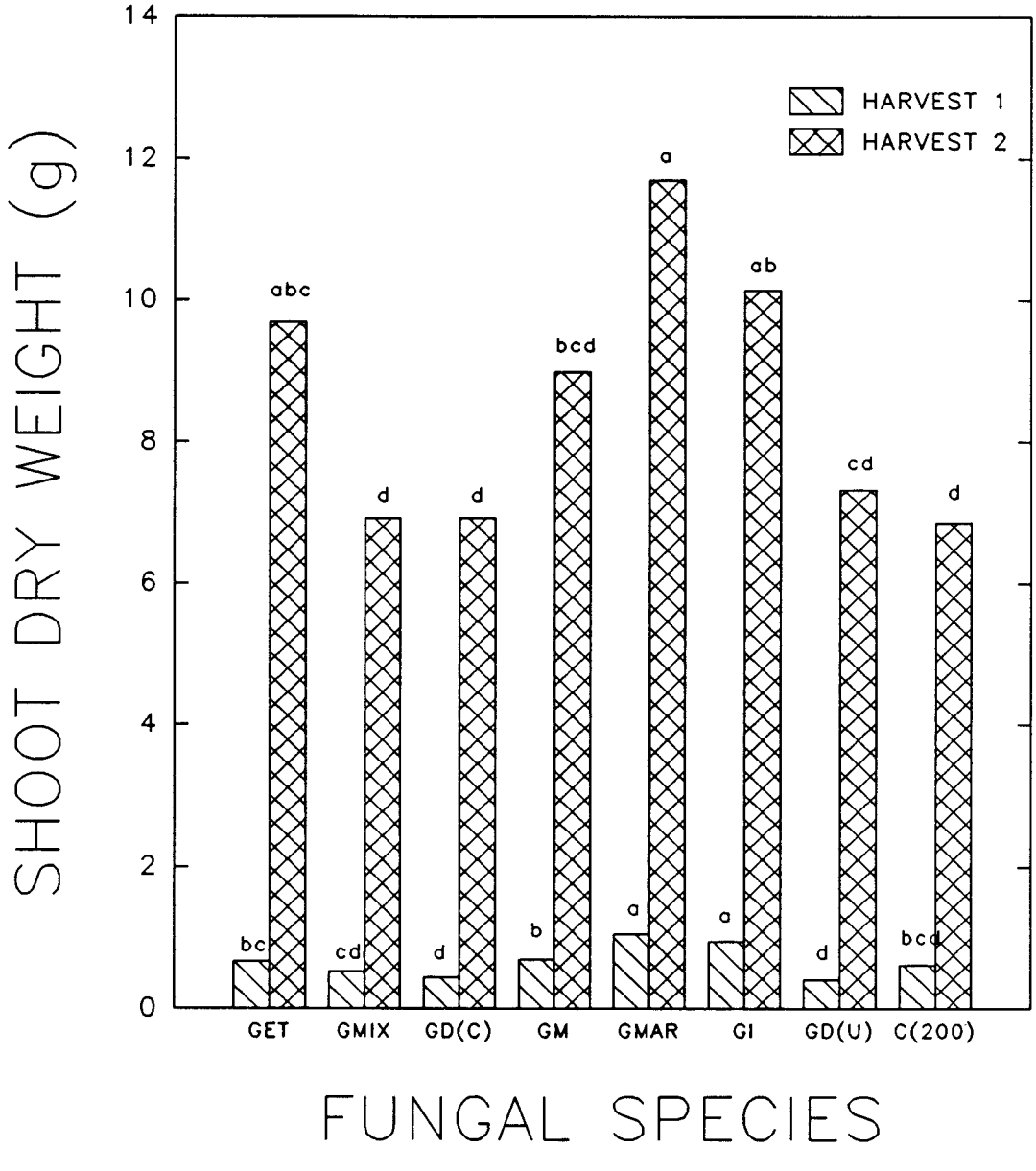

Figure 2-3. Mean shoot dry weight as influenced by VAM fungal treatments. Means within a harvest and with common letters are not significantly different ($P<0.05$).

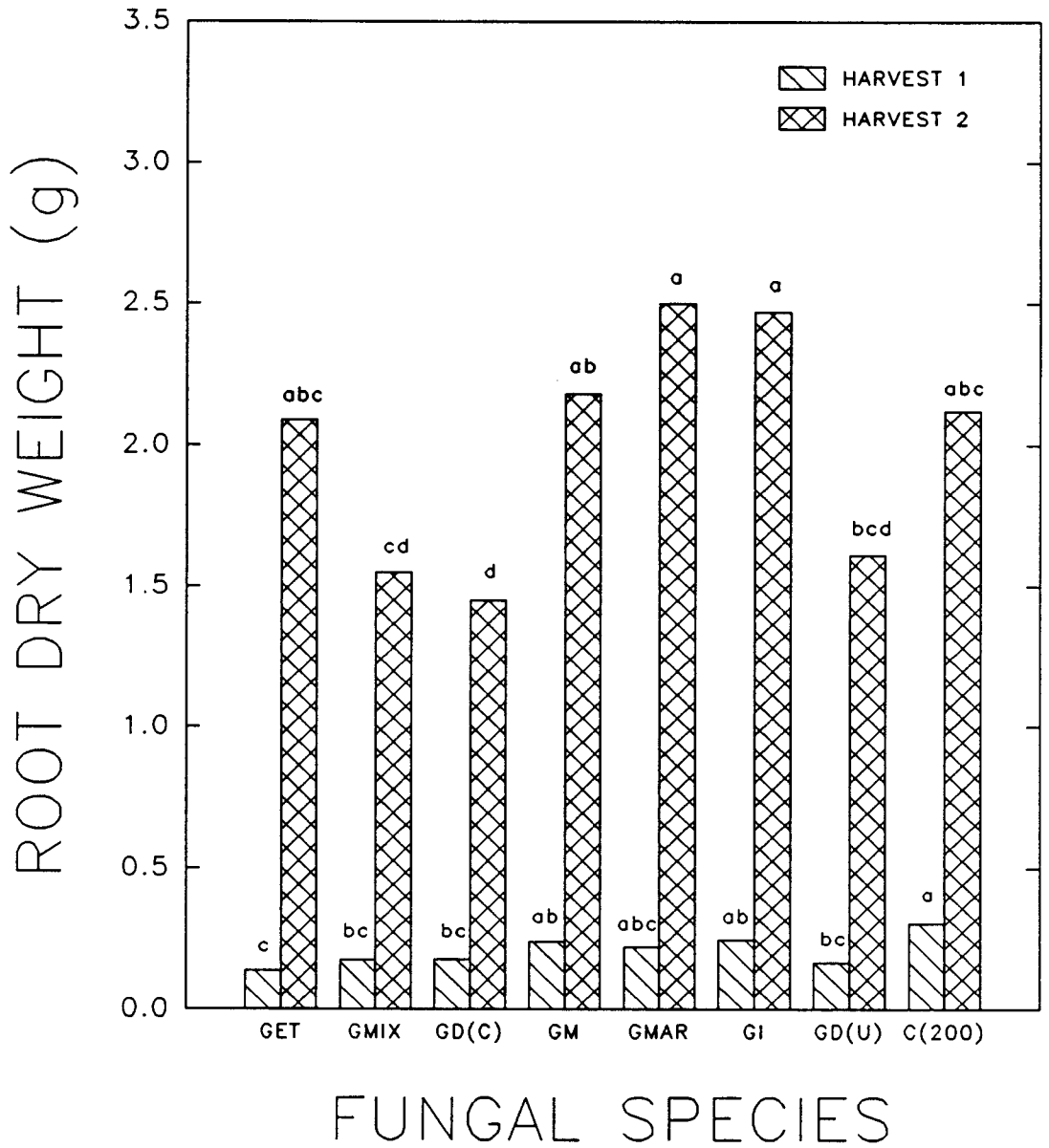

Figure 2-4. Mean dry root weights as influenced by VAM fungal treatments. Means within a harvest and with common letters are not significantly different ($P<0.05$).

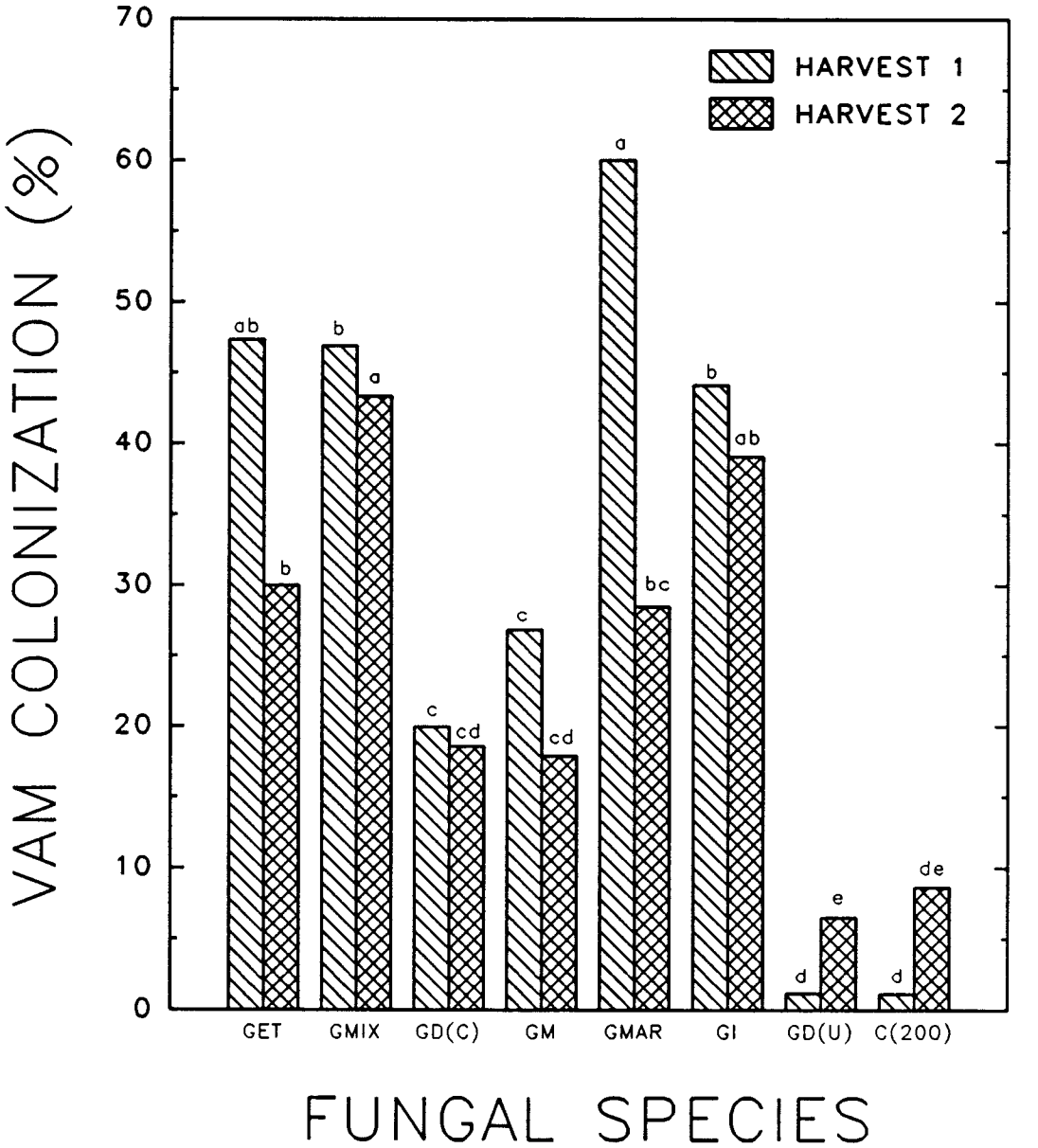

Figure 2-5. VAM colonization (% root length) of pigeon pea roots as influenced by VAM fungal treatment. Means within a harvest and with common letters are not significantly different ($P<0.05$).

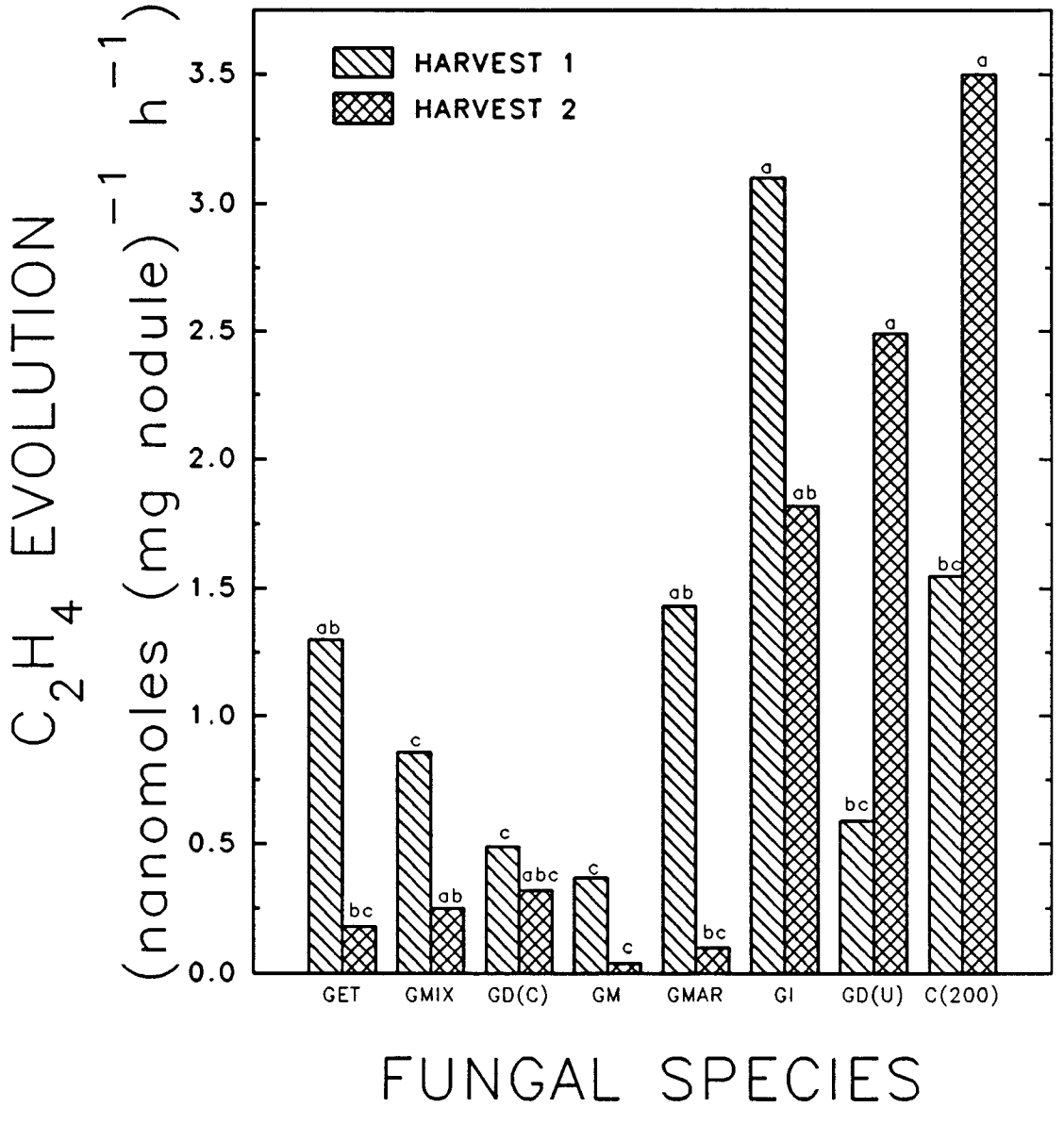

Figure 2-6. Mean C_2H_4 evolution values from the acetylene reduction assay as influenced by VAM fungal treatment. Means within a harvest and with common letters are not different ($P<0.05$).

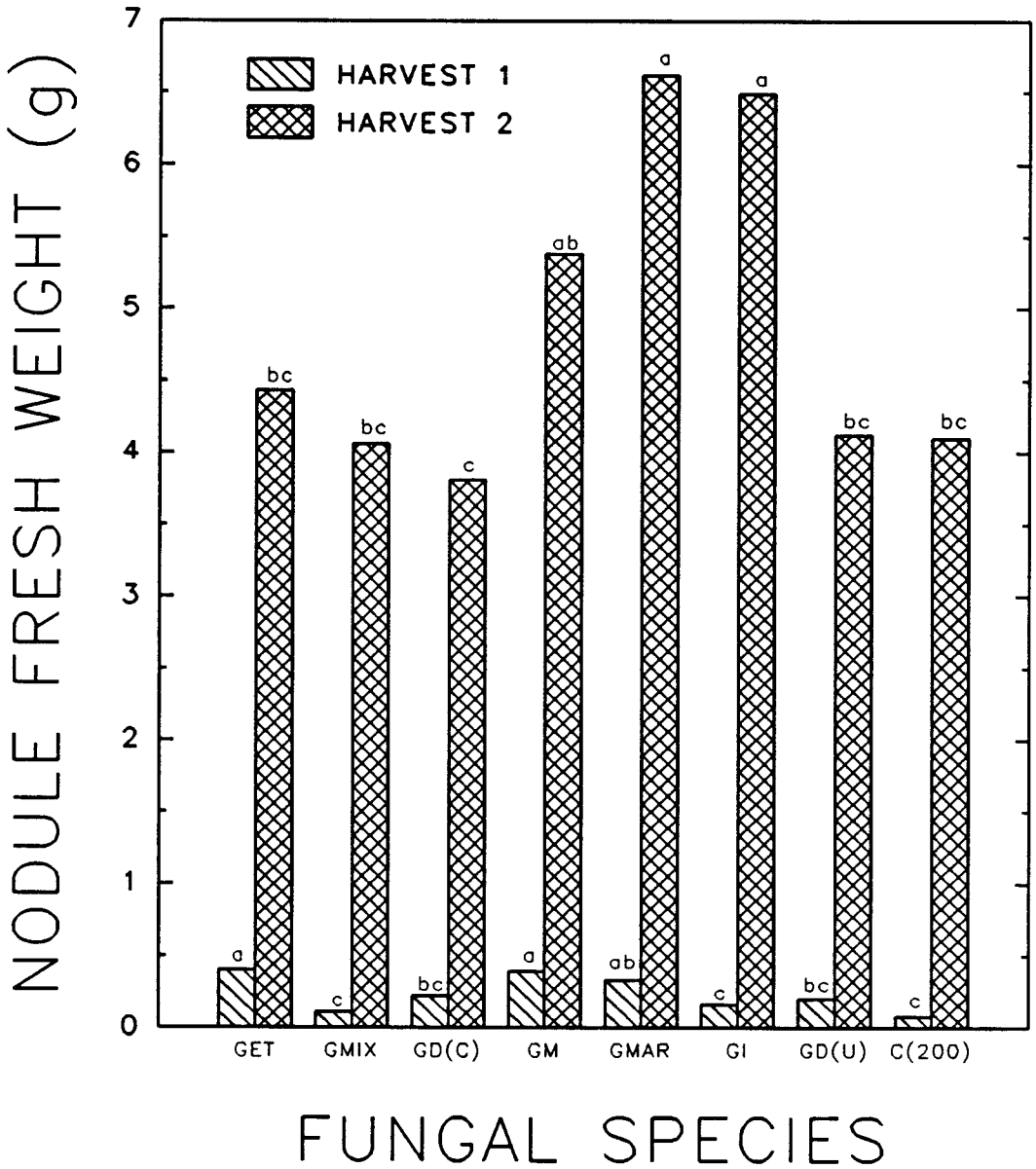

Figure 2-7. Nodule fresh weight as influenced by VAM fungal treatment. Means within a harvest and with common letters are not significantly different ($P<0.05$).

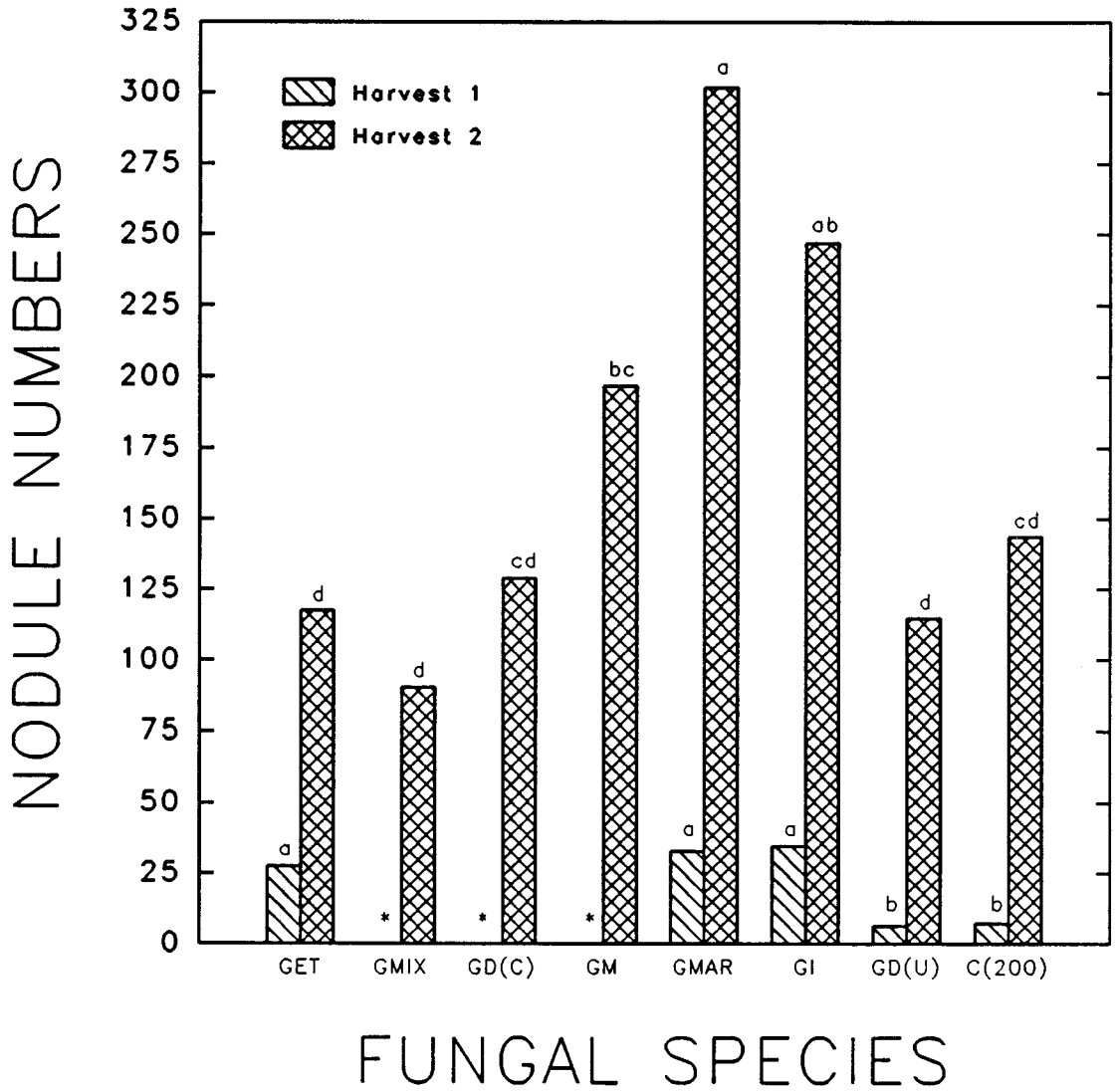

Figure 2-8. Nodule numbers as influenced by VAM fungal treatment. Means within a harvest and with common letters are not significantly different ($P<0.05$). * Indicates missing treatments.

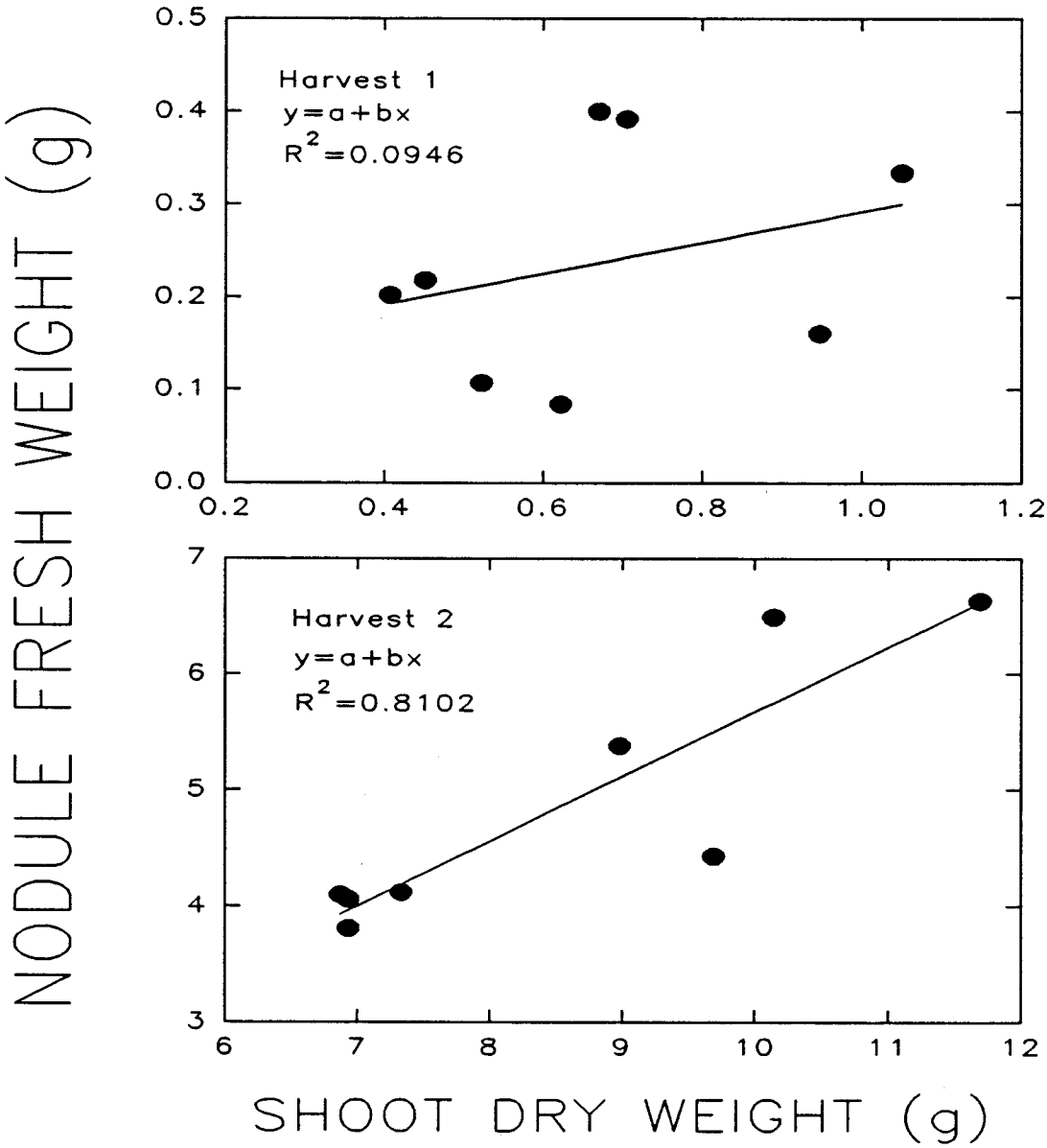

Figure 2-9. Shoot dry weight versus nodule fresh weight on plants inoculated with different VAM fungal isolates evaluated at harvest one (6 weeks) and harvest two (21 weeks).

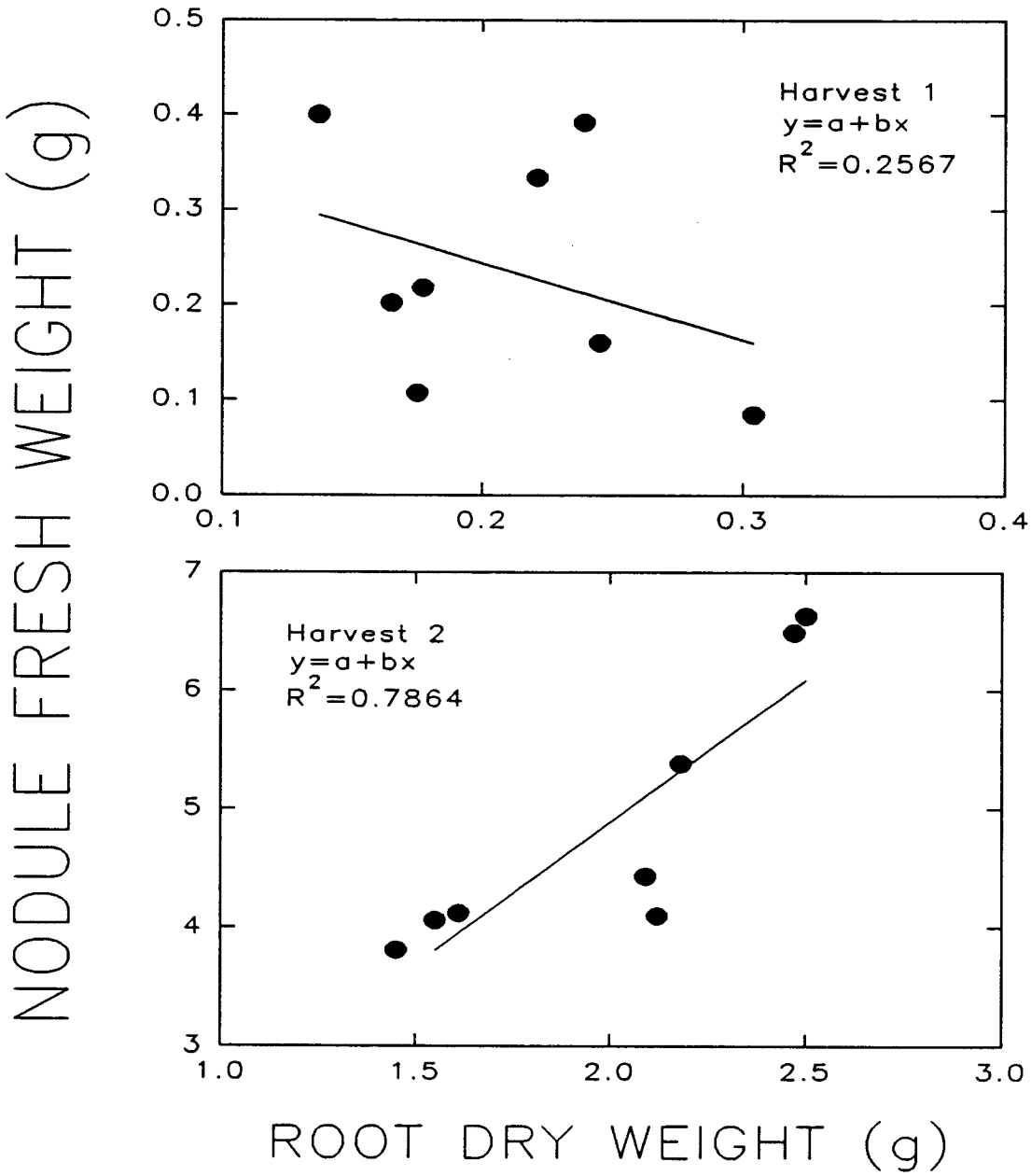

Figure 2-10. Root dry weight versus nodule fresh weight on plants inoculated with different VAM fungal isolates evaluated at harvest one (6 weeks) and harvest two (21 weeks).

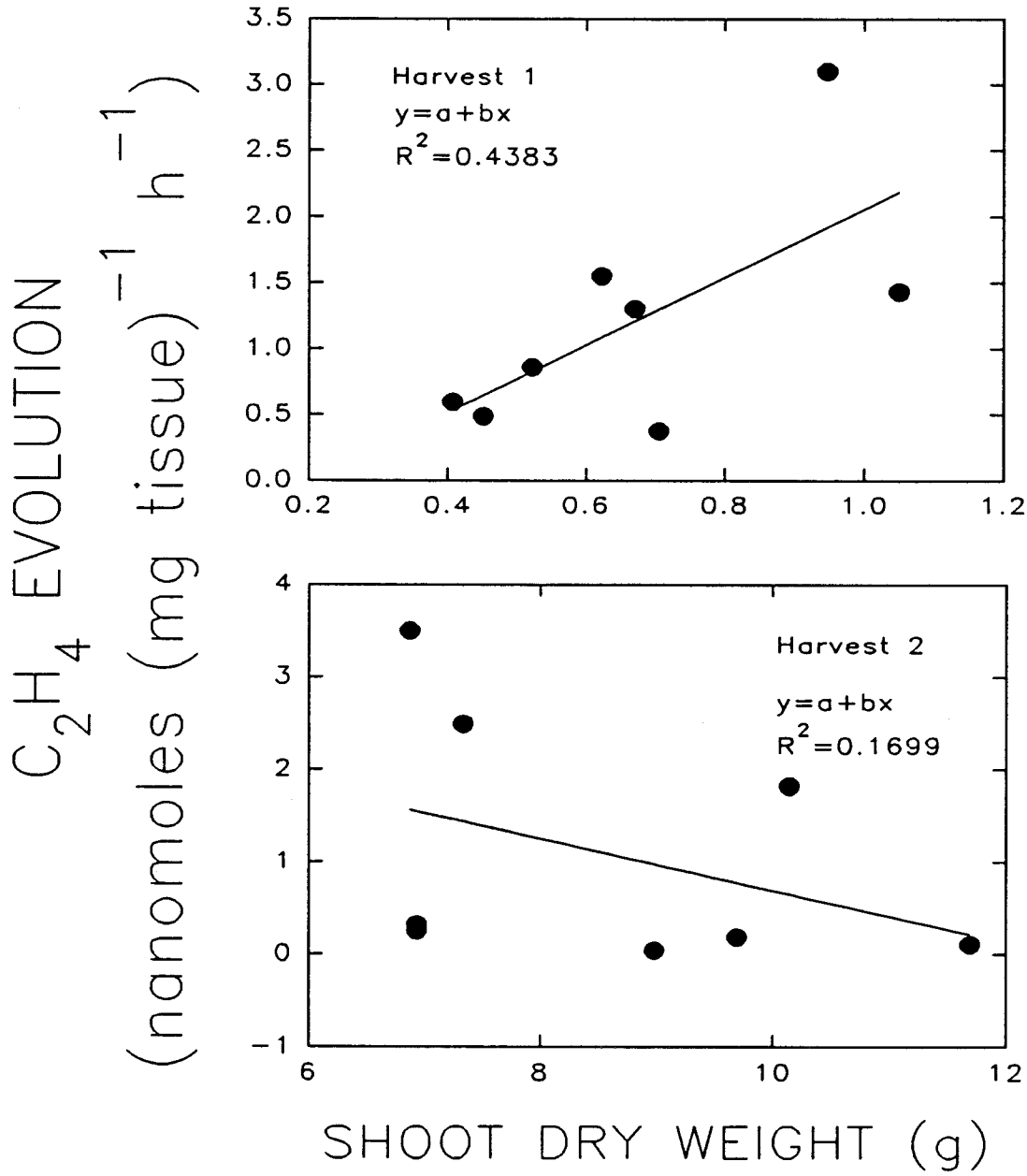

Figure 2-11. Shoot dry weight versus ethylene evolution on plants inoculated with different VAM fungal isolates evaluated at harvest one (6 weeks) and harvest two (21 weeks).

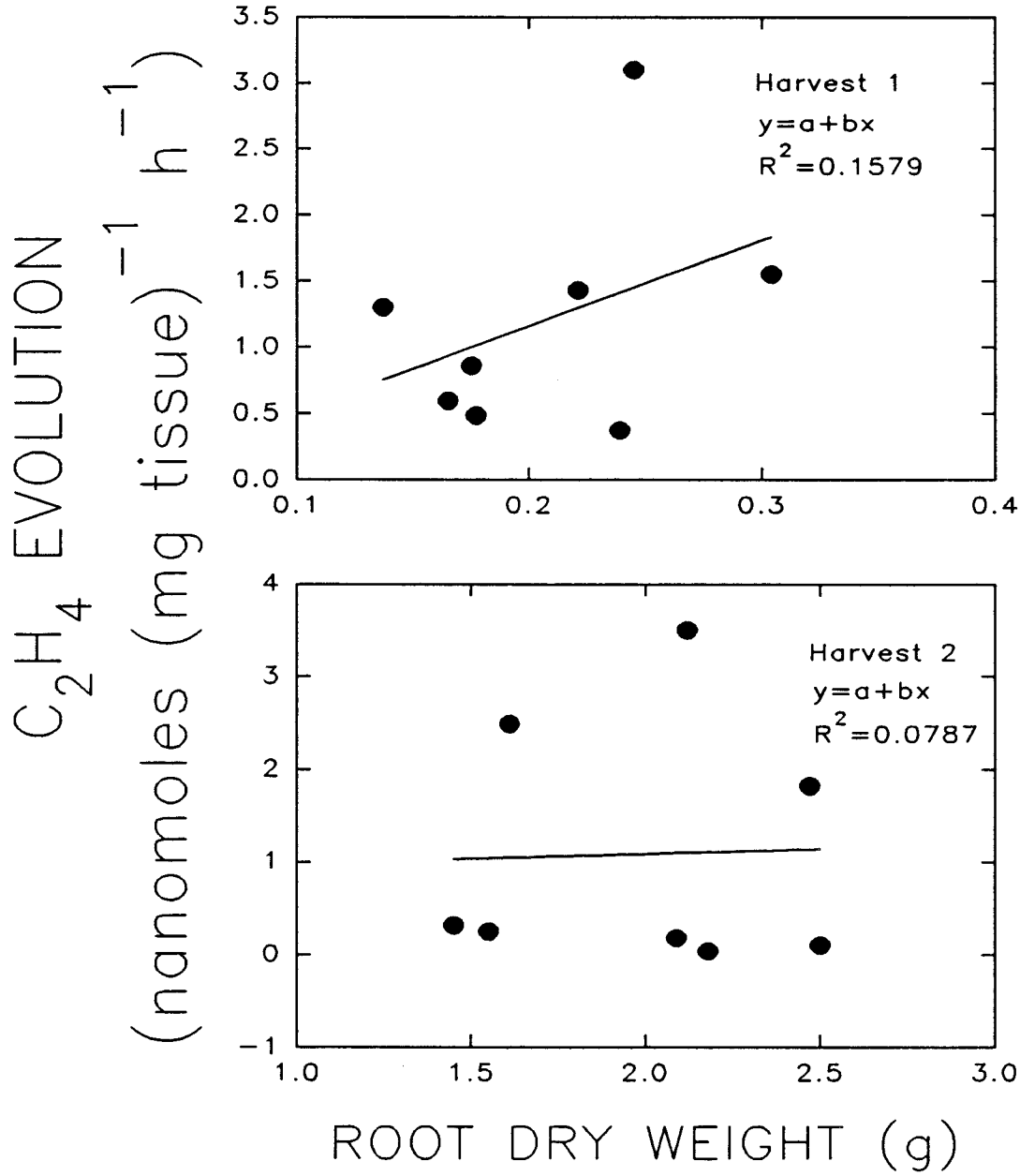

Figure 2-12. Root dry weight versus ethylene evolution on plants inoculated with different VAM fungal isolates evaluated at harvest one (6 weeks) and harvest two (21 weeks).

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CHAPTER 3

VARIATION IN VA MYCORRHIZAL FUNGUS ISOLATE INTERACTIONS WITH *RHIZOBIUM* ON PIGEON PEA (*CAJANUS CAJAN*): II. DROUGHT TOLERANCE OF PIGEON PEA

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SUMMARY

Legumes form a dual symbiosis with nodulating *Rhizobium* and VA mycorrhizal (VAM) fungi. Rhizobia in nodules fix N_2, and VA mycorrhizae help the plant tolerate environmental stresses including nutrient deficiency and drought. Often the dual symbiosis results in enhanced N_2 fixation and plant growth, depending on the degree of interendophyte compatibility. Our objectives were to document variation in response of pigeon pea (a relatively P-independent VAM host) to dual inoculation with an effective *Rhizobium* and different VAM fungi regarding host growth, number and size of rhizobial nodules, and host response to soil water deficits. In addition we wished to elucidate whether the production of extraradical hyphae was correlated with those symbioses on plants that continued CO_2 fixation under water deficits compared to those that did not.

In a multifactorial drought tolerance study with pigeon pea (Cajanus cajan) and seven VAM fungal isolates we observed growth increases that were independent of tissue P levels, and correlated to number and size of nodules formed and thus N_2 fixation. The non-VAM control and two VAM treatments formed very few nodules, whereas other VAM treatments formed many (75-100 per plant). Tolerance to soil water deficits was enhanced by some VAM strains more than others, but tolerance was not correlated with degree of nodulation. In a second multifactorial experiment with the same fungal isolates we observed, as in the first, some fungal isolates formed extensive VAM, but had little effect on nodulation; others formed relatively little VAM, yet dramatically enhanced nodulation. Root and shoot growth responded to VAM isolate in much the same way as in experiment one. The amount of extraradical hyphae was correlated with the capacity of VAM fungi to allow plants to maintain CO_2 fixation under soil water deficits. This response was more dramatic in larger than smaller plants.

The results support the contention that pigeon pea must be inoculated with Rhizobium and compatible VAM fungi to maximize nodulation and N_2 fixation. VAM fungi that enhance nodulation, however, may not be the best at increasing drought tolerance. In these studies, the VAM fungus Gigaspora margarita effectively did both.

INTRODUCTION

Over one-third of the earth's surface is classified as arid or semiarid because it is subject to permanent drought (Kramer, 1983). Furthermore, areas of the earth's surface where much of the world's

food supply is produced are influenced by periods of severe drought. Furthermore, monoculture cropping, common in major food producing nations, often results in rapid and dramatic disturbances in soil structure and thus soil water-holding capacity. The resulting soil water deficits, if occurring too rapidly, are stressful on new seedlings and cause loss of cell turgor and plant death. Consequently there is a need to understand plant growth under environmental water deficits. When environmental water deficits disturb normal functions, plants usually respond with a number of modifications which allow them to regain cell turgor and thereby, normal growth including osmotic adjustment, increased root to shoot ratios, reduced leaf area, thickened leaves, and stomatal closure. Understanding factors that influence these modifications and controlling them would result in increased plant tolerance and survival under water deficits.

The VA mycorrhizal symbiosis has been shown to influence plant response to water deficits (Safir et al., 1971, 1972; Levy and Krikun, 1980; Hardie and Leyton, 1981; Allen et al., 1981; Allen, 1982; Auge et al., 1986). Our increasing interest in VA mycorrhizae (VAM) and their ability to ameliorate plant responses to soil water deficits has resulted in a growing amount of literature. The general conclusion that has emerged is that VAM reduce whole plant resistance to water transport.

Safir et al. (1971) proposed four hypotheses to explain how mycorrhizae could increase hydraulic conductivity: (1) that VAM extraradical hyphae increase the total root water-absorptive surface

area and thereby enhance the amount of soil that could be explored by a VAM plant; (2) that VAM intraradical hyphae, which penetrate the root to the endodermis, provide a low resistance pathway for water movement across the root cortex; (3) that VAM hyphae enhance nutrient uptake (particularly P), which in turn decreases resistance to water movement within roots; and (4) that VAM symbiosis could increase root growth resulting in larger roots that could more effectively explore soil for water.

Safir et al. (1972), working with soybean (Glycine max), concluded that the enhanced P status of the VAM plant was the mechanism which altered root hydraulic conductivity. Levy and Krikun (1980) speculated that in their system (rough lemon (Citrus jambhiri)), alterations in root-shoot hormonal balance were responsible for influencing water relations and not root conductivity as Safir et al. (1972) had proposed. Hardie and Leyton (1981) observed with red clover (Trifolium pratense) that higher root hydraulic conductivity was at least partly due to greater root area (length and diameter) of mycorrhizal plants. They also speculated that hyphal growth in the soil had much to do with water relations. This conclusion was confirmed by Allen (1982) while studying the water relations of blue grama (Bouteloua gracilis) infected with the VAM fungus Glomus fasciculatus, and again by Hardie (1985) whose results showed that removal of extraradical hyphae disrupted the maintenance of transpirational flux in red clover (Trifolium pratense).

Much of the research on VAM water relations has been conducted on legumes (Safir et al., 1971, 1972; Aparicio-Tejo et al., 1980; Hardie and Leyton, 1981; Busse and Ellis, 1985; Bethlenfalvay et al., 1987, 1988). These studies provide a good basis for understanding the nature of this complex tripartite association including Rhizobium. It is difficult to generalize about the affects of VAM on water relations based on these studies because of the variety of environmental conditions under which they were done. For example we know very little about how different isolates or species of VAM may affect this tripartite association under similar experimental or environmental conditions, and we know even less of how the interaction will respond under drought conditions. The studies of Allen and Boosalis (1983), Stahl and Smith (1984), and Auge et al. (1986) provide some insight into the ability of different isolates and species of VAM to affect water relations of host plants under water deficits; however the host plants in their studies (Triticum aestivum, Agropyron smithii, and Rosa hybrida respectively) are physiologically much different from legumes which interact with nitrogen-fixing bacteria in addition to VAM.

One way different VA-mycorrhizal fungal isolates could differentially affect water relations would be by the production of varying amounts of extraradical hyphae. In previous experiments, we observed that the VAM fungus G. deserticola produced more extraradical hyphae than G. intraradices when in association with winter wheat (Triticum aestivum), green pepper (Capsicum annum), and western red cedar (Thuja plicata). Extraradical hyphae could be

very important in bridging the gap that occurs when the root shrinks away from the soil surface under conditions of soil water deficit.

The objectives of our study were to: (1) evaluate a number of VAM fungal isolates for their effect on the growth of pigeon pea, a relatively P-independent legume, and its association with a compatible Rhizobium; (2) evaluate these VAM isolates on pigeon pea for their ability to aid plants in maintaining CO_2 fixation under water deficit stress; and (3) elucidate whether the production of extraradical hyphae is correlated with those symbioses that can maintain CO_2 fixation under water deficits compared to those that cannot.

MATERIALS AND METHODS

EXPERIMENT ONE

Biological Materials

Seven VAM fungal isolates used in this experiment and their origins are listed in Table 3-1. Spore numbers were determined for each isolate and diluted with Willamette River sand to a spore density of 20 VAM spores ml^{-1} of inoculum. A 100 ml aliquot of inoculum containing 2000 spores was placed in a column beneath each transplanted seedling (Fig. 3-1).

The Rhizobium used was a Cowpea strain (P132-1 Arhar) isolated from Pigeon Pea (Cajanus cajan (L.) Millsp. var Corg-5) and 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 four days before inoculating germlings.

Pigeon Pea seeds were surface sterilized (10% NaClO for 5 min) and germinated in sterile distilled water at 27 C for two days. The seedlings were inoculated with Rhizobium by dipping them into the YMB Rhizobium culture. Numbers of colony forming bacteria per seedling were estimated by dilution plating bacteria washed from ten inoculated seedlings on Yeast Mannitol Agar (YMA) plates. Plates were incubated in the dark at 30 C for five days. The bacterial counts from treated seedlings were 1.5×10^9 CFU seedling^{-1}.

Growth Conditions

The planting medium used was a 1:1 mixture (v/v) of Willamette sandy loam (Ph 6.0) and river sand which contained: 0.02% total nitrogen, 10 mg kg^{-1} phosphorus, 74 mg kg^{-1} potassium, and 6.3 mequiv. calcium per 100 g of soil (Soil Test Laboratory, Department of Soil Science, Oregon State University).

To reduce microfloral differences between controls and VAM-inoculated plants, the Control inoculum was prepared from a Gigaspora margarita-pigeon pea pot culture sand/soil medium that proved beneficial to rhizobial symbiosis in a previous pigeon pea experiment. The VAM 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) prepared by filtering a slurry of nonpasteurized pigeon pea-Gigaspora-pot culture soil inoculum (10% by volume of the total control inoculum to be used)

through Whatman #1 paper to retain VAM 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 (22 C day, 18 C night) (Meyer and Linderman, 1986a).

Soil thermocouple psychrometers (Wescor soil psychrometers, model PCT55-15, WESCOR, Logan, Ut.) were placed horizontally into 4400 cm^3 pots (three randomly selected pots from each treatment to be assayed for CO_2 fixation) approximately 3.0 cm from the bottom and 5.1 cm horizontally into the pot (Fig. 3-2). To reduce soil thermocouple psychrometer sensitivity to heat gradients, soil water potential (Ψ_s) was monitored at predawn on a Dewpoint microvoltmeter (Wescor Inc. model HR-33T). Soil water potential was monitored daily for all treatments. Plants were maintained on greenhouse benches (22 C day, 18 C night) with supplemental lighting (high pressure sodium vapor lamps, 250μmol m^{-2} s^{-1} at 400-700nm) for a 16 h photoperiod. At the first trifoliate leaf stage, the surface of each pot was covered with coarse crushed quartz to retard evaporation.

Plant Nutrition and Growth

Initially plants were fertilized once a week with 100 ml of a P modified (20, 40 or 60 ppm P as NaH_2PO_4), low N, Long Ashton Nutrient Solution (LANS) beginning at the first trifoliate leaf stage (three weeks following transplant) (Table 3-2). At six weeks fertilization was increased to twice-weekly. Iron was provided as Fe citrate at 2

ml l^{-1} fertilization volume (3.6 ppm Fe). In addition pots were irrigated three times a week with 250 ml water.

At 6, 10, and 12 weeks, the affect of each VAM fungus isolate on plant growth was determined by measuring plant height and stem diameter.

Phosphorus Determination

At 12 weeks from transplanting, 12 plants of uniform size and vigor were selected from each treatment and randomly combined into four groups of three plants each. Eight plants from each treatment were discarded (four largest and smallest). From each plant a 1.72 cm^2 leaf disc was taken from the distal leaflet of the trifoliate leaf, three nodes from the terminal bud. Leaf discs in each group were pooled, and 100 mg fresh weight were dry ashed at 500 C for 3 h, then digested in 1 N HCl. The digest was filtered through Whatman #4 paper and diluted with 20 ml distilled water (Aziz and Habte, 1987). Phosphorus concentration was determined colorimetrically according to the molybdenum blue technique (Murphy and Riley, 1962) (Table 3-3).

On the basis of leaf P concentration, above ground plant height, stem diameter, and number of nodes, VAM treatments fell into three separate groups: Group 1, LAP (Low added P in LANS), Gimar and Gi; Group 2, (LAP), Get, Gm, and Gd(U); and Group 3, LAP, Gmix, GdC, and Ctl-high added P (60 ppm P in LANS).

Leaf CO_2 Fixation vs Soil Water Potential Assay

Net CO_2 fixation was monitored on one leaf, below the leaf from which P concentration was determined (Fig. 3-3), with a Portable Photosynthesis System (LI-COR Inc., Lincoln NE.; chamber volume used was 325.0 cm^3). Comparisons were made on VAM treatments within the previously defined groups. Immediately before measuring, a subject plant was placed under photosynthetically active radiation of 720 μmol m^{-2} s^{-1} (at leaf surface) from high pressure sodium vapor lamps. Radiation was filtered through 5 cm of water (to reduce heat load on the leaf caused by long wave radiation); all other light was excluded. Measurements were begun when CO_2 concentration inside the cuvette was approximately 350 ppm.

Plant Harvest

At 16 weeks, all treatments were harvested and leaflets used in the CO_2 fixation assay were removed, their fresh weight determined and leaf area measured in order to adjust CO_2 fixation for leaf area. Nodules were removed, counted and nodule dry weight determined as was dry weight of roots and shoots after drying at 70 C for 48 h.

VAM colonization was determined on half of the replicates by clearing roots overnight at 55 C in 10% potassium hydroxide and staining in trypan blue (0.05%) in lactoglycerol according to the methods of Phillips and Hayman (1970), and assaying for VAM colonization according to the method of Biermann and Linderman (1981).

Experimental Design

An eight x three factorial, completely randomized design was used, with eight fungal treatments (seven fungi and one non-VAM control) at three phosphorus (P) levels (20, 40, or 60 ppm P as NaH_2PO_4 in the LANS). There were twenty replicate plants per treatment.

Statistical Analyses

Results were analyzed according to a Multifactorial 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).

EXPERIMENT TWO

Biological Materials

The seven VAM fungal isolates previously listed (Table 3-1) were used in the second experiment. The VAM fungal inocula were obtained from pot cultures of pigeon pea grown in a 1:1 mixture of Willamette sandy loam soil and sand harvested from a previous experiment. Spore numbers were determined for each, and each was diluted as described in experiment one. A 100 g aliquot of inoculum containing 2000 spores was placed in a column beneath each transplanted seedling (Fig. 3-4).

The Rhizobium and cultural methods used are described in experiment one. Numbers of colony forming bacteria per seedling were

estimated by dilution plating the bacteria washed from five inoculated seedlings on Yeast Mannitol Agar (YMA) plates. Plates were incubated in the dark at 30 C for five days. Due to the size of the experiment, plants for two harvests and two groups within each harvest were planted one week apart. The bacterial counts from treated seedlings were: harvest 1 - first group - 0.83×10^6 CFU seedling^{-1}; harvest 1 - second group - 1.47×10^6 CFU seedling $^{-1}$; harvest 2 - first group - 83×10^6 CFU seedling^{-1}; harvest 2 - second group - 16.6×10^6 CFU seedling^{-1}.

Growth Conditions

The soil medium used was a 1:1 mixture of Willamette sandy loam (pH 6.0) and river sand which contained: 0.02% total nitrogen, 12 mg kg^{-1} phosphorus, 70 mg kg^{-1} potassium, and 8.5 mequiv. calcium per 100 g of soil (Soil Test Laboratory, Department of Soil Science, Oregon State University). To reduce microfloral differences between controls and VAM-inoculated plants, the control inoculum was prepared from Gigaspora margarita-pigeon pea pot culture soil that provided adequate Rhizobium nodules in a previous experiment. The VAM component of this inoculum was removed by pasteurization and the soil was inoculated with organisms (other than VA-mycorrhizal) as previously outlined. Seedlings were transplanted into 84 mm x 84 mm x 152 mm pots (volume 1080 cm^3).

Plant Nutrition and Growth

Plants were fertilized weekly with a modified Hoagland's solution (40 ppm P as NaH_2PO_4) (Table 3-4) beginning at the first trifoliate leaf stage (three weeks following transplant). The macronutrients were diluted 1:1 with tap water before fertilization. Iron was provided as Fe citrate at 2 ml per liter of the final fertilization volume. The non-VAM controls were fertilized with the modified Hoagland's, but with varying amounts of KNO_3. Final solutions for these controls contained 100, 150, 200, or 300 ppm nitrogen as KNO_3. Varying the N in the control feeding solutions was done to equalize plant size between some controls and VAM inoculated plants.

Experimental Design

Plants were completely randomized on greenhouse benches with 11 fungal treatments (seven fungi and four non-VAM controls). Plants for the first harvest all were inoculated with Rhizobium; plants for the second harvest were either inoculated or not with Rhizobium.

First Harvest

At six weeks, 20 replicates per treatment were harvested. Shoots were removed and dried (70 C for 48 h), and roots were reserved for assessment of VAM colonization, nodulation, and dry weights. The root samples were divided into two groups of 10 replications each. Root dry weights and nodule dry weights were determined on the first half. VAM colonization was estimated on the

second half as follows: Whole fresh roots were blotted dry (to touch), weighed and cut into 0.5 to 1.0 cm segments. Segments were cleared, stained, and examined for VAM colonization as described in experiment one. Twenty-five root segments per sample were examined for % root length with VAM colonization. The number of root segments examined per sample was determined statistically by examining 3 samples with 50 and 25 segment samples in each and comparing variability.

Second Harvest

At 21 weeks, a second group of plants was harvested. VAM colonization was assayed on 1-cm root segment subsamples taken from the bottom of the root system and from 2.5 cm below the crown. The subsamples were weighed to estimate their proportion of the total root system weight. The remaining root system of each plant, minus the VAM subsample was weighed fresh and oven dried at 60 C for 48 h, and dry weight was determined. The ratio of dry weight to fresh weight was used to determine the expected dry weight of the root aliquot removed for VAM determinations (to be added to the whole root dry weight). Shoot dry weights, nodule weights and numbers were determined as previously described.

The length of extraradical hyphae from VAM was determined as follows: three cores (6 cm deep, 1 cm diameter) were removed from each pot at an equal distance from plant and side of pot. Core samples from three pots were suspended in water, and blended at low speed for 15 sec., and dried by evaporation for 36 h. Two grams of

soil were taken from each pooled repetition and processed according to methods outlined in Abbott et al. (1984). A grid intersection method was used to estimate hyphal length (Newman, 1966).

Statistical Analyses

Plants in the first harvest were arranged 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 in the second harvest were also completely randomized and results were analyzed according to a Multifactorial Analysis Of Variance. Significant differences were compared as outlined above.

RESULTS

EXPERIMENT ONE

Growth Response

Growth curves for stem diameter and plant height along with root and shoot dry weights indicated that plants generally fell into three size groups. Plants inoculated with Gimar and Gi were among the largest plants (Figs. 3-4, 3-5, and 3-6). Plants inoculated with Gi exhibited an increase in the growth rate between 10 and 12 weeks. Those plants inoculated with Get, Gm, and Gd(U) were medium sized and plants inoculated with Gmix, Gd(C), and the non-VAM control were the smallest (Figs. 3-4, 3-5, and 3-6). Most VAM treatments caused

increased growth rates with time, with little variation except plants inoculated with Gi and Gd(U) that exhibited sharply increased growth between 10 and 12 weeks (Figs. 3-5 and 3-6). Non-VAM controls were the smallest plants despite receiving three times the amount of P as any VAM treatment. The addition of P to the non-VAM control seemed to affect root growth more than shoot growth, as control root systems were comparatively large whereas shoots were small and generally chlorotic. VAM colonization differed significantly from isolate to isolate as did the establishment and development of Rhizobium nodules (Fig. 3-8). Increased nodule number and nodule weight correlated well except for plants inoculated with Gd(U) where many small nodules formed (Fig. 3-8).

CO_2 Fixation vs Soil Water Potential

Plants within a group (determined by similarity in size and leaf P concentration) were compared for ability to fix CO_2 under soil water deficits. In the small plant group, the non-VAM control plants had higher CO_2 flux at a lower soil water potential than did plants inoculated with Gmix or Gd(C), but were the first to exhibit a shutdown in CO_2 fixation, and upon rewatering took three days to recover photosynthetic capability (Fig. 3-9). Photosynthesis for any given leaf was considered to have recovered when we could obtain measurable CO_2 fixation following a period of zero photosynthesis. Soil water potential in pots of Gmix and Gd(C) inoculated plants increased slightly. Soil thermocouples were placed low in the pot (Figs. 3-1, 3-2) and therefore a lag time in the response of soil

water potential to drying and rewetting cycles may have been due to water percolating through the soil profile and may not have reflected bulk soil water potential. Plants inoculated with Gd(C) continued to fix CO_2 after the non-VAM control or Gmix had stopped, and resumed CO_2 fixation faster than Gmix or non-VAM controls.

In medium sized plants, those inoculated with Get and Gm exhibited rapid changes in CO_2 fixation (Get dramatically so) as influenced by drought cycles (Fig. 3-10). Both recovered rapidly upon rewetting on day five, but stopped CO_2 fixation again by day seven. Gd(U) inoculated plants exhibited a reduction in CO_2 fixation from days one to three, but recovered by day six to a CO_2 flux that was slightly higher than the first reading and yet plants were exposed to a greater soil water deficit (Fig. 3-10).

In the large plant group, Gimar inoculated plants fixed significantly more CO_2 than Gi inoculated plants at similar soil water potential, and this occurred throughout all three drying and rewetting cycles (Fig. 3-11). Both plant treatments responded rapidly to rewetting, but Gimar inoculated plants recovered to greater CO_2 fixation levels, and on the third cycle CO_2 fixation dropped off gradually as opposed to a rapid decline in plants inoculated with Gi.

EXPERIMENT TWO

Growth response of pigeon pea to the different VAM treatments observed in experiment two was similar to observations in experiment one (Fig. 3-12). Growth differences observed in harvest one were

less dramatic and less variable than those at harvests two (Fig. 3-12). The addition of N to the non-VAM controls (rather than P) slightly ameliorated shoot growth, except the treatment with low added N (100 ppm N) which did not improve root growth (Fig. 3-12). In the second experiment, non-VAM control plants (low P, varied N) were greener and generally larger and healthier (less leaf loss) than were non-VAM control plants from the first experiment (low N, varied P).

vam colonization and extraradical hyphal growth

All VAM inoculated plants had a lower % of roots with VAM colonization at harvest two than harvest one (Fig. 3-13). The amount of colonization was also less at harvest two than occurred at harvest two of experiment one.

Mean extraradical hyphal lengths were only compared between treatments within groups that were of similar size and had been compared for CO_2 fixation vs soil water potential in experiment one. Plants in the large plant group inoculated with Gimar had significantly more extraradical hyphae than those inoculated with Gi (Fig. 3-14). In the medium size group, there were no significant differences in hyphal length, and in the small size group the only significant differences observed was an increase when added N was increased with non-VAM plants (Fig. 3-14).

DISCUSSION

Pigeon pea is generally considered to be unresponsive to large concentrations of added phosphate (Whiteman et al., 1985), and our results in experiment one confirmed this. Plant growth enhancement varied with VAM isolate independent of P level. In fact, the intermediate P level (40 ppm P) resulted in a decrease in root weight in all VAM treatments (Fig. 3-15); at 60 ppm root weight increased somewhat (compared to 40 ppm) in most treatments and markedly so for the non-VAM control. There is evidence to substantiate both growth enhancement (Allen et al., 1981; Hardie and Leyton, 1981; Huang et al., 1985; Bethlenfalvay et al., 1987) and growth depression (Abbott and Robson, 1985) in the early growth phase of VAM inoculated plants when compared to controls. Growth enhancement correlated well with number and size of nodules and, therefore, with the amount of N_2 fixed.

The effort was made to compare non-mycorrhizal control plants that were equal to VAM inoculated plants in size and nutrient status by varying P fertilization in experiment one and N fertilization in experiment two. In the first experiment we could not achieve a uniform plant size, and had to separate plants into groups with comparable size and relative tissue P concentration for measurement of CO_2 fixation. The need for, but difficulty in achieving and maintaining, comparable-sized mycorrhizal and non-mycorrhizal plants for water relations studies is clear. Using the data and visual information collected in experiment one, we decided in experiment two to vary N rather than P, and this resulted in control plants that

were healthier and larger than control plants of experiment one, and had roots comparable to five of the seven VAM fungal treatments. Future studies of the interaction between VAM and pigeon pea where there is concern for achieving uniform sized non-mycorrhizal control and VAM plants, should vary N instead of P fertilization regimes.

Positive association was found between extent of VAM colonization, nodule number and plant size in four fungal treatments but not Gmix, Gd(C), and Gd(U). The observed plant growth increase resulting from enhanced N_2 fixation as affected by Get, Gm, Gimar, and Gi concur with previous observations (Smith and Daft, 1977; Smith et al., 1979; Varma, 1979). VAM treatments resulting in reduced growth and nodule numbers (as was seen in Gmix and Gd(C)) are not as commonly reported, but have been observed by Bethlenfalvay et al. (1985). Gmix colonized heavily yet had little affect on nodulation and produced little growth enhancement. In contrast Gd(U) colonized very little yet enhanced growth and nodule numbers. Results of these two fungal treatments illustrate how misleading it can be to assume a positive correlation between VAM colonization and growth benefits from enhanced N_2 fixation.

The ability to continue CO_2 fixation under increasing soil water deficits and to recover that ability upon rewatering varied with VAM fungal isolate. Plants were separated into groups of similar size and foliar tissue P concentration. Plants within such groups had varied CO_2 fixation, therefore we believe that P nutrition was not the major factor in observed differences in CO_2 fixation. During the imposed drought and recovery periods (ranging from 8-16

d), no fertilization took place but we do not believe that P status of plants changed enough during that period to cause an effect (Graham et al., 1987). The observation that P nutrition is not necessarily a major influence on water uptake and CO_2 fixation under soil water deficits has been previously reported by Auge et al. (1986) and Sweatt and Davies (1984) in mycorrhizal studies.

Under our experimental conditions, it was apparent that different VAM fungal isolates increased CO_2 fixation by some mechanism other than improved N nutrition. The amount of CO_2 a plant can fix is controlled by stomatal opening, which in turn is controlled by cell turgor. Cell turgor is controlled by water availability and water availability is a function of hydraulic conductivity (Boyer, 1976). Safir et al. (1971) and Safir and Nelson (1985) have offered three hypotheses, other than direct influence of P nutrition, to explain increased conductivity or lowered resistance to water movement in VAM plants: (1) increased root to shoot (r/s) ratio; (2) an increased overall root surface area as provided by extraradical hyphae; and (3) intraradical hyphae that may act as a low resistance pathway through the root cortex. In this study root and shoot weights for treatments used in the CO_2 fixation assay were similar within each group with the exception of Gd(U) shoot weight in group two and root and shoot weight of the non-VAM control in group one (Fig. 3-7). In group three (Gimar and Gi) and group two (Get, Gm, and Gd(U)) the more successful VAM isolates (in terms of CO_2 fixation under soil water stress) had the lowest r/s values although a statistical analysis had not been done. Only in group one (Gmix,

Gd(C), and the non-VAM control) did the treatment with the highest r/s weight ratio (non-VAM control) fix the most CO_2 under stress. This treatment was also the first to shut down CO_2 fixation and the slowest to recover, possibly due to a much larger root system with which to extract water from the limited soil volume.

Our observed differences in CO_2 fixation under soil water deficits were not due to improved P nutrition, and at least in the case of groups two and three, were probably not correlated with r/s ratio. In previous experiments we observed that VAM colonization of G. deserticola and G. intraradices on winter wheat, green pepper, and western red cedar produced vastly different amounts of extraradical hyphae depending on the plant and fungal isolate. We used this knowledge in setting up experiment two to observe if a positive correlation existed between the production of extraradical hyphae and the ability to fix CO_2 under greater soil water deficit. The experimental conditions were the same as experiment one except that pot size was smaller and we fertilized non-VAM controls with a N modified Hoagland's solution and all treatments received twice the amount of P. The only striking difference was in group three where VAM isolate Gimar produced significantly more extraradical hyphae than Gi.

There is experimental evidence for increased water uptake from soil via direct hyphal flow (Hardie and Leyton, 1981; Allen, 1982; Hardie, 1985). However, given that not all experimental conditions were similar in our studies, we do not feel there is enough evidence to directly link extraradical hyphae with increased CO_2 fixation via

increased water uptake. We did not specifically test for a correlation between production of intraradical hyphae (by any one VAM isolate) and increased water movement and CO_2 fixation. However, when assaying for VAM colonization, we observed a variation between isolates in the amount of internal versus external hyphae (Figs. 3-8, 3-14). We believe that if the major mechanism of improved water movement is the production of intraradical hyphae, then we would expect isolates like Gmix and Gi (which colonized well) to be more effective than they were.

Another possible mechanism to account for variations in CO_2 fixation under soil water deficit might be that different isolates differentially affected the production or accumulation of plant hormones (eg. cytokinins, gibberellins, and abscisic acid) such that stomates remain open longer despite whole plant water status. Changes in r/s hormonal balance have been observed by Allen and coworkers (1980, 1982), and have been implicated in stomatal activity as observed by Levy and Krikun (1980).

Osmotic adjustment is another mechanism that allows plants to enhance water uptake, maintain turgor, and thus enhance CO_2 fixation under soil water deficits. That osmotic adjustment is an important contributing factor to the dehydration tolerance of pigeon pea has been documented (Flower and Ludlow, 1986), but to our knowledge there is no supporting evidence of VAM contributing to the osmotic adjustment of pigeon pea. Osmotic adjustment has been reported in rose, however (Auge et al., 1986).

Kramer (1983) defines drought as the absence of rainfall for a period of time long enough to result in depletion of soil water and injury to plants. He also points out that the length of time without precipitation necessary to cause plant injury depends on: the kind of plant; the water storage capacity of the soil; and the atmospheric conditions affecting the rates of evaporation and transpiration. In our opinion the length of time may be affected not only by those factors, but also mycorrhizosphere effects.

From the applied aspect, we believe that mycorrhizae may increase the length of time plants can survive under soil water deficits without suffering irreversible injury, and can speed up plant recovery following rewatering. That differing VAM isolates can drastically affect this period of time on a single host under similar experimental conditions is the value of this study. From the aspect of basic knowledge, we feel this study emphasizes the great variation that can occur between VAM isolates with respect to the degree of drought acclimation they confer on the host. In addition, perhaps no single mechanism by which VAM influence drought tolerance of a host can be extrapolated to other plant systems under a variety of experimental or field conditions.

Table 3-1. List of VAM fungal isolates and their origin.

VAM SPECIES	CULTURE ORIGIN	ABBREVIATION
Glomus etunicatum	Native Plants Inc.	*Get*
Glomus aggregatum/microcarpum	Lowell Young (ARS)	*Gmix*
Glomus deserticola	California via Lowell Young (ARS)	*Gd(C)*
Glomus mosseae	Native Plants Inc.	*Gm*
Gigaspora margarita	Native Plants Inc.	*Gimar*
Glomus intraradices	Native Plants Inc.	*Gi*
Glomus deserticola	Native Plants Inc.	*Gd(U)*

Table 3-2. List of compounds used in a modified Long Ashton Nutrient Solution (LANS).

Compound	Stock Concentration g l^{-1}	Amount of Stock $(100\ l)^{-1}$
KNO_3	40.4	500.0 ml
$MgSO_4 \cdot 7H_2O$	73.6	500.0 ml
$Ca(NO_3)_2 \cdot 4H_2O$	94.4	500.0 ml
$NaH_2PO_4 \cdot H_2O$	36.8	low P-217.4 ml med P-434.8 ml high P-652.2 ml
Trace elements		0.1 ml l^{-1} fertilization solution
H_2O		100 l
Citrate solution †		2.0 ml l^{-1}
Trace Elements Stock Solution		
$MnSO_4 \cdot H_2O$		16.9 g
$CuSO_4 \cdot 5H_2O$		2.5 g
$ZnSO_4 \cdot 7H_2O$		2.9 g
H_3BO_3		31.0 g
NaCl		59.0 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$		0.88 g
make up to 100 ml distilled water		

† Two ml Ferric Citrate solution added just before fertilizing

Table 3-3. Mean leaf disc tissue P content.

Fungal Species	Phosphorus Treatment	Mean Leaf Disc Phosphorus Content (%) †
Get	low P	0.18
Gmix	low P	0.39
Gd(C)	low P	0.28
Gm	low P	0.27
Gimar	low P	0.17
Gi	low P	0.23
Gd(U)	low P	0.17
Ctl	high P	0.26

† Leaf disc tissue P content values were used in connection with plant size (stem caliper and plant height) to determine plant groupings for subsequent CO_2 fixation assay.

Table 3-4. List of compounds used in a modified Hoagland's solution.

MACRONUTRIENTS (diluted 1:1 in final solution)	g $(100\ l)^{-1}$
K_2SO_4	27.5
$MgSO_4 \cdot 7H_2O$	49.0
KH_2PO_4	17.62
K_2HPO_4	22.4
$CaSO_4$	81.6
$CaCl_2 \cdot 2H_2O$	6.0
MICRONUTRIENTS (1 ml l^{-1} added to final solution)	**g l^{-1}**
H_3BO_3	0.23
$MnSO_4$	0.12
$ZnSO_4 \cdot 7H_2O$	0.22
$CuSO_4 \cdot 5H_2O$	0.08
$Na_2MnO_4 \cdot 2H_2O$	0.02
$CoCl_2 \cdot 6H_2O$	0.04
$NiCl_2 \cdot 6H_2O$	0.04

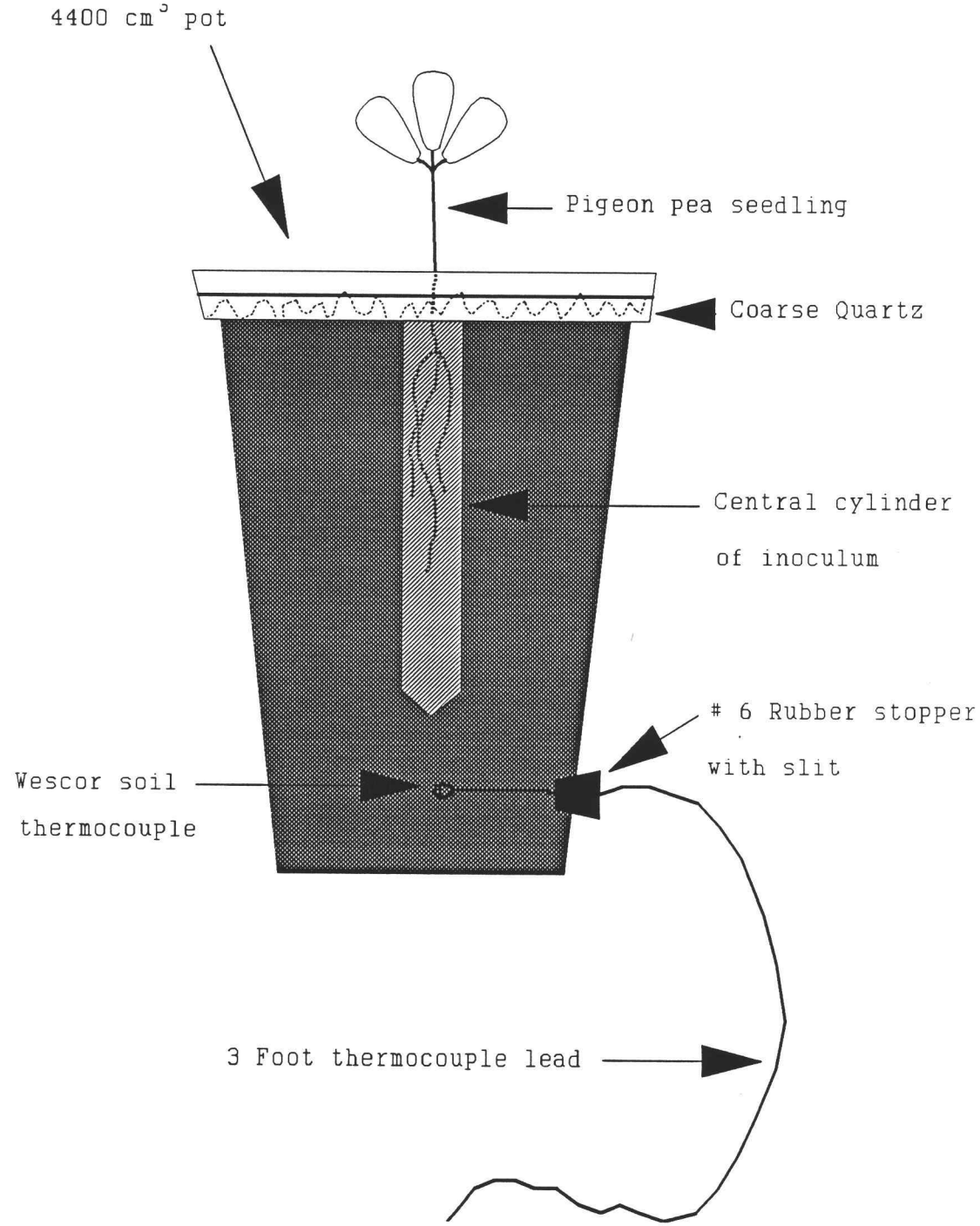

Figure 3-1. Pot design for experiment one with central cylinder of VAM fungal inoculum.

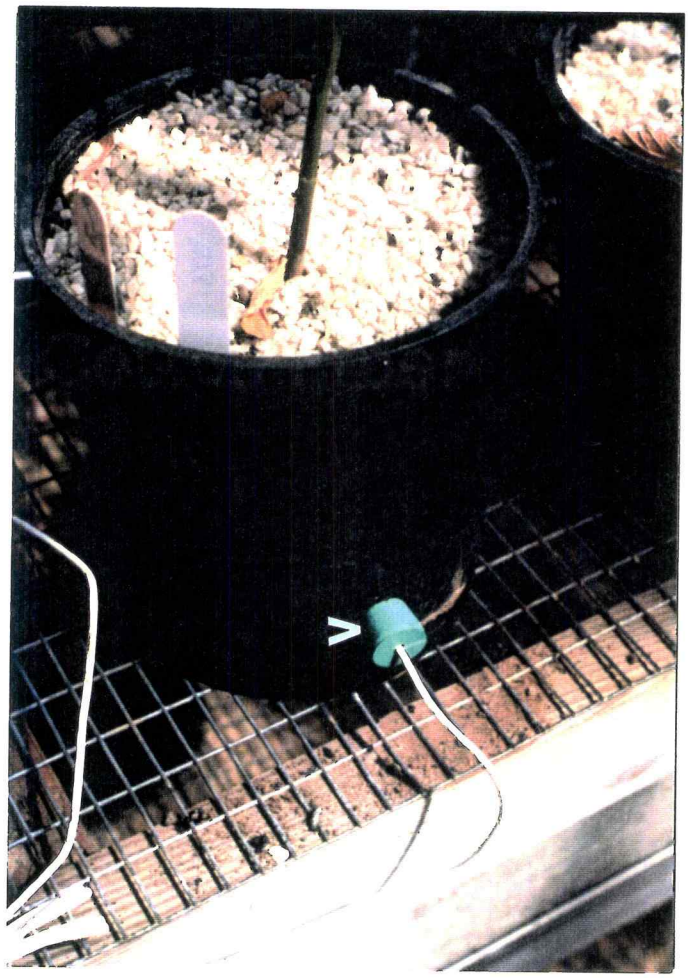

Figure 3-2. Photograph showing experiment pot set-up with soil thermocouple psychrometer in place.

Figure 3-3. Photograph showing portable CO_2 infrared gas analyzer. Measured leaf is highlighted.

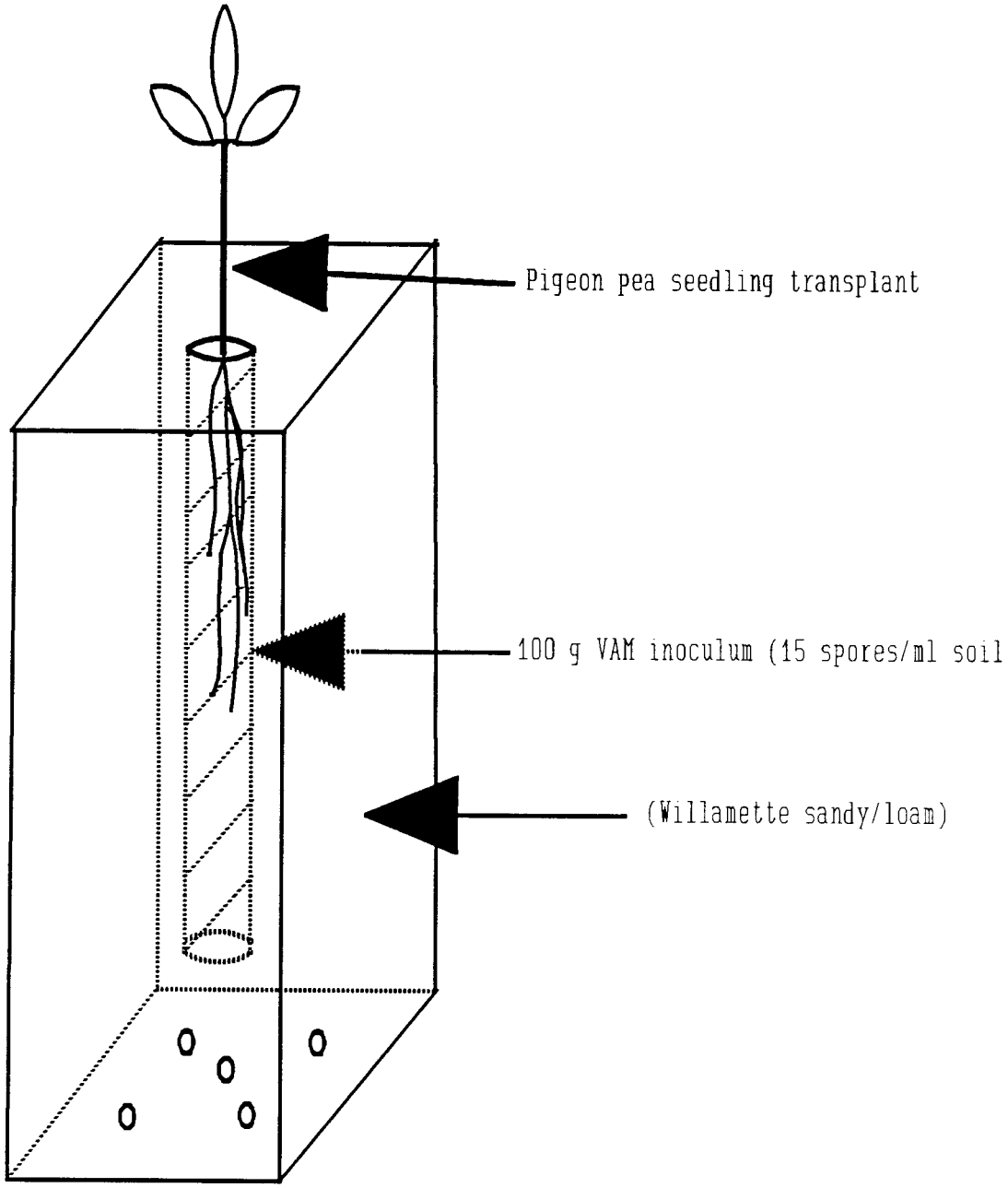

Figure 3-4. Pot design for experiment two showing central cylinder of VAM fungal inoculum.

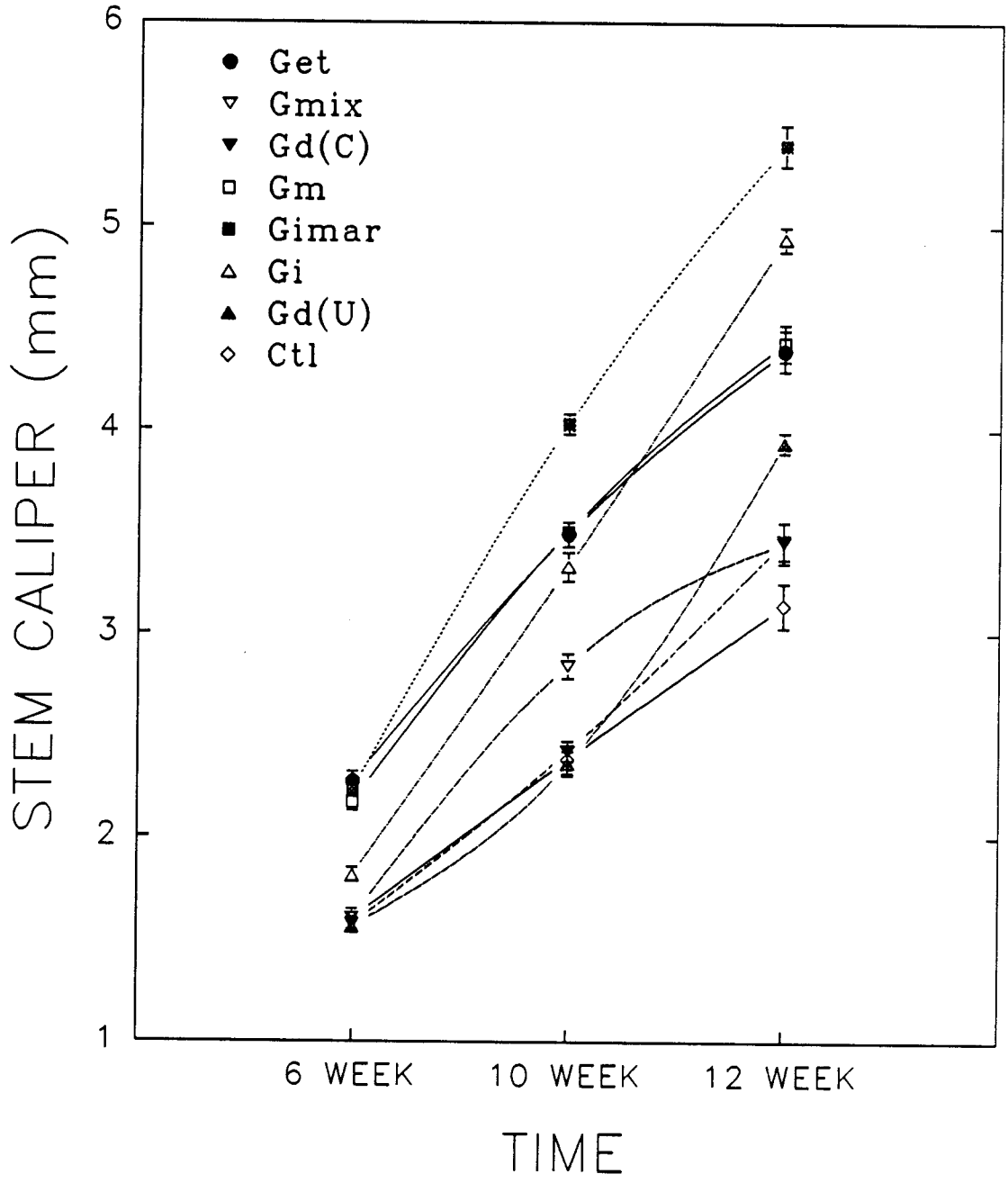

Figure 3-5. Pigeon pea stem diameter over time as influenced by VAM fungal isolates.

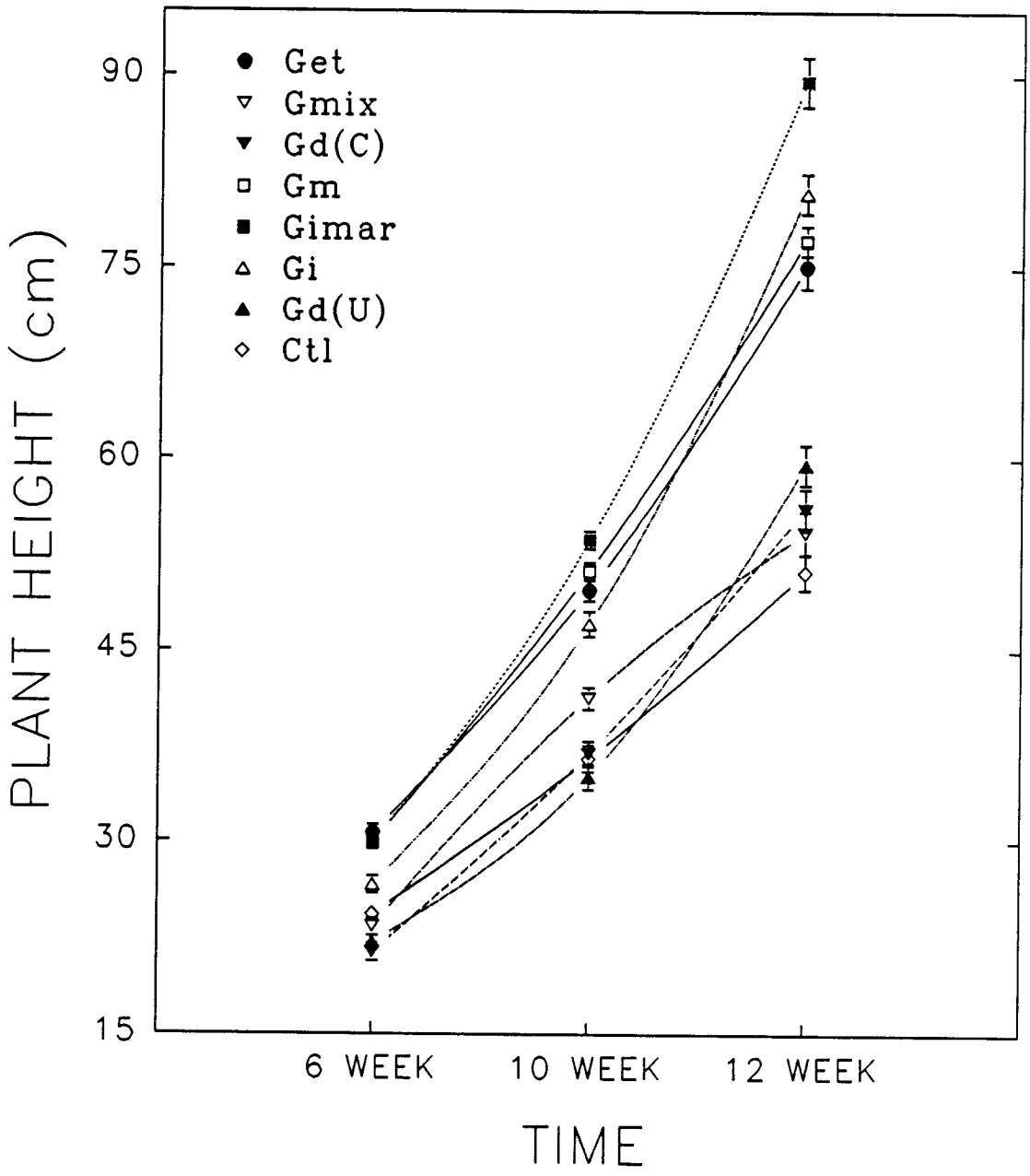

Figure 3-6. Pigeon pea stem height over time as influenced by VAM fungal isolates.

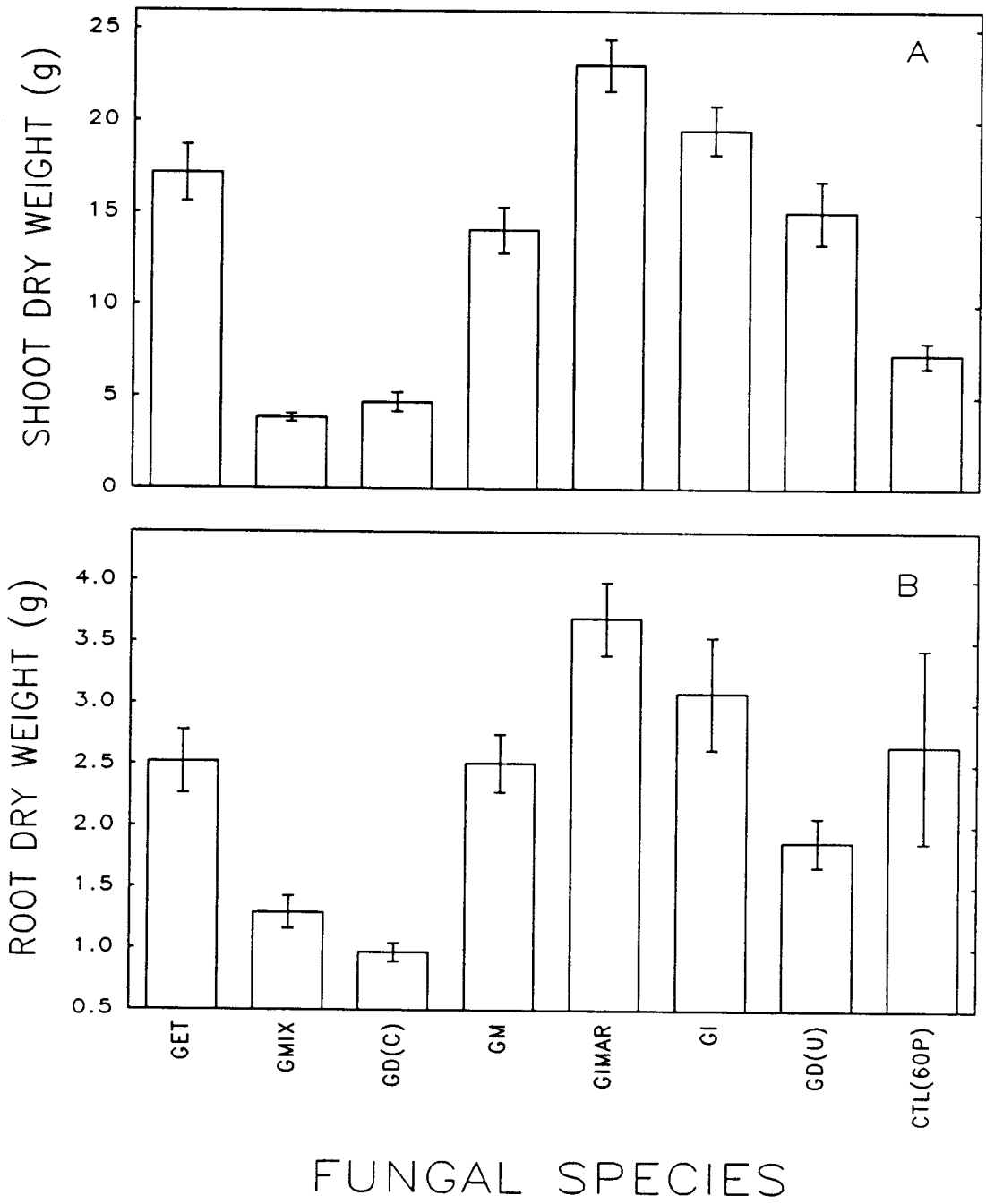

Figure 3-7. Mean shoot and root dry weight (standard error $P<0.05$) as influenced by VAM fungal isolate.

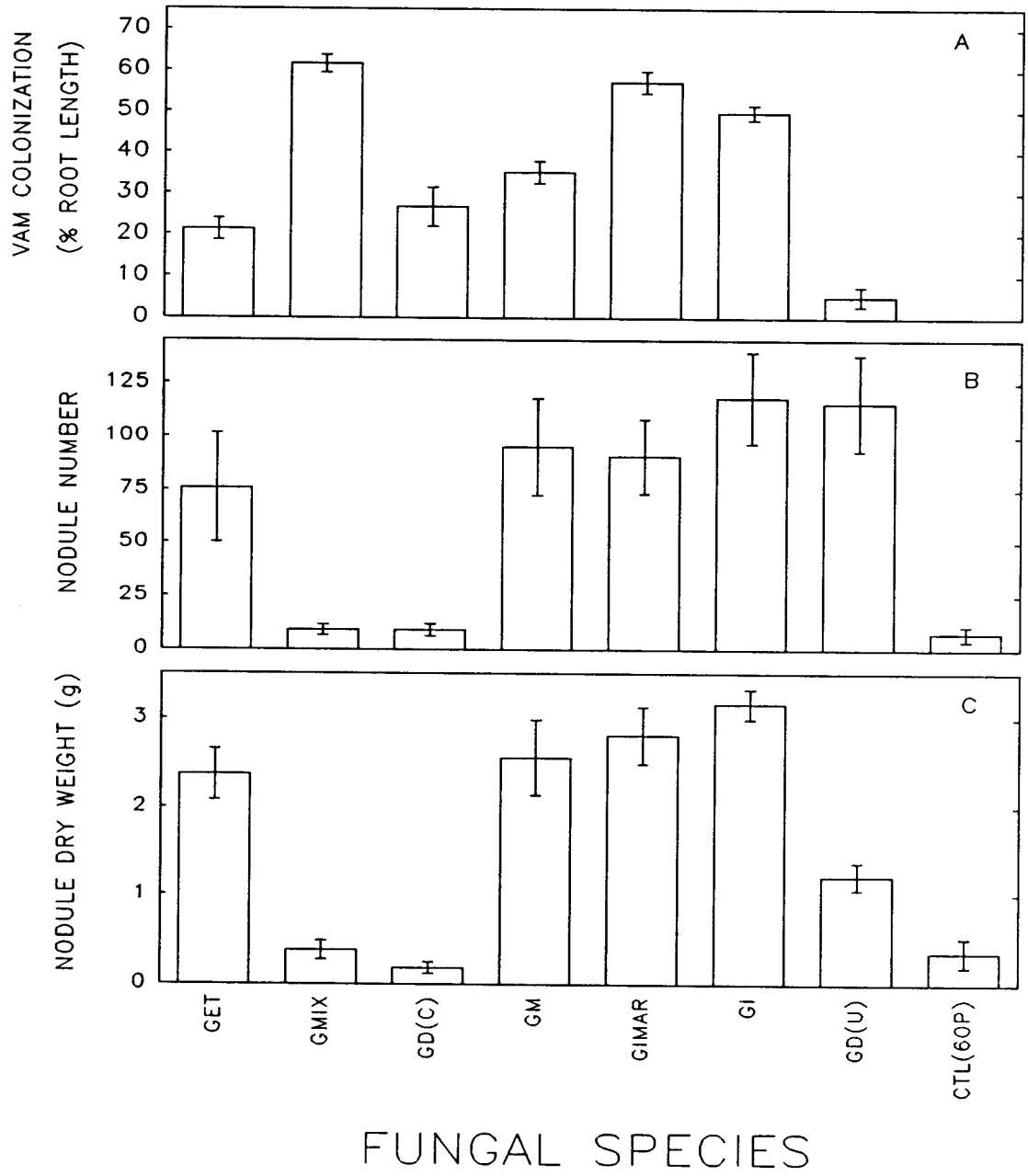

Figure 3-8. Mean VAM colonization (A), nodule number (B), and nodule dry weight (C) as influenced by VAM fungal isolate (standard error $P<0.05$).

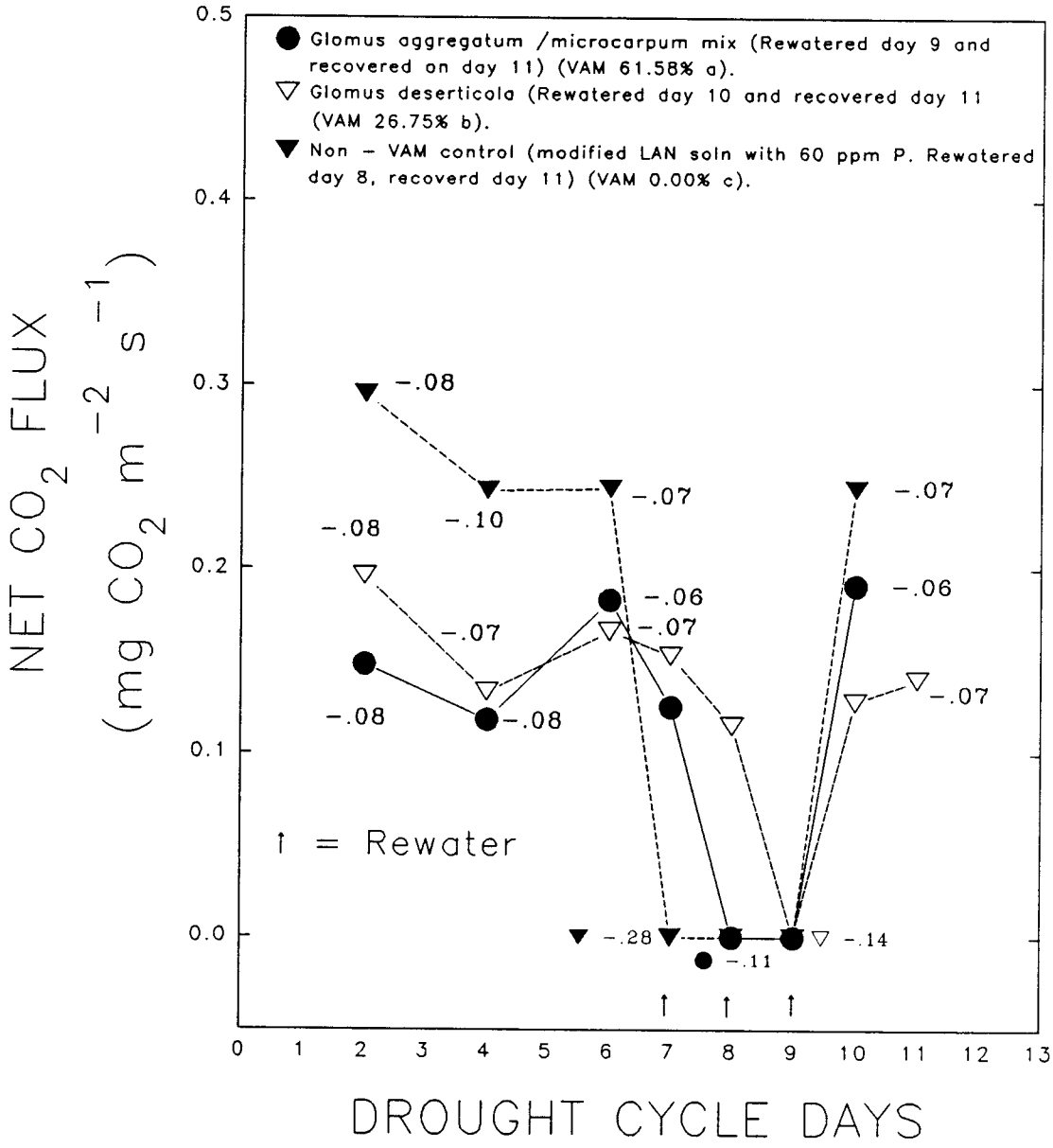

Figure 3-9. Mean net CO_2 flux values for 3 fungal treatments during drought cycles. Some soil water potential (ψ_w) values are also presented if differences in CO_2 flux values were significant. G. deserticola (C) plants were rewatered day 9 and continued fixing CO_2 until terminated on day 11.

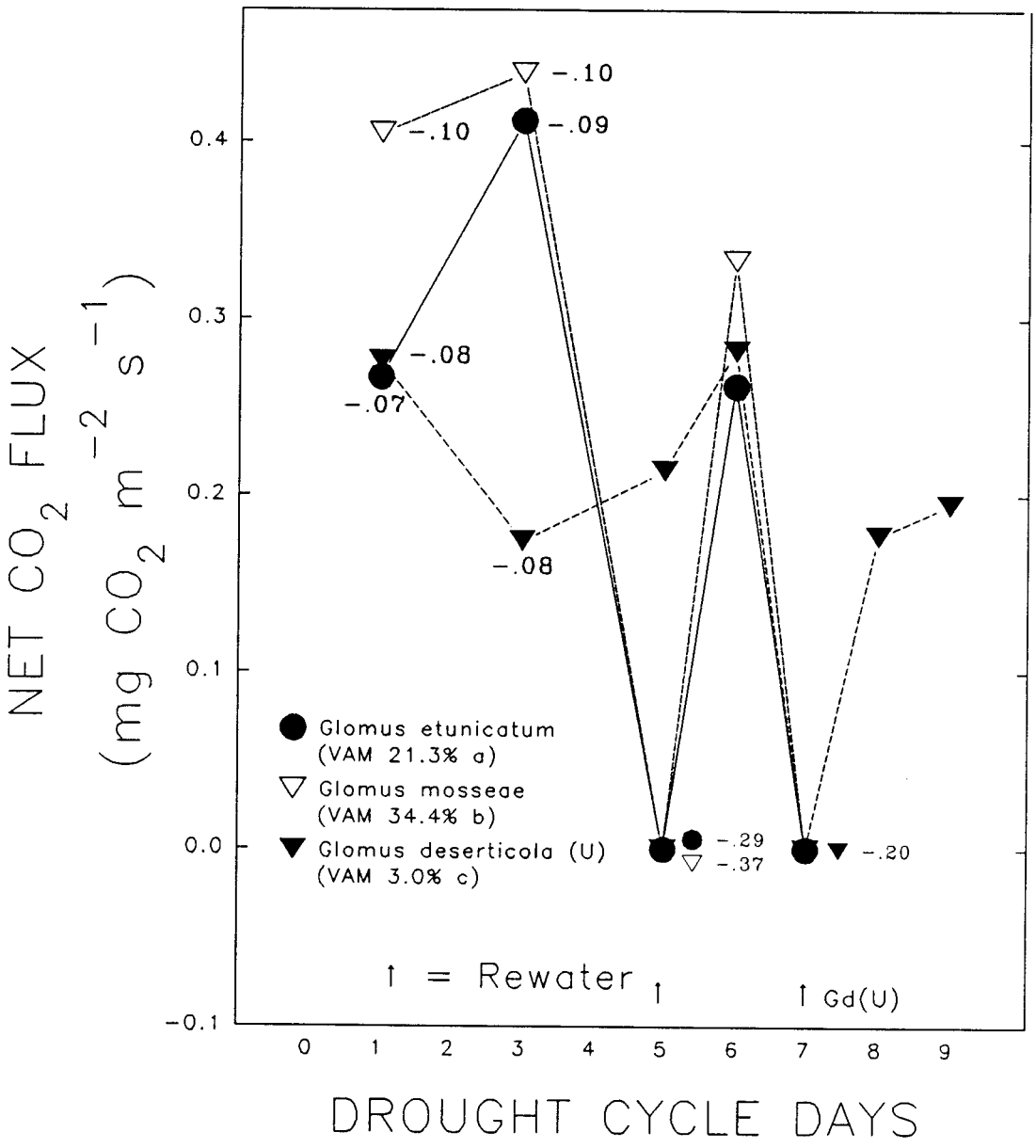

Figure 3-10. Mean net CO_2 flux values for 3 fungal treatments. Some soil water potential (ψ_w) values are also presented if differences in CO_2 flux values were significant. G. etunicatum and G. mosseae were rewatered following readings on day 5 and were terminated on day 7. G. deserticola (U) was rewatered on day 7 and terminated on day 9.

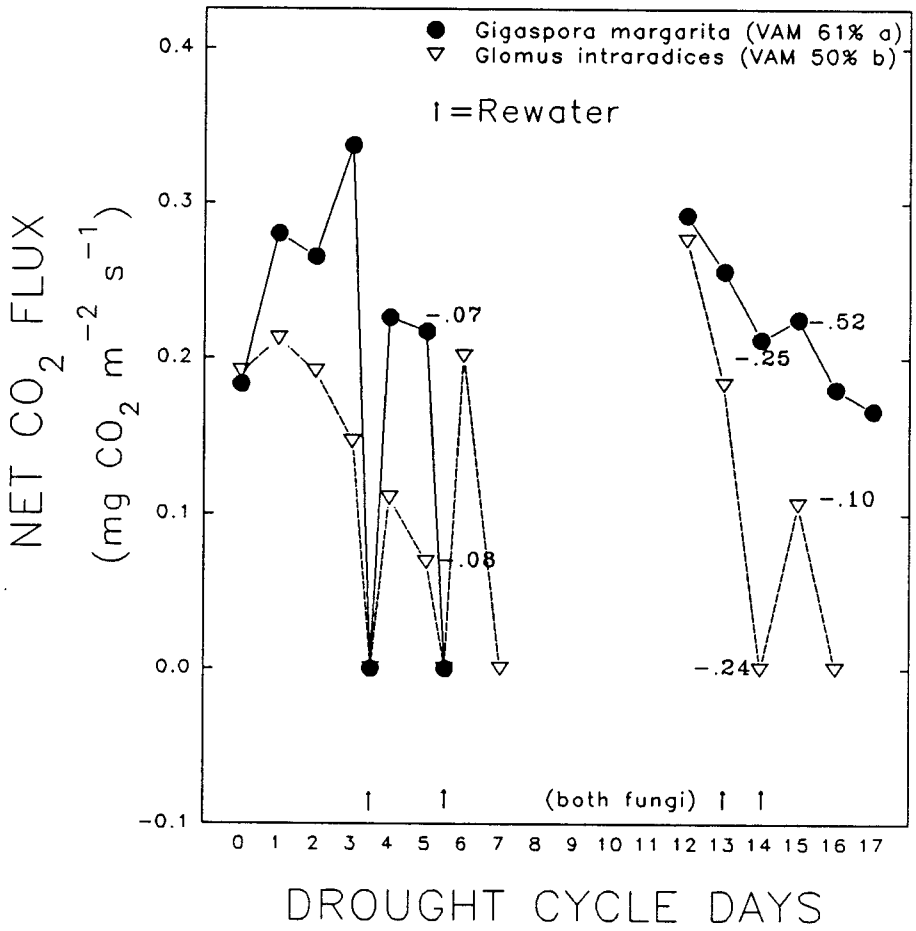

Figure 3-11. Mean net CO_2 flux values for two fungal treatments during three drought cycles. Some soil water potential (ψ_w) values are also presented if differences in CO_2 flux values were significant. Gig. margarita (Gimar) and G. intraradices (Gi) plants were rewatered on day 3. Gi plants were rewatered on day 5 and readings terminated on day 7. Gimar readings were terminated on day 5. A third cycle for both treatments was begun on day 12. Gi plants were rewatered on day 14 and the plants discontinued valid CO_2 fixation on day 16. Gimar plants were rewatered on day 15 and terminated on day 17.

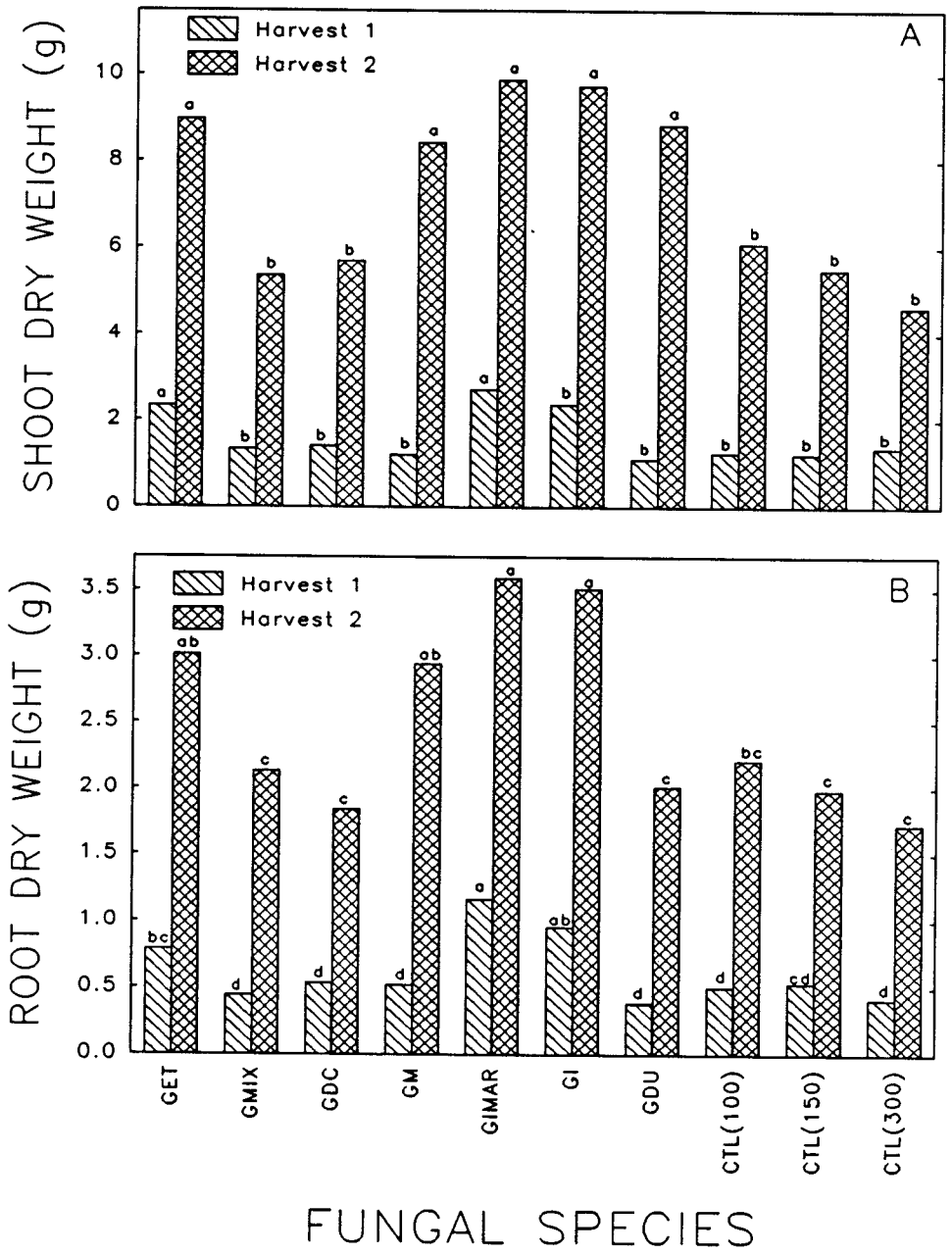

Figure 3-12. Mean Shoot and root dry weight as influenced by VAM fungal treatment. Means within a harvest and with common letters are not different ($P<0.05$).

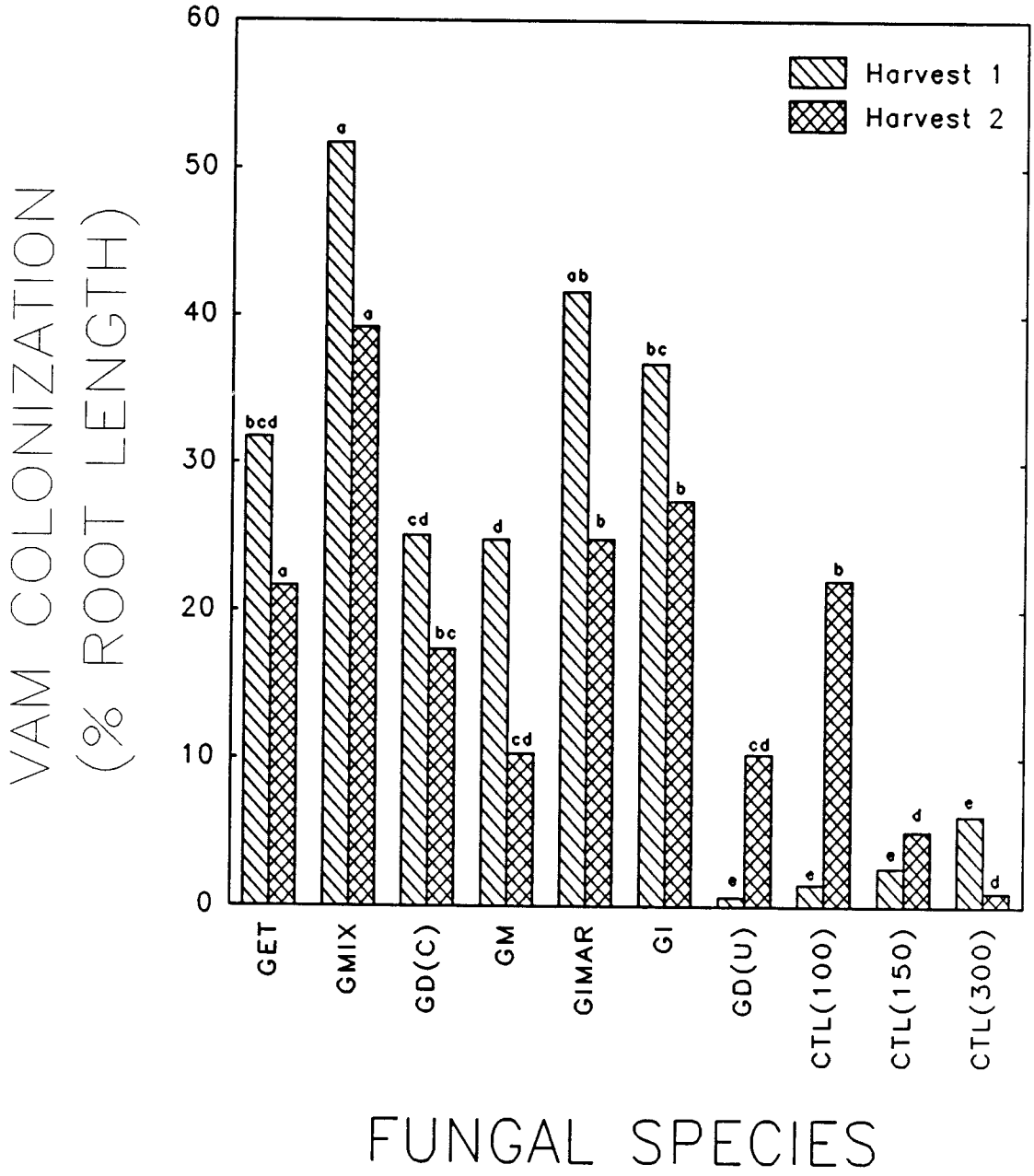

Figure 3-13. Mean VAM colonization (% root length) as influenced by VAM fungal treatment. Means within a harvest and with common letters are not different ($P<0.05$).

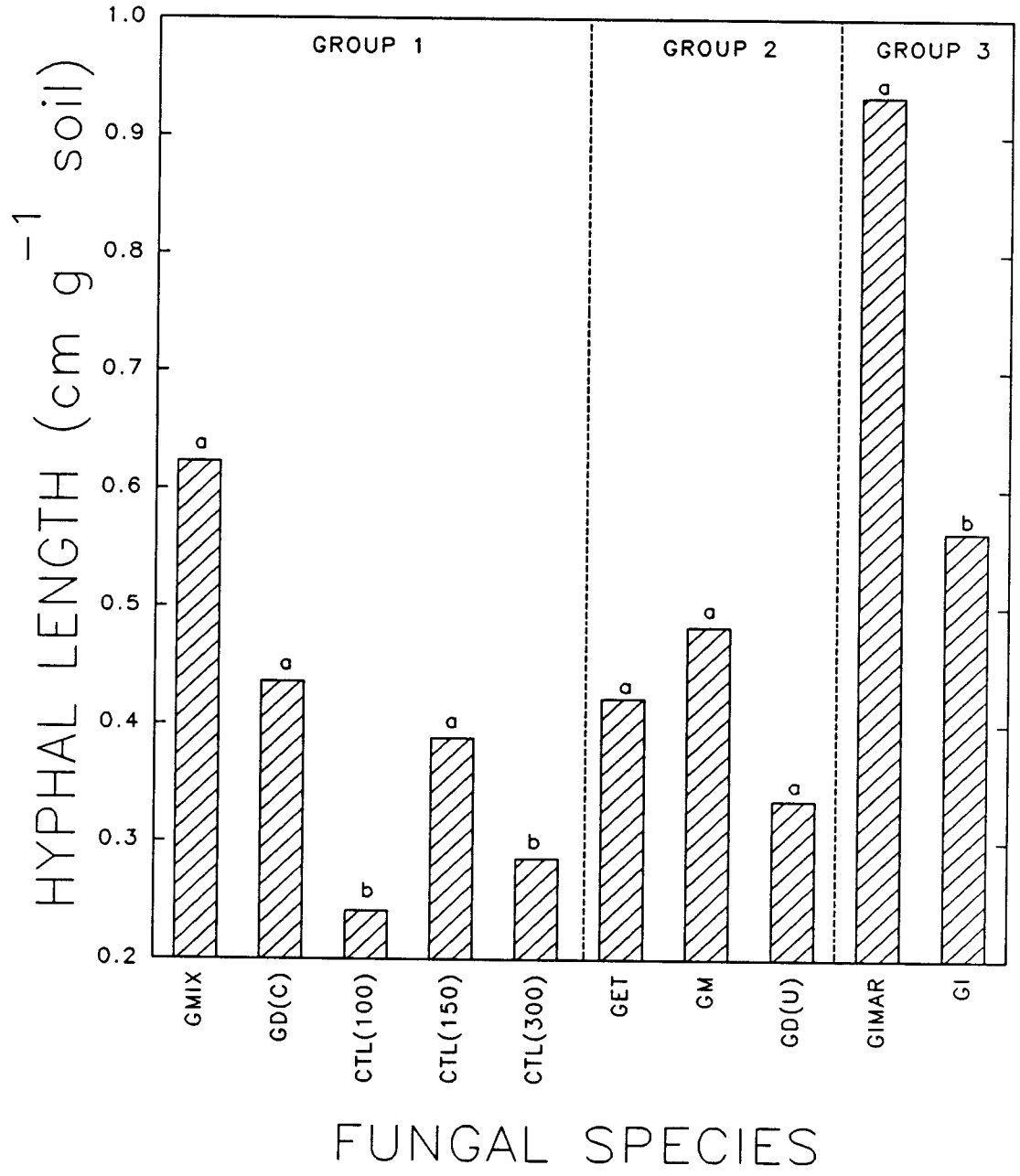

Figure 3-14. Mean hyphal length values as influenced by VAM fungal treatment. Means within a group and with common letters are not different ($P<0.05$).

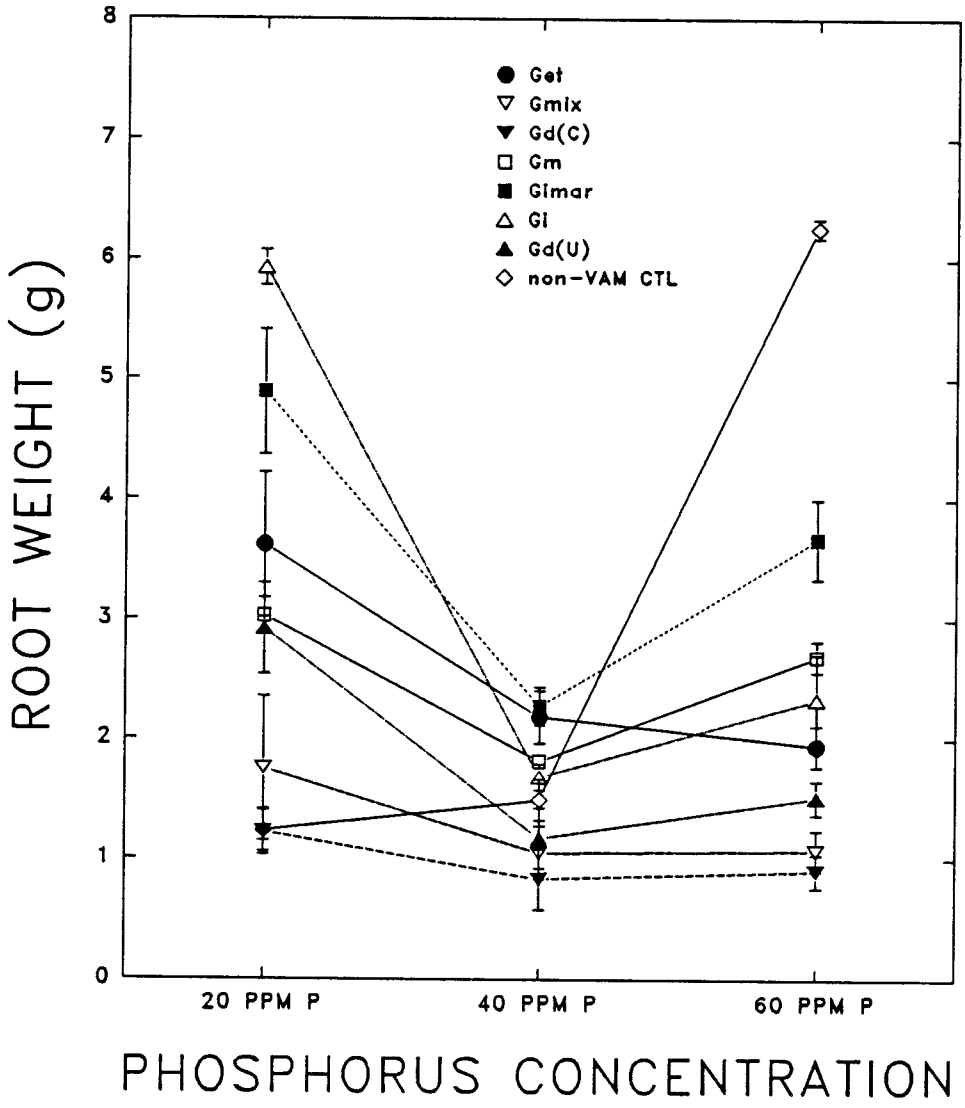

Figure 3-15. Root dry weight at varied P concentrations for different VAM fungal treatments. Standard error bars are presented ($P<0.05$).

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APPENDIX

Appendix Table A-1. Winter wheat shoot and root dry weight (g) means as influenced by the significant interaction between placement and VAM fungal isolate at three weeks.

VAM Isolate	Placement		
	Band	Column	Dispersed
Shoot			
Glomus deserticola	0.036b †	0.044a	0.033b
Glomus intraradices	0.035b	0.035b	0.037b
Root			
Glomus deserticola	0.033ab	0.038a	0.026bc
Glomus intraradices	0.025bc	0.021c	0.025bc

† Means for shoot or root weight followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-2. Winter wheat root dry weight (g) means as influenced by the significant interaction between placement and VAM fungal isolate at six weeks.

	Placement		
VAM Isolate	Band	Column	Dispersed
Root			
Glomus deserticola	0.260b †	0.386a	0.178b
Glomus intraradices	0.200b	0.161b	0.227b

† Means for root dry weight followed by a common letter are not significantly different (P<0.05).

Appendix Table A-3. Green pepper shoot dry weight (g) means as influenced by the significant interaction between VAM fungal isolate and inoculum density at three weeks.

	Density		
VAM Isolate	Ctl	Low Density	High Density
Shoot			
Glomus deserticola	0.260b †	0.386a	0.178b
Glomus intraradices	0.200b	0.161b	0.227b

† Means for shoot dry weight followed by a common letter are not significantly different (P<0.05).

Appendix Table A-4. Green pepper shoot dry weight (g) means as influenced by the significant interaction between inoculum placement and inoculum density at six weeks.

	Concentration		
Placement	Ctl	Low Density	High Density
Band	1.45cd †	1.80bc	2.50a
Column	1.53bcd	1.96b	1.95b
Dispersed	1.88bc	1.14d	1.63bc

† Means for shoot dry weight followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-5. Green pepper root dry weight (g) means as influenced by the significant interaction between inoculum placement and inoculum density at 10 weeks.

	Concentration		
Placement	Ctl	Low Density	High Density
Band	0.323c †	0.540ab	0.614a
Column	0.450bc	0.353bc	0.432bc
Dispersed	0.432bc	0.413bc	0.392bc

† Means for root dry weight followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-6. Green pepper shoot dry weight (g) means as influenced by the significant interaction between placement, VAM fungal isolate, and inoculum density at 10 weeks.

		Concentration		
	Placement	Ctl	Low Density	High Density
	Band	0.775e †	1.301ab	1.425a
Glomus deserticola	Column	0.786e	1.126bcd	1.248abc
	Dispersed	1.086bcd	0.793e	0.764e
	Band	0.775e	0.908de	0.906de
Glomus intraradices	Column	0.727e	0.705e	0.748e
	Dispersed	0.770e	0.701e	0.910cde

† Means for shoot dry weight followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-7. Western red cedar root dry weight (mg) means as influenced by the significant interaction between placement, VAM fungal isolate, and inoculum density at five weeks.

		Density	
VAM Isolate	Placement	Ctl	VAM
	Band	4.24efgh †	4.68defghi
Glomus deserticola	Column	4.80defghi	3.82ghi
	Dispersed	6.00bcdefg	6.78abcdef
	Band	8.10ab	5.22cdefghi
Glomus intraradices	Column	5.58bcdefghi	4.00fghi
	Dispersed	3.18i	4.64defghi
	Band	5.50bcdefghi	6.64bcdef
Phipps Nursery	Column	3.78ghi	7.48abc
	Dispersed	9.38a	5.12cdefghi
	Band	7.28abcd	4.58efghi
Olympic Peninsula	Column	5.9bcdefgh	3.3hi
	Dispersed	5.72bcdefghi	5.12cdefghi

† Means for root dry weight followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-8. Western red cedar VAM colonization (% root length) means as influenced by the significant interaction between inoculum placement and VAM fungal isolate at eight weeks.

	Placement		
VAM Isolate	Band	Column	Dispersed
Glomus deserticola	5.20b †	8.67a	4.13bc
Glomus intraradices	7.47a	7.73a	2.27d
Phipps Nursery	0.93e	3.20cd	1.73de
Olympic Peninsula	0.10f	0.53ef	0.13f

† Means for VAM colonization followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-9. Western red cedar shoot dry weight (mg) means as influenced by the significant interaction between inoculum placement, VAM fungal isolate and inoculum density at eight weeks.

		Density	
VAM Isolate	Placement	Ctl	VAM
	Band	7.77kl †	13.09efghi
Glomus deserticola	Column	8.97jkl	13.50efgh
	Dispersed	9.54ijkl	10.38ghijkl
	Band	11.5fghijk	10.4fghijkl
Glomus intraradices	Column	12.47efghi	7.64kl
	Dispersed	7.231	13.04efghij
	Band	23.72ab	23.13abc
Phipps Nursery	Column	9.63hijkl	14.27ef
	Dispersed	15.64de	10.11ghijkl
	Band	22.64abc	20.58bc
Olympic Peninsula	Column	19.43cd	13.92efg
	Dispersed	24.88a	11.85efghij

† Means for shoot weight followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-10. Western red cedar VAM colonization (% root length) means as influenced by the significant interaction between inoculum placement and VAM fungal isolate at 10 weeks.

	Placement		
VAM Isolate	Band	Column	Dispersed
Glomus deserticola	6.2cd †	7.5cd	14.24bcd
Glomus intraradices	21.62ab	15.46bc	15.72bc
Phipps Nursery	26.08a	10.26cd	4.52d
Olympic Peninsula	22.90ab	27.34a	10.15cd

† Means for VAM colonization followed by a common letter are not significantly different (P<0.05).

Appendix Table A-11. Western red cedar shoot dry weight (mg) means as influenced by the significant interaction between VAM fungal isolate and inoculum density at 10 weeks.

	VAM Isolate			
Density	Glomus deserticola	Glomus intraradices	Phipps Nursery	Olympic Peninsula
Ctl	20.69bc †	15.53c	20.29bc	44.73a
VAM	17.65bc	35.17ab	33.56ab	34.13a

† Means for shoot dry weight followed by a common letter are not significantly different (P<0.05).

Appendix Table A-12. Western red cedar shoot dry weight (mg) means as influenced by the significant interaction between inoculum placement and VAM fungal isolate at 10 weeks.

	VAM Isolate			
Density	*Glomus deserticola*	*Glomus intraradices*	Phipps Nursery	Olympic Peninsula
Band	13.18cd †	34.84abc	54.42a	43.08ab
Column	20.69bcd	21.93bcd	16.85cd	43.90ab
Dispersed	23.79bcd	19.27bcd	10.51d	31.31abcd

† Means for shoot dry weight followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-13. Western red cedar shoot dry weight (mg) means as influenced by the significant interaction between inoculum placement and inoculum density at 10 weeks.

	Inoculum Placement		
Inoculum Density	Band	Column	Dispersed
Ctl	30.18bc †	21.31d	24.16cd
VAM	42.39a	30.34b	18.28d

† Means for shoot dry weight followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-14. Western red cedar root dry weight (mg) means as influenced by the significant interaction between inoculum placement, VAM fungal isolate, and inoculum density at 10 weeks.

		Density	
VAM Isolate	Placement	Ctl	VAM
	Band	10.76ghi †	6.20hi
Glomus deserticola	Column	10.85ghi	7.50ghi
	Dispersed	14.92defghi	14.24efghi
	Band	14.94defghi	21.62bcdef
Glomus intraradices	Column	11.38ghi	15.46defghi
	Dispersed	11.82fghi	15.72defgh
	Band	17.08cdefg	26.08ab
Phipps Nursery	Column	7.44ghi	10.26ghi
	Dispersed	9.72gh	4.52i
	Band	24.18abcd	22.90abcde
Olympic Peninsula	Column	11.62ghi	27.34ab
	Dispersed	29.88a	10.15ghi

† Means for root dry weight followed by a common letter are not significantly different ($P<0.05$).

Appendix Table A-15. Pigeon pea VAM colonization (% root length) at four weeks as influenced by the main factors, VAM fungal isolate and inoculum density.

VAM Fungus	VAM Colonization (% root length)
Glomus etunicatum	29.99b †
G. aggregatum/microcarpum mix	42.58a
Glomus deserticola (C)	8.64d
Glomus mosseae	33.76b
Gigaspora margarita	19.64c
Glomus intraradices	16.72c
Glomus deserticola (U)	2.19d
VAM Concentration	
2.5 spores/ml	18.95b
5.0 spores/ml	18.59b
10.0 spores/ml	29.58a

† Means followed by common letters are not significantly different ($P<0.05$).

Appendix Table A-16. Pigeon pea shoot dry weight (g) at 4 weeks as influenced by the interaction between VAM fungal isolate and inoculum density.

	Spores ml^{-1} Inoculum		
VAM Isolate	2.5	5.0	10.0
Glomus etunicatum	0.161h †	0.153h	0.167h
Glomus aggregatum/microcarpum mix	0.256cdef	0.357a	0.262bcdef
Glomus deserticola (C)	0.227efg	0.240defg	0.269bcde
Glomus mosseae	0.227efg	0.218efgh	0.195fgh
Gigaspora margarita	0.257bcdef	0.254defg	0.334ab
Glomus intraradices	0.153h	0.303abcd	0.321abc
Glomus deserticola (U)	0.372a	0.328abc	0.180gh

† Means followed by common letters are not significantly different ($P<0.05$).

Appendix Table A-17. Pigeon pea root dry weight (g) at 12 weeks as influenced by the interaction between Rhizobium and VAM fungal isolate.

VAM Isolate	- Rhizobium	+ Rhizobium
Glomus etunicatum	1.61abc †	1.82a
Glomus aggregatum/microcarpum mix	1.10e	0.88fg
Glomus deserticola (C)	1.19de	0.47h
Glomus mosseae	1.13e	1.08ef
Gigaspora margarita	1.42cd	1.23de
Glomus intraradices	1.26de	1.79ab
Glomus deserticola (U)	1.05efg	0.84g

† Means followed by common letters are not significantly different (P<0.05).

Appendix Table A-18. Pigeon pea shoot dry weight (g) at 12 weeks as influenced by the interaction between Rhizobium and VAM fungal isolate.

VAM Isolate	- Rhizobium	+ Rhizobium
Glomus etunicatum	4.60ab †	5.18a
Glomus aggregatum/microcarpum mix	3.38cd	2.43g
Glomus deserticola (C)	3.32cde	1.22h
Glomus mosseae	2.77efg	2.84defg
Gigaspora margarita	4.21b	4.00bc
Glomus intraradices	3.05defg	4.38b
Glomus deserticola (U)	3.10def	2.49fg

† Means followed by common letters are not significantly different (P<0.05).

Appendix Table A-19. Pigeon pea VAM colonization (% root length) at 12 weeks as influenced by the interaction between Rhizobium and VAM fungal isolate.

VAM Isolate	- Rhizobium	+ Rhizobium
Glomus etunicatum	20.85de †	19.85de
Glomus aggregatum/microcarpum mix	47.16a	42.52ab
Glomus deserticola (C)	17.54ef	29.35c
Glomus mosseae	14.06ef	18.69ef
Gigaspora margarita	28.76c	38.56b
Glomus intraradices	41.58ab	27.10cd
Glomus deserticola (U)	13.66ef	12.71f

† Means followed by common letters are not significantly different ($P<0.05$).

Appendix Table A-20. Pigeon pea VAM colonization (% root length) at 12 weeks as influenced by the interaction between VAM fungal isolate and inoculum density.

	Spores ml^{-1} Inoculum		
VAM Isolate	2.5	5.0	10.0
Glomus etunicatum	24.01defgh	14.70ijk	20.81ghij
Glomus aggregatum/microcarpum mix	32.85cd	47.64a	52.44a
Glomus deserticola (C)	32.42cdef	23.61fghi	15.67hijk
Glomus mosseae	18.92hij	15.88hijk	15.23ijk
Gigaspora margarita	31.86cde	29.11defg	38.68bc
Glomus intraradices	33.40cd	43.63ab	28.47def
Glomus deserticola (U)	9.90k	19.64ghij	10.15k

† Means followed by common letters are not significantly different ($P<0.05$).

Appendix Table A-21. Growth characteristics, VAM colonization, leaf P concentration and Rhizobium growth characteristics of treatments used in CO_2 fixation assay.

Treatment	Dry weight (g)		VAM	Leaf P	Nodule	Nodule
	Root	Shoot	%	%	Number	Dry weight (g)
††Group 3-Low P						
Gimar	4.9a †	20.6a	47a	0.17a	45.2a	3.22a
Gi	5.9a	17.9a	46a	0.23a	96.7a	3.45a
Group 2-Low P						
Get	3.6a	15.8ab	22b	0.18b	75.5b	2.82a
Gm	3.0a	17.3a	36a	0.27a	138.2a	2.71a
Gd(U)	2.9a	13.2b	5c	0.17b	150.5a	1.16b
Group 1-Low P						
Gmix	1.7a	3.7a	57a	0.39a	15.2a	0.68a
Gd(C)	1.2a	3.8a	28b	0.28b	9.5a	0.43a
High P						
Ctl	6.3b	6.2b	0c	0.26b	22.8a	1.12a

† Means within a column and size group followed by a common letter are not significantly different ($P<0.05$).

†† Low P = 20 ppm P; High P = 60 ppm P.