STUDIES ON VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI IN THE GROWTH IMPROVEMENT OF PUERARIA PHASEOLOIDES BENTH.

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Dedicated to my Parents

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Certificate

This is to certify that this thesis entitled, **Studies on Vesicular-Arbuscular Mycorrhizal Fungi in the Growth Improvement of *Pueraria phaseoloides* Benth.,** is an authentic record of the research work carried out by **Mrs. Kochuthresiamma Joseph**, under my scientific supervision and guidance at the Rubber Research Institute of India, Kottayam, in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** of the Mahatma Gandhi University, under the Faculty of Science and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.

Kottayam
January 1997

Dr. R. Kothandaraman
(Supervising Guide)

Declaration

I hereby declare that this thesis entitled, **Studies on Vesicular-Arbuscular Mycorrhizal Fungi in the Growth Improvement of *Pueraria phaseoloides* Benth.,** has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles for recognition.

Kottayam
January 1997

Kochuthresiamma Joseph

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I cannot claim that this thesis is free from errors, omissions and digressions, and deformities if any, that remains are mine and mine alone.

Kochuthresiamma Joseph

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Chapter 1

Introduction

1 INTRODUCTION

Natural rubber (*Hevea brasiliensis* Muell. Arg.) is an important cash crop in our country. It occupies a key position among plantation crops in India. Hevea rubber is cultivated in 5.16 lakh ha and the annual production of rubber is 4.72 lakh tonnes. India is the sixth largest rubber growing country in the world and ranks fourth in natural rubber production. The narrow strip of peninsular South India along the Western ghats accounts for 96 per cent of rubber production in the country. The rubber plantation industry has achieved tremendous strides in the field of its expansion and productivity during the past four decades. The demand of natural rubber is expected to exceed 7 lakh tonnes by AD 2000, but whether the production would be able to meet the requirements is a pertinent question.

A number of package of practices has been introduced to augment the production of natural rubber. The lacuna in production verses consumption could be filled up only by scientific methods of cultivation. Establishment of a suitable cover crop is one of such scientific methods of production. Cultivation of leguminous cover crop is essential and important in maintaining the fertility of rubber growing soils. The advantages of cover crop include fixation of atmospheric nitrogen, prevention of soil erosion, suppression of weed growth and improvement of physico-chemical properties and biological activities of soil. Leguminous cover crops help in reducing the use of costly synthetic nitrogenous fertilisers. They further help in early maturity and increased yields in rubber.

Pueraria phaseoloides Benth. is more popular among the various cover crops. Strenuous efforts are being made to increase the growth and nitrogen fixing capacity of this cover crop. The leguminous creeper, the root nodule bacterium—*Bradyrhizobium* sp. and Vesicular-Arbuscular Mycorrhiza (VAM) constitute the tripartite symbiosis in the fixation of atmospheric nitrogen. In order to augment the efficacy of symbiosis, one or more of the components of the tripartite system has to be manipulated. VAM association is unique in leguminous plants like *P. phaseoloides*. VAM fungi vastly vary in their symbiotic effectiveness and there could be endophyte preference for the host. Apart from differences in colonisation, the plant growth responses also differ between fungal species. The factors affecting these are, *inter alia*, their preferences for particular soils or host plants, effectiveness in stimulating plant growth, competitive ability, tolerance to added fertilisers and fungicides and so on.

The species and population of VAM fungi in a given soil are determined by a number of factors like physico-chemical characters of soil, crop and it's management practices, etc. A right combination of plant and VAM is highly essential for maximum benefit.

The most apparent and important benefit of VAM association with leguminous plant is the absorption of phosphates from soil. Legumes generally have less extensive root system as compared to other plants and many of them are poor foragers of soil phosphates. Phosphate is an important macronutrient for leguminous plants due to its role in energy transfer during the process of nitrogen fixation. A good supply of phosphorus is also essential for effective nodulation which could be accomplished by VAM association. Nodules are generally known to possess higher concentration of phosphorus than root tissues. The stimulation of

nodulation and nitrogenase activity are often attributed to increased phosphorus status of mycorrhizal plants.

VAM association not only augments the nitrogen and phosphorus level in leguminous plants but also increases photosynthesis, levels of amino acids, protein fraction and the activity of the enzyme—alkaline phosphatase (Gianinazzi-Pearson and Gianinazzi, 1978, 1984). Even the biochemical constituents of root exudates of plants colonised by VAM fungi are reported to have changed and such phenomenon favours root nodulation by *Bradyrhizobium* sp. as well as rhizosphere microbial population (Linderman, 1992).

In addition to phosphorus, VAM association also augments the uptake of zinc and copper which ultimately results in the increased plant growth (Gilmore, 1971; Timmer and Leyden, 1978; Bowen, 1980).

The level of available phosphorus in the soil is one of the factors which determines the root colonisation by VAM fungi. Though soil phosphorus level increases the VAM formation, it is detrimental to root colonisation at higher levels. Rock phosphate being the major phosphatic fertiliser used in rubber plantations it is obvious to study the safe level of this fertiliser for the formation of VAM and maximum P uptake.

VAM symbiosis exists amidst of both beneficial and harmful microorganisms and they influence each other. Dual inoculation of leguminous plants with VAM and one or more of the beneficial organisms like *Bradyrhizobium*, non-symbiotic nitrogen fixing bacteria and phosphate solubilising bacteria are practiced in many crops.

Rubber growing soils are generally acidic and highly eroded with low phosphorus. The applied phosphates are fixed either as aluminium or ferric phosphates and therefore, necessitate adequate supplementation of phosphatic fertilisers. It is essential that the input cost of rubber cultivation be minimised. This will help in the sustainable development of rubber cultivation which means improving the quality and quantity within the carrying capacity of supporting ecosystem. A dynamic concept of carrying capacity would imply in operation terms the conservation of natural ecosystem as well as their continuous improvement. Once a state of ecological balance has been attained, rubber production could be a sustainable event.

Selection of suitable VAM fungi for *P. phaseoloides* would be of much agronomic importance in rubber cultivation. Farmers are also becoming increasingly aware that crop productivity could be improved by VAM inoculation.

Though VAM association is reported in *P. phaseoloides*, the effect of different species on the growth, nodulation and nitrogen fixation was not studied. Therefore, a detailed study on various aspects of VAM development and its effect on *P. phaseoloides* was carried out with the following objectives.

1) Distribution of VAM fungi in rubber plantations and selection of an efficient species for *P. phaseoloides*.
2) Time course study for VAM colonisation and plant response.
3) Effect of different levels of rock phosphate on VAM formation, growth and nutrient content of *P. phaseoloides*.
4) Interaction of VAM with other beneficial microorganisms and their effect on VAM development, growth and nutrient content of *P. phaseoloides*.
5) Developing a technique for mass production of VAM fungi 'for *P. phaseoloides*.

Chapter 2

Review of Literature

2 REVIEW OF LITERATURE

Establishment and maintenance of a ground cover in rubber plantations is an accepted agromanagement practice. Many years of extensive research has shown the various beneficial effects of leguminous cover plants. These include improvement of physical and chemical properties of the soil (Soong and Yap, 1976), the growth and yield of rubber tree (Watson, 1961; Watson *et al.*, 1964; Wycherley and Chandapillai, 1968; Pushparajah and Chellappah, 1969). Additionally it brings down the cost of maintenance of rubber plants.

An ideal cover crop is expected to have maximum desirable characters like enhanced nitrogen fixation, fast growth, tolerance to frequent cuttings, shade and drought, as well as free from pests, diseases and competition with rubber for nutrients and water. *Pueraria phaseoloides* is cultivated extensively as cover crop in many rubber growing countries including India.

P. phaseoloides is specific for particular strains of *Bradyrhizobium* sp. (Bogdan, 1977) and species of VAM fungi (Sieverding, 1990) which implies that selection of one or both may help in establishing this cover crop in spite of occasional failures.

Nature of VAM and their distribution

Mycorrhizae or fungus roots is a symbiotic association between certain fungi and plant roots and is an essential phenomenon in most of plants except in families

like Cruciferae, Chenopodiaceae and Caryophyllaceae. Among different groups of mycorrhizae, VAM is more common in cultivated plants.

VAM association is geographically ubiquitous and occurs in plants in artic, temperate and tropical regions including dense forest, open wood land, scrub, savanna, grass lands, heaths and sand dunes and semideserts. The occurrence within these systems varies according to localised environmental conditions and plant cover (Hayman, 1982). Nicolson (1960) demonstrated that both the per cent VAM root infection in dune grasses and the amount of external mycelia were related to the ecological succession stage. Koske *et al.* (1975) reported external VAM mycelium in grasses bind the soil particles and thus contributing to dune stability. Redhead (1968) found VAM in all 15 exotic and 44 out of 51 indigenous plant species that he examined in a low land tropical rain forest in Nigeria. In a deciduous wood land in England, every plant examined except the ectomycorrhizal trees had VAM (Hayman, 1982).

Members of the families, Fabaceae and Poaceae are normally mycorrhizal. VAM association in plants of Fabaceae family is considered to be the most essential one, as it helps P uptake, the essential nutrient in symbiotic nitrogen fixation. VAM development in Fabacious plants were reported to vary considerably. *Stylosanthes* sp. and *Trifolium* sp. usually have dense VAM infection whereas *Lupins* have little or none (Morley and Mosse, 1976; Trinick, 1977).

Eversince Mosse (1957), Gerdemann (1964) and Baylis (1967) in the last few decades demonstrated VAM could increase P uptake from soil by plants, researchers have been trying to manipulate this 'phosphorus sparing' effect of mycorrhizae in agriculture (Hayman, 1978; Tinker, 1978).

Gerdemann (1975) reported that certain plant species require mycorrhizae to a much greater extent than do others and this is usually referred to as "mycorrhizal dependency", which is the degree to which plant is dependent on the mycorrhizae to produce its maximum growth or yield at a given level of soil fertility. Mycorrhizal dependency of a plant species can be altered by many factors including genotype and soil P. In the case of wheat cultivars, Azcon and Ocampo (1981) observed that mycorrhizal dependency was affected by the root weight, and root/shoot dry weight ratio rather than by concentration of N, P, K, Ca and Mg in the tissues. Pope *et al.* (1983) have reported a wide range of mycorrhizal dependency in plants and were significantly influenced by fungal species. Mycorrhizal dependency values were greatest for *Fraxinus pennsylvanica* followed by *Liriodendron tutipifera* and *Platanus occidentails* and among the 6 VAM fungi (*Glomus mosseae*, *G. fasciculatum*, *G. etunicatum*, *G. macrocarpum*, *G. epigaeum* and *Gigaspora margarita*), inoculation of *G. macrocarpum* resulted in the highest mycorrhizal dependency for all tree species.

A great variation in dependency on mycorrhizae was observed among forage legume species. The total uptake of all elements by non-mycorrhizal legumes and uptake of P, N, and K by non-mycorrhizal grasses correlated inversely with mycorrhizal dependency (Saif, 1987).

Isolation and selection of effective VAM fungi

Isolation and maintenance of VAM fungi under sterile condition is highly essential in research. Hayman (1982) reviewed different methods adapted for isolating VAM fungi and stated that isolation by single spore technique is comparatively superior to other techniques like soil sieving, infected roots, infected plants and baiting.

Selection of effective strains of VAM fungi for crops is a pre-requisite to obtain optimum or maximum response under defined soil environmental conditions. In soybean, some fungal isolates depressed the growth, a few of them gave appreciable growth increase and others did not respond (Carling and Brown, 1980). Sweet gum seedlings inoculated with *G. fasciculatum*, *G. mosseae*, *G. etunicatum* and a mixture of species of *Glomus* and *Gigaspora* showed significant increase in plant height, leaf, stem and root weight over uninoculated controls (Kormanik *et al.*, 1981).

Habte and Aziz (1985) reported that under both sterile and unsterile conditions, *G. fasciculatum* improved the growth of *Sesbania grandiflora* compared to *G. mosseae*. In strawberry, there was no correlation between per cent root infection and stimulation of growth whereas there was significant correlation in apple, asparagus, leek and oat (Plenchette *et al.*, 1982). In white oak seedlings (*Fraxinus americana*) inoculated with different VAM fungi, growth differed between each fungal species but the differences decreased after 82 days of growth. However, dry weight remained higher with *G. epigaeum*, *Glomus* sp. and *G. monosporum* (Furlan *et al.*, 1983). Borges and Chaney (1988) concluded that the differences in growth response to mycorrhizal inoculation could be due to inherent differences in efficacy, fungal aggressiveness and amount of infection or combination of these factors. The best root and shoot growth, nodule formation and infection intensity of *Leucaena* sp. was observed with *G. fasciculatum*, *G. mosseae* and *G. etunicatum* (Huang *et al.*, 1983), whereas Nalini *et al.* (1986) reported that VAM fungal species belonging to *Glomus*, *Gigaspora*, *Acaulospora* and *Sclerocystis* inoculated to *Leucaena* cultivars varied in their ability to improve shoot dry weight. Bagyaraj *et al.* (1989) found that local isolate of *G. mosseae* was the best mycorrhizal fungus for *Leucaena* cultivars—K28, K67, and K72 for obtaining healthy, vigorously

growing seedlings. Sreenivasa (1992) studied the effect of *G. fasciculatum*, *Gi. margarita*, *A. laevis*, *S. dussi* and a local isolate, *G. macrocarpum* on the growth of chilli (*Capsicum annuum*) and concluded that the local isolate *G. macrocarpum* was superior over the other isolates in promoting the growth.

Reena and Bagyaraj (1990) also reported that the most efficient VAM for inoculation of *Acacia nilolica* was *G. mosseae* (ICRISAT) followed by *G. caledonium* and for *Calliandra calothyrsus* it was *G. velum* and *G. morredum*.

Blaszkowski (1993) made a detailed study on the effect of five species of *Glomus* on wheat. He observed that all the fungi increased dry weight and *G. caledonicum*, was the most effective. While studying on the effect of different isolates of mycorrhizal fungi Janson and Linderman (1993) recorded variations due to VAM species in the growth, nodulation and N_2 fixation in pigeon pea. Jadhav and Patil (1995) also proved the growth variation of groundnut under the influence of different species of VAM fungi and emphasised the need for strain selection of VAM fungi which are most effective.

Crop response to mycorrhizal inoculation

Growth

Various laboratory and green house experiments have demonstrated that VAM inoculation can improve growth and nutrition of crop plants (Hayman, 1980; Smith *et al.*, 1986; Lin and Hao, 1988; Raju *et al.*, 1990). Enhanced dry weight of mycorrhizae inoculated plants were shown in various crops (Pope, 1980; Jakobson, 1983; Potty, 1988).

Studies conducted by Mosse (1957) in apple leaf bud cuttings, Daft and Nicolson (1966) in tobacco, tomato and maize; Pope (1980) in *P. occidentalis* and Lamar and Davey (1988) in green ash (*F. pennsylvanica*) showed increased height of mycorrhizal plants compared to non-mycorrhizal plants.

Singh *et al.* (1992) reported that VAM colonisation in soil increased root and shoot length and number of leaves per rough lemon seedlings. The root shoot ratio was wider in non-mycorrhizal plants compared to mycorrhizal plants (Baon *et al.*, 1994).

Potty (1988) and Rai (1990) showed an increase in yield of cassava and potato respectively in mycorrhizal plants.

Increased biomass production was reported upon mycorrhizal inoculation in leguminous plants like cowpea (Godse *et al.*, 1978), groundnut (Krishna *et al.*, 1982), blackgram, pigeonpea and cowpea (Manjunath and Bagyaraj, 1984), chickpea (Champawat, 1989), tropical kudzu (*P. phaseoloides*) (Sieverding, 1990) and *Vicia faba* (Ishac *et al.*, 1994).

Enhanced nodulation, nitrogen fixation and biomass production upon VAM inoculation are well established in *P. phaseoloides* and *Stylosanthes guyanensis* (Waidhyanatha *et al.*, 1979), *Leucaena leucocephala* (Purcino *et al.*, 1986), *A. mangium* and *Albezia falcataria* (Dela Cruz *et al.*, 1988), *Cicer arietinum* (Singh and Tilak, 1989) and *Centrosema pubescens* and *Medicago sativa* (Crush, 1974; Smith and Daft, 1977). An increase in height of legume trees, *A. mangium* and *A. falcataria* was reported by Dela Cruz *et al.* (1988). Rice bean (*Vigna umbellata*) inoculated with *G. fasciculatum* and *Rhizobium* sp. in a P deficient soil significantly increased VAM colonisation, nodulation and yield of plants (Kaur and Singh, 1985).

Joshi *et al.* (1991) showed in 60 days old groundnut a significant positive correlation between per cent root mycorrhizal infection and number of nodules.

Mineral content

It has been documented that mycorrhizal roots can absorb phosphate from soils of low phosphorus status at a greater rate per unit length of roots than non-mycorrhizal controls (Sanders and Tinker, 1971; Gupta *et al.*, 1990). Jalali and Thareja (1980) showed an increased uptake of phosphate and dry matter production in pearl millet and wheat. Pairunan *et al.* (1980) observed an increased uptake of phosphate in subterranean clover by inoculation with *G. mosseae*.

Studies by Powell (1981) showed an increase in seed phosphate content by 35 per cent in barley by mycorrhizal inoculation. Mycorrhizal formation is also known to increase the coefficient of utilisation of P fertilisers by 5-10 folds and nitrogen and potassium fertilisers by 3-5 folds in sycamore (*Acer pseudoplatanus*) (Kabre *et al.*, 1982). Jensen (1982) showed that the increased uptake of nutrients and yields of barley depended on the species of VAM inoculated. Seedlings inoculated with VAM fungi did not show any significant increase in foliar P and N concentration though the growth was significantly enhanced (Backhaus *et al.*, 1986).

Increased uptake of nutrients in leguminous plants is much conspicuous and phisologically important (Bowen, 1980; Krishna *et al.*, 1982). Krishna and Bagyaraj (1984) reported enhanced uptake of P, Zn, K, Mg, Cu, Fe and Mn contents in groundnut by VAM fungi. Saif (1987) reported that growth and uptake of minerals such as P, N, K, Ca, and Mg was improved due to VAM inoculation in forage legumes grown in sterilised soil. Inoculation of VAM increased N

concentration, N content, P concentration and P content of the tree legumes *A. mangium* and *A. falcataria* (Dela Cruz *et al.*, 1988).

Biochemical constituents

Nemac and Guy (1982) reported that the leaves of VAM inoculated citrus seedlings contained greater amount of total soluble sugars, sucrose, reducing sugars, starch and non-structural carbohydrates per gram of tissues than uninoculated controls in low P soil (9-12 ppm). Doss *et al.* (1988) found higher levels of starch grains and insoluble protein in root cap cells of finger millet upon VAM inoculation. Prasad and Bilgrami (1995) found an increase in volume of juice and per cent of sucrose content in cane shoots of VAM inoculated plants compared to VAM free plants.

Increased accumulation of free amino acids in mycorrhizae infected plants at later stages of plant growth has been observed by Young *et al.* (1972) and Nemec and Meredith (1981). The increased amino nitrogen concentration and protein levels in mycorrhizal plants were also reported by Krishna and Bagyaraj (1983) in groundnut and increased protein content in soybean plants by Chang *et al.* (1992).

It has been repeatedly observed that other than the mineral uptake mycorrhizal association enhanced the tolerance of plants to altered environmental conditions and to a lesser extent increases the resistance to soil borne plant pathogenic fungi. Phenolic substances like phytoalexins are synthesised when soybean root is colonised by VAM (Morandi *et al.*, 1984). Lakshmanan *et al.* (1987) reported the enhanced accumulation of phytoalexin in cowpea infected with VAM fungi compared to uninoculated control.

Phenol deposition in mycorrhizal tomatoes has been reported to impede the infection and spread of *Fusarium* sp. (Dehne and Schönbeck, 1979).

Krishna (1981) showed that the concentration of *ortho* dihydroxy phenols in the roots of mycorrhizal peanut plants at different stages of plant growth ranged from 87-130 mg g^{-1} fresh weight of the root sample while in non-mycorrhizal plants it ranged from 62-85 mg g^{-1}. Dehne (1982) reported an increase in synthesis of metabolites like lignin, ethylene, and phenols which may contribute to the protective efforts of mycorrhizal roots to pathogens. Krishna and Bagyaraj (1983) reported the formation of *ortho* dihydroxy phenols as inhibitory compounds by the interaction of mycorrhizae and pathogen *Sclerotium rolfsii* in peanut. Kanakadurga and Rama Rao (1995) reported higher levels of phenols in shoot system of VAM infected plants which would implicate high levels of disease resistance.

Chlorophyll content and photosynthetic rate

VAM fungi depend on plants for carbohydrate and the increased carbon requirement by mycorrhizal plants were compensated by a higher photosynthetic rate as shown in *Citrus aurantium* (Johnson, 1984) and in faba beans (Pang and Paul, 1980; Kucey and Paul, 1981, 1982).

Pang and Paul (1980) showed that the mycorrhizal and non-mycorrhizal faba beans plants fixed 44 mg C g^{-1} dry matter day^{-1} and 36 mg C g^{-1} dry matter day^{-1} respectively over the 48 h labelling period.

An increase in chlorophyll content in VAM inoculated tomato plants was reported by Dehne and Schönbeck (1975). Enhanced chlorophyll content and photosynthetic rates of VAM inoculated grass (*Bouteloua gracillis*) was observed by Allen *et al.* (1981).

Dual inoculation of leguminous plants with *Rhizobium* and VAM was found to enhance chlorophyll content and photosynthetic rates in *Cyamopsis* sp. (Neeraj and Ajit Varma, 1995) along with increased leaf area in mung bean (Panwar and Thakur, 1995) compared to inoculated plants with either of these micro symbionts.

Nitrogen fixation

The tripartite relationship of the leguminous plants with *Rhizobium* and VAM was found to stimulate nodulation and enhance nitrogen fixation rates, measured as acetylene reduction activity by two folds in french bean (*Phaseolus vulgaris*) (Daft and El-Giahmi, 1974) pea nut (Daft and El-Giahmi, 1976) and *Medicago sativa* (Smith and Daft, 1977). The stimulation of nodulation and nitrogenase activity is attributed to increased phosphorus status of mycorrhizal plants (Smith and Daft, 1977). Smith and Daft (1978) reported that there is a sequence whereby VAM fungi first stimulate nodule bacteria and their activity by increasing tissue P concentration resulting in enhanced nitrogen fixation, consequently higher plant growth. A time course trial with soybean has shown that VAM increases nitrogenase activity rapidly (140-197%) between 40 and 80 days while no stimulation could be detected in non-mycorrhizal plants (Subha Rao and Krishna, 1988). They also found that increasing P addition eliminated mycorrhizal effects on growth at 0.25 g KH_2PO_4 kg^{-1} soil, nodulation at 0.5 g KH_2PO_4 kg^{-1} soil and nitrogenase activity at 1.0 g KH_2PO_4 kg^{-1} soil.

The stimulation of nitrogenase activity due to VAM inoculation was reported in many crops including *T. subterranium* (Smith *et al.*, 1974), faba bean (Kucey and Paul, 1982), *L. leucocephala* (Purcino *et al.*, 1986), soybean (Vejsadova *et al.*,

1988; Vejsadova *et al.* 1992; Tilak *et al.*, 1995), mung bean (Panwar and Thakur, 1995), etc.

Effect of VAM inoculation on plant growth at different intervals

Rapid and maximum colonisation is the most important determining factor of mycorrhizal response (Menge, 1983). VAM root infection usually reaches a maximum towards the end of the growing season (Hayman, 1970), following a three phase pattern of growth (Sutton, 1973). With field grown *Phaseolus* beans and soybeans, Sutton (1973) noted an initial lag phase of 20-25 days, attributed to rapid root growth of the seedlings and the time required for spore germination, germ tube growth and penetration of the host plant root. In the second phase lasting, 30-35 days, extensive mycorrhizal development coincided with most shoot growth and copious spread of external mycelium leading to multiple infection. During the third phase, from host fruiting to senescence, the proportion of mycorrhizal to non-mycorrhizal roots remained constant. Studies by Sullia and Chandranath (1991) in *Phaseolus radiatus*, chickpea and *Vigna sinensis* had shown that all the three plant species were colonised 15 days after sowing in field plots. There was an increase in the number of vesicles and arbuscules upto 30-45 days followed by a decline to almost zero after 120 days.

Factors influencing the natural distribution of VAM fungi in soil

VAM population in cultivated lands is affected by various, soil, plant and environmental factors as well as various agricultural and horticultural practices.

Plant

Plant species vary widely with regard to VAM colonisation. Some are heavily infected, some only moderately, whereas others such as swedes and sugar beet have almost no infection. In Southern Spain, maize, *Phaseolus* beans, and grape vine were consistently heavily mycorrhizal, olives were variable and tomatoes consistently fairly lightly infected even when present at the same site as the first three (Hayman *et al.*, 1976). The presence or absence of a host plant obviously plays a larger role and the affinity of a host plant to VAM endophyte will determine the degree of colonisation and sporulation. Joseph *et al.* (1988) reported that the different leguminous cover crops in the same field differed in VAM infection and spore production.

Soil type

Type of soil is one of the important factor which influences the VAM association and distribution (Kehri *et al.*, 1987; Sivasaravanan and Sundaram, 1995).

Soil pH

Soil pH significantly influences VAM activity (Kruckelmann, 1975). Hayman and Mosse (1971) obtained VAM formation in *Coprosma robusta* in soils with pH 5.6 to 7.0 and not in acid soils of pH 3.3 to 4.6. Mosse (1972a) observed considerably high efficiency of VAM by increasing soil pH. However, very high and low pH decreased VAM infection. Zhaobin (1988) reported that *Glomus* sp. infected cotton roots ramified extensively and formed more hypae at high soil pH conditions.

Much VAM infection but few spores were reported in acid hill grass lands in Northern England (Sparling and Tinker, 1978), mid-Wales (Hayman and Mosse, 1979), Western United States and Canada (Molina *et al.*, 1978). Abbott and Robson (1977) reported that the distribution of different types like 'honey coloured sessile' and 'yellow vacuolate' spores in Western Australia was related to soil pH.

However, Mohan Kumar and Mahadevan (1987) could not find any definite correlation between soil pH and VAM development as well as sporulation.

Effect of fertilisers

Changes in soil fertility due to application of mineral fertilisers or organic matter markedly affect the activity of soil mycorrhizal population in terms of the amount of root infection and number of resting spores produced (Hayman, 1982).

A negative effect of phosphatic fertiliser on mycorrhizal population in soil was observed by Hayman (1975), and on mycorrhizal infection was shown by Jasper *et al.* (1979) in various crops. Excess phosphorus reduced the root infection and spore production by VAM in *Abelmoscus esculentus* (Krishna and Bagyaraj, 1982) cardamom (Rohini Iyer *et al.*, 1988) and blackgram (Umadevi and Sitaramaiah, 1990). Krucklemann (1975) reported that fertilisers may have a positive effect on VAM if the initial soil fertility is very low. Krucklemann (1975) also found that application of phosphate (0-220 Kg P ha^{-1}) for seven years did not affect the frequency of VAM spores in soil.

Jasper *et al.* (1979) reported that mycorrhizal infection declined with increasing plant soil phosphate status. Although more spores were found at intermediate levels of P, neither the relative abundance nor infection levels were

consistently related to P application (Hayman, 1982). Ryan *et al.* (1994) reported that higher levels of soluble P have negative effect on VAM colonisation but insoluble rock phosphate did not decrease levels of VAM.

The effect of VAM inoculation on plant growth varied at different levels of P application. Powell and Daniel (1978) reported that P from poorly soluble rock phosphate may only be available to mycorrhizal plants.

Inoculation with mycorrhizal fungus was found to increase growth of citrus seedlings (Krikum and Levy, 1980), cotton, cowpea and finger millet (Bagyaraj and Manjunath, 1980), apple seedlings (Hoepfner *et al.*, 1983) and tomato plants (Khaliel and Elkhider, 1987) in low phosphorus soil.

Powell (1984) reported growth response to mycorrhizal inoculation in case of onion, citrus and some legumes, even in soils with moderate to high P status.

Studies by Bagyaraj and Sreeramulu (1982) in chilli and Govinda Rao *et al.* (1983) in finger millet had shown that plants given half the recommended level of P with selected mycorrhizal fungal inoculation were comparable to inoculated plants given full level of P fertiliser. Stimulation of growth and nutrient uptake were found to depend on species of VAM fungi (Strong and Davies, 1982; Kormanik, 1985).

Mosse *et al.* (1976) found that legumes inoculated with appropriate *Rhizobium* strain in the most P deficient soil nodulated only where mycorrhizae are present.

Increased growth, nodulation and yield were reported due to VAM inoculation at various range of phosphate in soybean (Ross, 1971), *Calopogonium caeruleum* (Ikram *et al.*, 1987; Ibrahim *et al.*, 1988), *S. capitata*, *C. macrocarpum* and *P. phaseoloides* (Arias *et al.*, 1991) compared to uninoculated control. The mycorrhizal response were pronounced at low P levels.

Interactions with other microorganisms

It is well documented that mycorrhizal fungi improve plant growth and enhance microbial activity in the rhizosphere of mycorrhizal plants (Bagyaraj and Menge, 1978).

The beneficial interaction of phosphate solubilising microorganisms, *Bacillus circulans*, *B. polymyxa* and *B. megaterium* with VAM fungi in P deficient soils amended with rock phosphate, resulting in increased P nutrition, growth and yield of crops were reported (Raj *et al.*, 1981; Krone *et al.*, 1985; Heggo and Barakah, 1993, Das *et al.*, 1995).

Synergistic effect on growth of tomato with inoculation of *G. fasciculatum*, *Beijerinckia mobilis* and *Aspergillus niger* have been reported (Manjunath *et al.*, 1981). Similar beneficial effects in tomato plants was also reported with *Azotobacter* strains (Bagyaraj and Menge, 1978; Mohandas, 1987; Azcon, 1989).

The tripartite symbiotic association of leguminous plants helps them available with two vital elements i.e., P and N_2. Inoculation with selected efficient strains of rhizobia and VAM fungi improved nodulation, N_2 fixation, growth and P nutrition in some forage grains and legumes (Bagyaraj *et al.*, 1979; Munns and Mosse, 1980; Redente and Reeves, 1981), pigeon pea and cowpea (Manjunath and Bagyaraj,

1984), *Leucaena* (Nalini *et al.*, 1986), soybean (Kumutha and Santhanakrishnan, 1995) and *A. lebbek* (Kaushik and Kaushik, 1995).

Studies in legumes had also shown that addition of rock phosphate or phosphate solubilising microbes along with VAM and *Bradyrhizobium* stimulated root infection and nodulation, N_2 and P uptake, available soil P, plant growth, grain yield, etc. in various crops (Pal *et al.*, 1989; Pathiratna *et al.*, 1990; Singh and Singh, 1993; Tilak *et al.*, 1995).

Mass multiplication of VAM fungi

Inoculation with VAM fungi is known to result in drastic improvements in plant growth under conditions of limited nutrient supply (Abbott and Robson, 1982; Menge, 1983). Menge (1984) explained the importance of mass culturing and field application of VAM. Sudan grass, bahia grass, guinea grass, cenchrus grass, clover, strawberry, sorghum, maize, onion, coprosma and coleus have all been studied for their suitability in producing VAM inoculum (Bagyaraj, 1988). Sreenivasa and Bagyaraj (1988) reported that rhodes grass (*Chloris gayana*) is the best host for mass production of *G. fasciculatum*. *Gi. margarita* is reported to infect onion better than lettuce and *Nardus stricta* is good for sporocarp production by *G. mosseae* (Hayman, 1982).

From the foregoing review of literature it becomes apparently clear that crop plants are highly benefited by VAM association. If properly applied to crops, VAM inoculum would improve crop productivity and the fertility status of soil.

Chapter 3

Materials and Methods

3 MATERIALS AND METHODS

3.1 Distribution and isolation of VAM

3.1.1 Collection of soil samples

Soil samples were collected from sixteen locations of rubber growing area (Fig. 1) to study the natural distribution of VAM fungi. Soil was dug out with a trowel to a depth of 15 cm after scrapping away the top 1 cm layer of soil. Samples were collected from 20 different places in each area, pooled and homogenised. Representative samples were taken in polythene bags, labelled and stored at 2°C until they were processed further. From this, 10 lots of 50 g soil were wet sieved for taking spore count and isolation of spores (Hayman, 1982).

3.1.2 Determination of soil pH

pH of the samples was determined in 1:2.5 (v/v) soil:water solution in a Philips pH meter.

3.1.3 Enumeration of VAM spore population

The spores were collected by wet sieving as detailed by Gerdemann and Nicolson (1963). A quantity of 50 g of soil was suspended in 200 ml of luke-warm water. Heavier particles were allowed to settle for a few seconds and the suspension was decanted through a 710 μm sieve to remove the larger particles of organic matter. The residue was resuspended in more water and sieving was repeated. The

suspension that passed through this sieve was saved and stirred to resuspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid decanted through 250 μm sieve. The suspension that passed through this sieve was again collected and the sieving was repeated using 105 μm sieve and 45 μm sieve. The larger particles of organic matter were caught on the top sieves of higher pore size. The soil particles and spores collected in 105 μm and 45 μm sieves were taken in 100 ml conical flasks separately. The suspension in each flask was shaken thoroughly and allowed to settle for 30 seconds. The spores present in these suspensions were trapped on a nylon mesh, with 45 μm pore size placed on a marked petridish and the number of spores were counted by observing under a stereoscopic microscope.

3.1.4 Identification of spores

Spores of common species of VAM were identified using synoptic keys to the genera and species of Zygomycetous mycorrhizal fungi by Trappe (1982) and photographic slide collection illustrating features of the endogonaceae by Hall and Abbott (1981).

3.1.5 Isolation of VAM fungi

Spores of fungi were taken in water and they were picked up with a fine capillary pipette under a dissecting microscope using fine needles to separate organic matter. Thirty different spore types were isolated based on their morphology and they were surface sterilised using 2 per cent chloramine T and streptomycin sulphate (200 $\mu g\ ml^{-1}$) for 20 minutes and washed in several changes of sterile water. A single spore was placed in the neck region of a funnel assembly (Plate 1) filled with

sterilised sand and established seedlings of *Sorghum bicolor* (Nicolson, 1967). The seedlings maintained in the funnel for 4 weeks were transferred to mud pots containing sterilised 1:1 sand soil mix and maintained in a glass house. After two months a portion of the soil was removed and checked for purity. Pure isolates of selected VAM were multiplied under sterile condition. *P. phaseoloides* plants raised under similar conditions were also inoculated by VAM fungi for confirming their efficacy.

Soil sand mix (1:1 w/w) in mud pots (15 cm diameter) was steam sterilised for 2 h for two consecutive days and used for this purpose.

3.1.6 Establishment of stock plants (Hayman, 1982)

The root system of uniformly well infected sorghum seedlings together with the adhering soil were finely chopped and used as the starter inoculum. Sterile soil in pots were inoculated with 5-10 per cent of the starter inoculum as a layer of two inches below the soil level and surface sterilised seeds of *S. bicolor* were planted. The seedlings were periodically watered with sterile water for 90 days. Rock phosphate 0.75 g and urea 0.25 g bag^{-1} were applied after 25 days of establishment.

3.1.7 Establishment of P. phaseoloides

To break dormancy, seeds of *P. phaseoloides* were taken in a glass beaker and added concentrated sulphuric acid just sufficient to form a coat over the seeds and kept at constant stirring for 10 minutes. It was then washed thoroughly with tap water to remove acid.

MAP OF KERALA STATE

Figure 1. Sites of soil sample collection

Plate 1 **Funnel assembly for VAM isolation studies**

Bradyrhizobium sp. isolated from *P. phaseoloides* was grown in yeast extra mannitol broth (Annexure 1) for 3 days and mixed with the acid treated seeds c *P. phaseoloides*. Such treated seeds were pellatised with required quantity o calcium carbonate and planted in soil taken in polythene bags.

3.2 Selection of VAM

The relative efficiency of 11 different VAM fungi representing four genera on root colonisation, nodulation, nitrogenase activity, biochemical contents and growth of *P. phaseoloides* was studied under sterile conditions and the competitive ability of VAM fungi was confirmed under unsterile conditions too.

3.2.1 Sterile condition

The experiment was conducted in polythene bags of 15 cm diameter using steam sterilised sandy loam soil of pH 5.2 containing total nitrogen 0.26 per cent, available potassium 2.74 mg g^{-100}, available phosphorus 0.225 mg g^{-100} and organic carbon 1.7 per cent. Each poly bag contained 4 kg of soil. Eleven VAM fungi obtained from the earlier experiment were used. A quantity of 15 ml of inoculum containing about 300 spores and infected roots (85%) was uniformly distributed below 2 cm of top soil in polythene bags. The treatments included are

T_1 Inoculated with *Gigaspora calospora*

T_2 Inoculated with *Sclerocystis* sp.

T_3 Inoculated with *Glomus monosporum*

T_4 Inoculated with *G. boreale*

T_5 Inoculated with *G. macrocarpum*

T_6 Inoculated with *G. epigaeum*

T_7 Inoculated with *G. multicule*

T_8 Inoculated with *G. flavisporum*

T_9 Inoculated with *G. fasciculatum*

T_{10} Inoculated with *Acaulospora scrobiculata*

T_{11} Inoculated with *A. laevis*, and

T_{12} Uninoculated control.

Acid treated *P. phaseoloides* seeds inoculated with *Bradyrhizobium* sp. were sown in polythene bags. Five plants were maintained per bag. The treatments were replicated thrice in RBD design. The bags were irrigated with sterile water periodically to maintain field capacity and harvested 50 days after sowing.

3.2.2 Studies under unsterile condition

The experimental conditions were the same as that of sterile condition except sterilisation. The experiment was repeated with soil containing an indigenous population of 140 VAM spores in 50 ml. In this study tap water was used for watering the plants. The plants were harvested after 50 days of growth and observation were made for various parameters as given below.

3.2.2.1 Shoot length

The height of the main shoot was measured from the ground level to the tip of the terminal bud.

3.2.2.2 Root length

The length of the main root from collar to the tip was measured.

3.2.2.3 Dry weight

The root and shoot portions of the plants were separated. The root portion was washed gently to remove all the adhering soil particles in running tap water. Both the portions were gently pressed in folds of filter paper to remove excess moisture. The fresh weight was determined and the samples were wrapped in paper and kept in a hot air oven at 105°C for 24 h, removed, cooled in desiccator and reweighed.

3.2.2.4 Nodule number and dry weight

After 50 days of growth in the polythene bags the number of nodules and dry weight of nodules were determined. Plants were removed carefully from the bags with their root system and nodules intact. The nodules with roots were washed, separated and counted. Samples were dried in an oven at 105°C till constant dry weight was attained.

3.2.2.5 Per cent of mycorrhizal infection (Phillips and Hayman, 1970)

The roots were cut into 1 cm bits, washed gently in tap water without disturbing the external mycelium. The samples were heated to about 90°C for 1 h in 10 per cent potassium hydroxide solution in a waterbath. It was rinced four times in tap water and acidified by immersing for five minutes in 2 per cent hydrochloric acid. The acid was poured off and added 0.05 per cent cotton blue in lactophenol.

The roots were boiled in this stain for three minutes. The stain was poured off and added with lactophenol and kept over night to destain the host tissues and examined under a microscope for mycorrhizal infection.

Mycorrhizal colonisation was expressed using the following formula,

$$\text{Per cent colonisation} = \frac{\text{Number of root segments with VAM}}{\text{Total number of root segments examined}} \text{ X } 100$$

The root segment was considered mycorrhizal even if one of the three structures, i.e., hyphae, arbuscules or vesicles was present.

3.2.2.6 VAM spore count

The spores were collected by wet sieving and decanting method and spore counts were taken as described earlier.

3.2.2.7 Ethyl alcohol extraction of plant materials (Chandramohan *et al.*, 1967)

Leaves of the plants were collected, chopped and used for ethyl alcohol extraction after removing excess moisture by blotting them between folds of filter paper. Exactly 1 g of the chopped material was plunged into 20 ml of boiling 80 per cent ethyl alcohol, extracted for 5 minutes on a hot waterbath and cooled in running tap water. The material was homogenised by grinding in a porcelain mortar with pestle and squeezed through two layers of cheese cloth. The residue was reextracted with ethyl alcohol and the extracts pooled. The final volume was adjusted to 20 ml with 80 per cent ethyl alcohol. The residue after drying was used for the estimation of starch.

3.2.2.8 Quantitative estimation of total phenols

Total phenols were estimated by employing Folin-Ciocalteu reagent (Bray and Thorpe, 1954) (Annexure 2).

Folin-Ciocalteu reagent was diluted with equal quantity of water. One ml of this reagent was added to 1.0 ml of the alcohol extract in a 25 ml marked boiling tube followed by a 2 ml of 20 per cent sodium carbonate and the mixture was heated in a boiling waterbath for exactly one minute. The blue colour was diluted to 25 ml with glass distilled water. Reagent blank was maintained with 1.0 ml of distilled water instead of ethyl alcohol extract. The percentage of light transmittance was determined in a 'Spectronic-20' colorimeter at 725 nm. Total phenols were calculated from a standard curve plotted using catechol.

3.2.2.9 Quantitative estimation of ortho dihydroxy phenols

Ortho dihydroxy phenols were estimated by the method described by Johnson and Schaal (1952) employing Arnow's reagent. To 1 ml of the alcoholic extract in a 25 ml marked boiling tube, 1 ml of 0.5 N hydrochloric acid, 1 ml of Arnow's reagent prepared by dissolving 10 g of sodium nitrate and 10 g of sodium molybdate in 100 ml of glass distilled water and 2 ml of 1 N sodium hydroxide were added. The volume was raised to 25 ml with distilled water and the light pink colour was read in the 'Spectronic-20' colorimeter at 522 nm. Reagent blank contained 1 ml of distilled water in the place of ethyl alcohol extract. *Ortho* dihydroxy phenols were calculated from a standard curve prepared using catechol.

3.2.2.10 Determination of reducing sugars

Reducing sugars content in the alcohol extract was determined by the Nelson's (1944) method.

To 1 ml of alcohol extract in a 25 ml marked boiling tube, 1 ml of mixture of reagent 'A' and 'B' (Annexure 2) prepared by mixing 25 parts of reagent 'A' with 1 part of reagent 'B' was added. The mixture was heated for 20 minutes in a boiling waterbath, cooled in tap water and 1 ml of the Arsenomolybdate reagent was (Annexure 2) added. The solution was thoroughly mixed and diluted to 25 ml with glass distilled water. Reagent blank contained 1 ml of distilled water in the place of ethyl alcohol extract. The resulting blue colour was read in a 'Spectronic-20' colorimeter at 497 nm. Glucose was used as standard and the results are expressed as glucose equivalent.

3.2.2.11 Determination of non-reducing sugars

Non-reducing sugars present in the alcohol extract were first hydrolysed to reducing sugars (Inman, 1962) and then estimated. Exactly 1 ml of the alcohol extract was taken in a boiling tube and evaporated to dryness on a waterbath. One ml of glass distilled water and 1 ml of 1 N sulphuric acid were added to the residue. The mixture was hydrolysed by heating at 49°C for 30 minutes over a waterbath. The solution was neutralised with 1 N sodium hydroxide using methyl red indicator.

Total sugars content of the hydrolysed samples was estimated by the Nelson's method. Non-reducing sugars were calculated by substrating the reducing sugar value from that of total sugars and were expressed in glucose equivalents.

3.2.2.12 Determination of amino nitrogen

Amino nitrogen was determined by the ninhydrin method of Moore and Stein (1948).

To 1 ml of the alcohol extract in a boiling tube, 1 drop of methyl red indicator was added and the extract was neutralised with 0.1 N sodium hydroxide, if necessary. To this solution 1 ml of ninhydrin reagent was added, mixed thoroughly by shaking and aluminium caps were placed on the tubes. The mixture was heated for 20 minutes in a waterbath. The tubes were removed cooled in running tap water, 5.0 ml of diluent solution was added and the contents thoroughly mixed. The purple colour of the solution was read in a 'Spectronic-20' colorimeter at 475 nm. Blanks consisted of 1 ml of distilled water in the place of alcohol extract. Amino nitrogen was calculated from the standard graph prepared using glutamic acid.

3.2.2.13 Quantitative estimation of starch

Starch in the samples was estimated by the method of Sumner and Somers (1949).

Two hundred mg of finely powdered 80 per cent alcohol insoluble residue, dried in an oven at 60°C for two consecutive days, were placed in a glass stoppered 100 ml Erlenmeyer flask. Three ml of 6 N hydrochloric acid were added to the flask and steamed in an autoclave at 110°C for 1 h. The flasks were cooled and the solution was neutralised by using 1 N sodium hydroxide. The volume was raised to 25 ml with distilled water. An aliquot of 1 ml was withdrawn and glucose was estimated by Nelson's (1944) method. The amount of starch was determined by multiplying the amount of estimated glucose by the factor 0.9.

3.2.2.14 Extraction and estimation of chlorophyll (Arnon, 1949)

A quantity of 1 g fresh leaves were cut into small pieces and homogenised in a mortar with excess acetone, with a pestle. The supernatant was decanted and filtered on a Buchner funnel through Whatman No. 42 filter paper. Sufficient quantity of 80 per cent acetone was added and repeated the extraction. The filtrates were pooled and the volume was made to 100 ml in a volumetric flask. The absorbance of the extract at 645 and 663 nm was determined for determination of total chlorophyll.

The chlorophyll content was calculated on a fresh weight basis employing the following formula,

$$\text{Total chlorophyll (mg/g)} = \frac{20.2\ A_{645} + 8.02\ A_{663}}{a \times 1000 \times w} \times V$$

where,

a - length of path light in the cell

v - volume of the extract in ml, and

w - fresh weight of the sample in g

3.2.2.15 Determination of nitrogen

Total nitrogen in the sample was determined by microkjeldahl method (Jackson, 1962).

The samples were dried at 70°C for 48 h and powdered. Fifty mg of the powdered sample dried at 105°C for 6 h was transferred into a digestion flask and digested with 2 g of potassium sulphate, 40 mg of mercuric oxide and 2.4 ml of concentrated sulphuric acid. Gently heated the flask until frothing ceased and heating continued more strongly until the solution was cleared. After cooling 10 ml

of distilled water was added and warmed to dissolve the solute material. Blanks were prepared using reagents alone.

Estimation

The digested sample was transferred into the distillation flask. A quantity of 2 ml sodium hydroxide-sodium thiosulphate mixture prepared by dissolving 50 g of sodium hydroxide and 5 g of hydrated sodium thiosulphate in 100 ml of water was added and steam distilled. The liberated ammonia was collected into 5 ml of 4 per cent boric acid solution (in water), containing 2-3 drops of methyl red-bromocresol green indicator (prepared by mixing five parts of 0.2 per cent alcoholic bromocresol green solution with one part of 0.2 per cent alcoholic methyl red solution). The distillate was titrated against 0.02 N hydrochloric acid. The end point was chosen as the appearance of green colour. The blank digest was also run in the same way. Nitrogen in the sample was calculated by employing the factor, 1 ml of 1 N acid is equivalent to 14 mg of nitrogen.

3.2.2.16 Estimation of phosphorus (Jackson, 1962)

Phosphorus content in the samples was estimated using an auto analyser.

A quantity of 0.5 g of the sample previously dried at 105°C for 6 h was transferred into a silica dish and allowed to form ash in a muffle furnace at 500-550°C for half an hour. The dishes were allowed to cool and the ash was carefully moistened with distilled water. Adding 5 ml of 6 N hydrochloric acid, the content was digested for 1 h over a waterbath. After cooling, it was transferred to a 100 ml standard flask and made upto the mark. This solution was used for the determination of phosphorus.

The calibration curve for phosphorus was obtained using 10 and 20 ppm standards of $NaH_2PO_4.2H_2O$.

3.2.2.17 Estimation of potassium

Potassium was also estimated using auto analyser, using sample solution prepared in the same way for estimation of phosphorus. The quantity of K in the sample was calculated referring to a standard graph already prepared with potassium phosphate.

3.2.2.18 Photosynthetic activity

Carbon dioxide exchange rate was measured by a closed system of Infrared Gas Analyser (Portable Photosynthetic System, LI 6200 LICOR, Nebraska, USA). Fully expanded mature leaf was inserted to the chamber as identical to natural position and exposed to sunlight for measurement. All measurements were done between 08.30 and 09.30 A.M. in the second half of January. The ambient temperature was 27 $\pm$ 2°C. Relative humidity was approximately 60 per cent and light intensity was 1000 $\pm$ 200 μmol $m^{-2}s^{-1}$. After gas exchange measurements, leaves were removed from each plant and area was recorded by leaf area meter (LI 3000, LICOR).

3.2.2.19 Nitrogenase activity (Turner and Gibson, 1980)

P. phaseoloides plants grown in polythene bags were uprooted after 50 days of growth. The adhering soil particles were removed gently without damaging the root system and nodules. Roots were excised from the shoot. With intact nodules the roots were incubated in a glass container closed air tightly with a rubber stopper.

Ten per cent of the air in the container was removed with a gas tight syringe and an equal volume of acetylene was injected. The system was incubated at room temperature (28 $\pm$ 2°C) for 1 h. A quantity of 0.5 ml gas sample was withdrawn and injected into a Shimadzu 9A gas chromatograph fitted with a flame ionisation detector (FID) and stainless steel column of 80-100 mesh Porapak N (column temperature 75°C, oven temperature 100°C). Nitrogen was used as carrier gas. The Acetylene Reduction Activity (ARA) (ethylene production) was calculated by measuring ethylene peaks. Correction for endogenous ethylene production and admixture of any ethylene in the acetylene gas was suitably applied and the results are expressed as follows:

$$\mu \text{ moles } C_2H_4 \text{ produced plant}^{-1} \text{ h}^{-1} =$$

$$\frac{\text{Sample ethylene after 1 h}}{\text{Standard ethylene after 1 h}} \times \frac{\text{Acetylene at 0 time}}{\text{Acetylene after 1 h}} -$$

$$\frac{\text{Sample ethylene at 0 time}}{\text{Standard ethylene at 0 time}} \times \frac{\text{Gv(ml) - VCF X VPM}}{22.4\ (T_1\text{-}T_0)h}$$

where,

Gv - Gas volume of the container

VCF - Vacuum correction factor

VPM - Ethylene concentration (Standard sample = 105)

T_1-T_0 - Difference in sampling intervals

3.3 Response of *P. phaseoloides* to VAM Inoculation at Different Intervals

Response of *P. phaseoloides* plants to VAM inoculation at different intervals of growth was studied in unsterile soil. The plants were raised in polythene bags as mentioned earlier. A set of 20 bags was inoculated with *A. laevis*, another set of 20 bags was inoculated with *G. fasciculatum* and a third set of 20 bags was treated as uninoculated controls. Five plants were maintained in each bag. The plants in the bags were arranged in RBD and they were irrigated with tap water. Plants from 4 bags in each set were harvested at an interval of 10 days, i.e., 10, 20, 30, 40 and 50 days after sowing.

Observations were made on:

1. Root colonisation
2. Shoot weight
3. Root weight
4. Nodule number
5. Nodule weight
6. Nitrogenase activity
7. VAM spore count in soil
8. Total phenols
9. OD phenols
10. Reducing sugars
11. Non-reducing sugars
12. Starch
13. Amino nitrogen, and
14. N, P and K content in tissues.

3.4 Studies on *P. phaseoloides* Inoculated with *G. fasciculatum* and *A. laevis* at Different Levels of Rock Phosphate Application

Plants were raised in polythene bags containing unsterile soil as mentioned earlier. Five plants were maintained in each bag. Two isolates of VAM, i.e., *G. fasciculatum* and *A. laevis* were used in this study. Phosphate in the form of rock phosphate (RP) was applied at three different levels of recommended dose (150 kg ha^{-1}). The treatments imposed are as follows.

Treatments

T_1 No VAM inoculation and no RP application.

T_2 *G. fasciculatum* inoculation without RP application.

T_3 *A. laevis* inoculation without RP application.

T_4 No VAM inoculation at 50 per cent recommended level of RP application.

T_5 *G. fasciculatum* inoculation at 50 per cent recommended level of RP application.

T_6 *A. laevis* inoculation at 50 per cent recommended level of RP application.

T_7 No VAM inoculation at 100 per cent recommended level of RP application.

T_8 *G. fasciculatum* inoculation at 100 per cent recommended level of RP application.

T_9 *A. laevis* inoculation at 100 per cent recommended level of RP application.

Rock phosphate was added to the soil and thoroughly mixed at the time of sowing *P. phaseoloides*. There were three replications for each treatment and the bags were arranged in RBD. The plants were irrigated with tap water and sampled after 50 days.

Observations were recorded on:

1. Root colonisation
2. Shoot length
3. Shoot weight
4. Root length
5. Root weight
6. Nodule number
7. Nodule weight
8. Nitrogenase activity
9. NPK of shoot and root
10. VAM spore count in soil

3.5 Impact of *Azotobacter* sp., *Beijerinckia* sp. and *Bacillus circulans* on Root Colonisation by *G. fasciculatum* and Growth, Nutrient Content and Rhizosphere Microbial Population of *P. phaseoloides*

The VAM isolate, *G. fasciculatum* was used in this study. The bacteria used are the non-symbiotic nitrogen fixing bacteria, *Azotobacter* sp. and *Beijerinckia* sp. and the phosphate solubilising bacteria, *B. circulans*. *P. phaseoloides* plants were raised in unsterile soil in polythene bags and inoculated with VAM and other bacteria as per the treatments. Five plants per bag were maintained. The treatments imposed are:

Treatments

T_1 No mycorrhizae and no associative bacteria

T_2 *G. fasciculatum* alone

T_3 *Azotobacter* sp. alone

T_4 *Beijerinckia* sp. alone

T_5 *B. circulans* alone

T_6 *G. fasciculatum* and *Azotobacter* sp.

T_7 *G. fasciculatum* and *Beijerinckia* sp.

T_8 *G. fasciculatum* and *B. circulans*

Bacterial cultures in the form of broth were mixed with the soil as 10 ml kg^{-1} of soil. *Bradyrhizobium* sp. treated *P. phaseoloides* seeds were sown in soil containing 50 per cent of recommended level of rock phosphate. The plants were irrigated with tap water. After 50 days of growth the plants were sampled and the following observations were taken.

1. Root colonisation
2. Shoot length
3. Shoot weight
4. Root length
5. Root weight
6. Nodule number
7. Nodule weight
8. Nitrogenase activity
9. NPK of shoot and root
10. VAM spore count in soil
11. Microbial population

Enumeration of microbial population

The populations of total saprophytic bacteria, fungi, actinomycetes, *Azotobacter* sp., *Beijerinckia* sp. and phosphobacteria in rhizosphere soil samples of *P. phaseoloides* in the different treatments were estimated by serial dilution method using soil extract agar, Martin's rose bengal streptomycin agar, Kenknight's agar, Jensen's agar, Becking's agar and apatite agar respectively (Annexure 1).

3.6 Screening of Plants for Mass Multiplication of VAM Fungi

Spores of *G. fasciculatum* were isolated by wet sieving and decanting technique (Gerdemann and Nicolson, 1963) and brought to pot cultures through funnel technique using sorghum plants. Further multiplication of VAM was carried out using four host plants, *viz.*, *S. bicolor*, *Zea mays*, *P. phaseoloides* and *Pennisetum polystygon*. Vermiculite and soil mixture (1:1 ratio) were used as growing media. Five replications were maintained. Soil samples and roots of plants were collected after 40 days and spore count as well as percentage root infection were taken as outlined in 3.1.3 and 3.2.2.5.

Chapter 4

Experimental Results

4 EXPERIMENTAL RESULTS

4.1 Distribution and Isolation of VAM

4.1.1 Occurrence of VAM in rubber growing soils

The results of the survey of VAM population in soils at different rubber growing regions (Table 1) show that irrespective of soil pH, all soils contained VAM fungi. Maximum VAM spore population was recorded in Palakkad soil the pH of which was 5.8 and the minimum population in Kottayam soil having a pH of 4.5. Positive correlation was observed with respect to soil pH and VAM spore count (R = 0.61 at 5 per cent level).

Irrespective of soil pH, the per cent of different species of *Glomus* was more in all soils followed by *Acaulospora* spp. and *Sclerocystis* spp. Population of *Gigaspora* spp. was very low in all the soils. Among different soils examined, Palakkad soil contained more of *Glomus* spp. Compared to other soils *Acaulospora* spp. were more in Kottayam soil.

4.1.2 Selection of VAM fungi

The sorghum plants inoculated with spores of different VAM fungi established well and the growth was comparatively better than the uninoculated plants. Out of 30 plants receiving different species of VAM spores, 11 plants showed augmented growth characters. Upon examination of the roots for VAM

colonisation, all the 11 plants under treatments recorded above 25 per cent infection under aseptic condition. The roots of these plants started entering the nutrient reservoir flasks of funnel assembly after 30 days of establishment. When the 11 isolates of VAM fungi were inoculated on *P. phaseoloides*, same results were obtained. This indicated the efficacy of these 11 VAM fungi in improving the root infection as well as growth in funnel assembly.

4.1.3 Spore identification

Eleven isolates of VAM used in the present study were belonging to the following genera i.e., *Gigaspora*, *Sclerocystis*, *Acaulospora* and *Glomus*.

Description of spores

(i) ***Gigaspora calospora*** (Plate 2)

Spores are globose to subglobose measuring 200-500 μm at maturity with smooth surface having multiple wall layers with thick outer layer over two or more thin separable inner layers. Subtending bulbous hyphae are orange brown with a diameter of 30-50 μm.

(ii) ***Sclerocystis*** sp. (Plate 3)

Chlamydospores are formed in sporocarps with spores arranged in a single layer around a central plexus of glebal hyphae. Individual spores measuring 90-120 μm are light yellow in colour.

Table 1

Distribution of VAM fungi in different rubber growing soils

Location	Soil pH	Spore count ml^{-50}	Per cent distribution of VAM fungi				
			Glomus spp.	*Acaulospora* spp.	*Sclerocystis* spp.	*Gigaspora* spp.	Unidentified
Nagercoil	5.6	407	75	10	10	2	3
Thiruvananthapuram	5.5	374	72	13	8	1	6
Punalur	5.2	413	70	12	10	2	6
Kodumon	5.2	326	76	10	8	2	4
Chethackal	5.0	258	72	15	8	2	5
Kottayam	4.5	214	66	22	8	2	2
Mundakayam	4.8	369	79	12	7	1	1
Thodupuzha	5.0	344	68	20	8	2	2
Kalady	5.2	274	74	10	10	3	3
Pudukad	5.3	314	70	14	12	1	3
Palakkad	5.8	428	82	10	4	2	2
Pullengode	5.0	230	76	10	11	1	2
Kinalur	5.8	318	79	11	7	1	2
Kalpatta	5.5	330	70	15	10	2	3
Iritty	5.2	296	77	12	6	3	2
Mangalore	5.0	262	74	13	7	2	4

Plates 2-12

Spores of different species of VAM

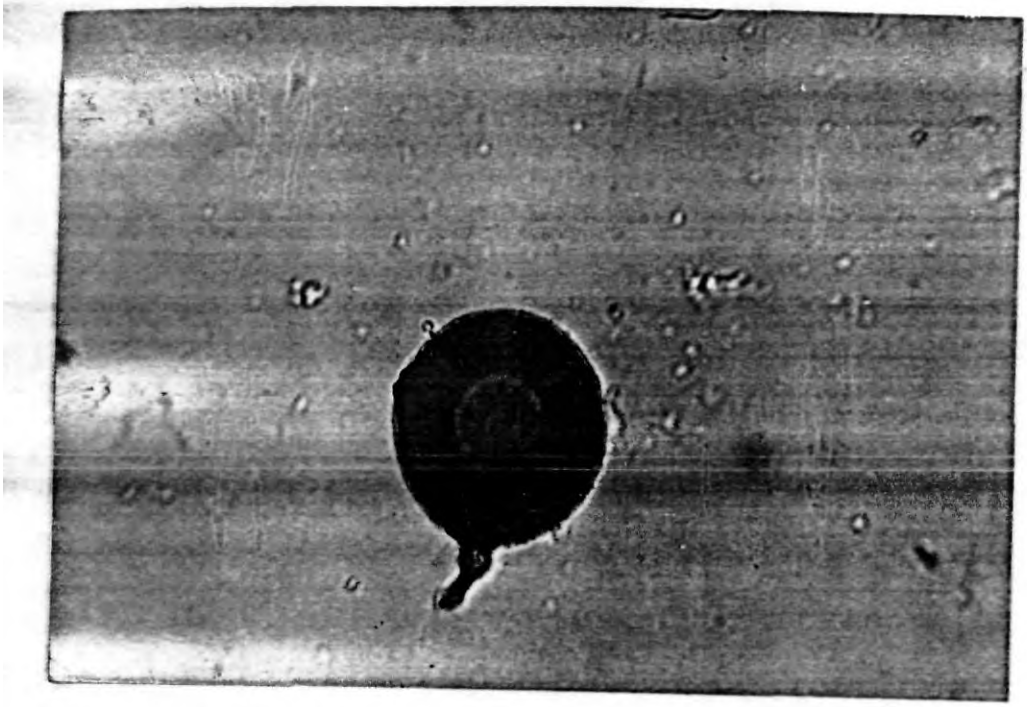

Plate 2 *Gi. calospora*

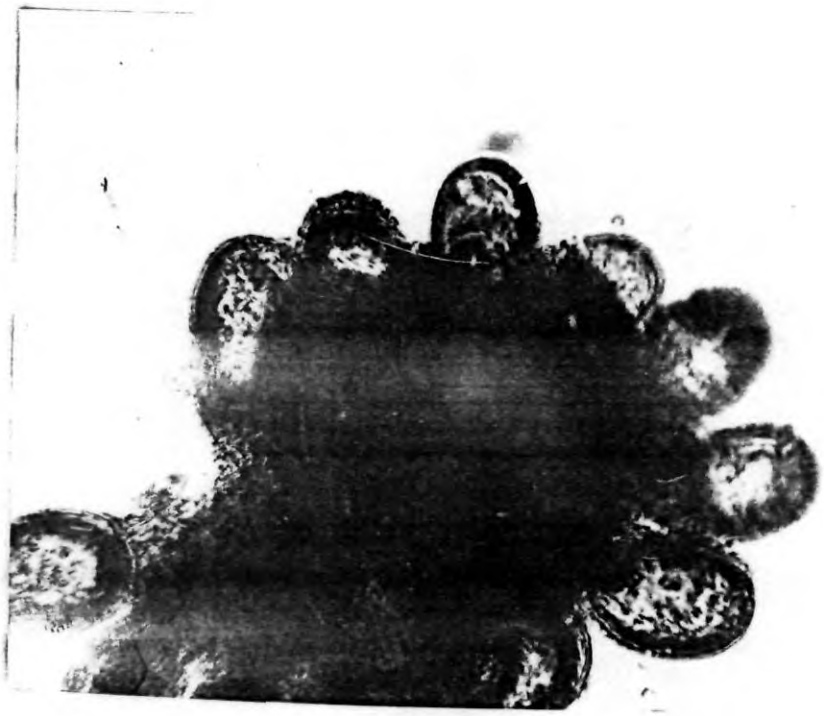

Plate 3 *Sclerocystis* sp.

(iii) ***Glomus monosporum*** (Plate 4)

Spores are globose or subglobose 125-300 μm. Spore surface is dull roughened with minute spines or fractures in hyaline matrix. Double wall layers with outer thinner than inner is present. Outer layer is hyaline and the inner layer is yellow to brown. Wall thickness is 9-12 μm. Subtending hyphae is hyaline.

(iv) ***Glomus boreale*** (Plate 5)

Spores are ellipsoid measuring 125-150 μm with single wall layer. Wall is orange brown in colour with a thickness of 5-8 μm. Single cylindric subtending hyphae is hyaline and spore separation is by plugging.

(v) ***Glomus macrocarpum*** (Plate 6)

Spores are subglobose to ovate measuring 100-200 μm with smooth surface and single wall layer having a thickness of 5-20 μm. Spores are yellowish brown. Subtending hyphae is single and cylindrical at the point of attachment. Wall colour is yellowish brown. Closure at spore wall is by wall thickening.

(vi) ***Glomus epigaeum*** (Plate 7)

Spores are subglobose or ovate, bright yellow measuring 95-140 μm, smooth with double wall layers, outer thinner than inner and the outer layer is hyaline. Inner wall is yellow to brown with a wall thickness of 9-12 μm.

The subtending hyphae is hyaline and cylindric. The pore at the point of attachment of subtending hyphae is occluded by a septum like plug.

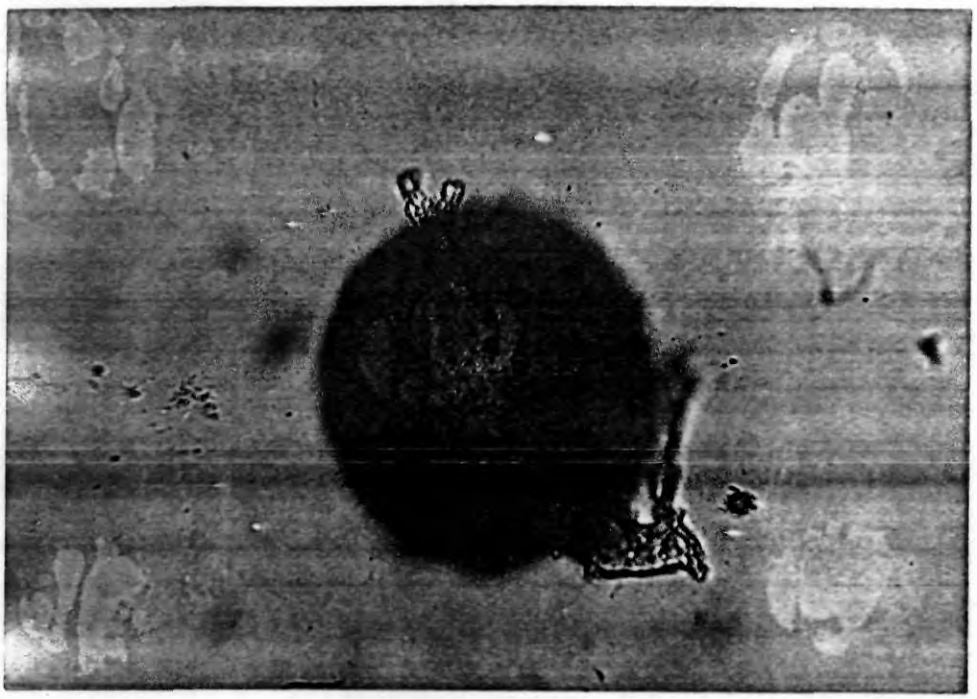

Plate 4 *G. monosporum*

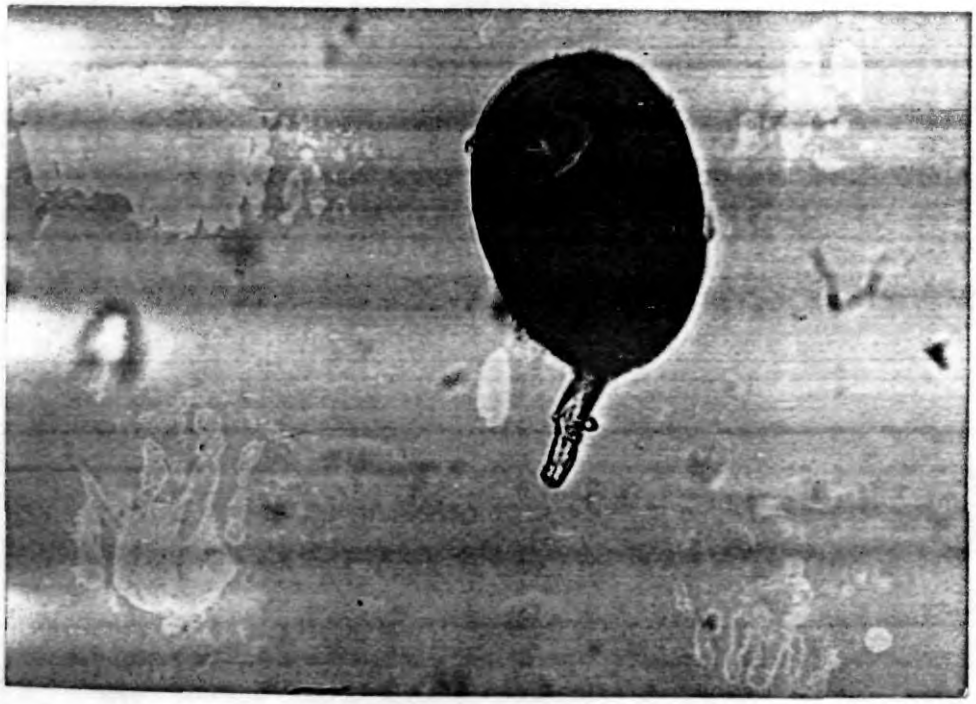

Plate 5 *G. boreale*

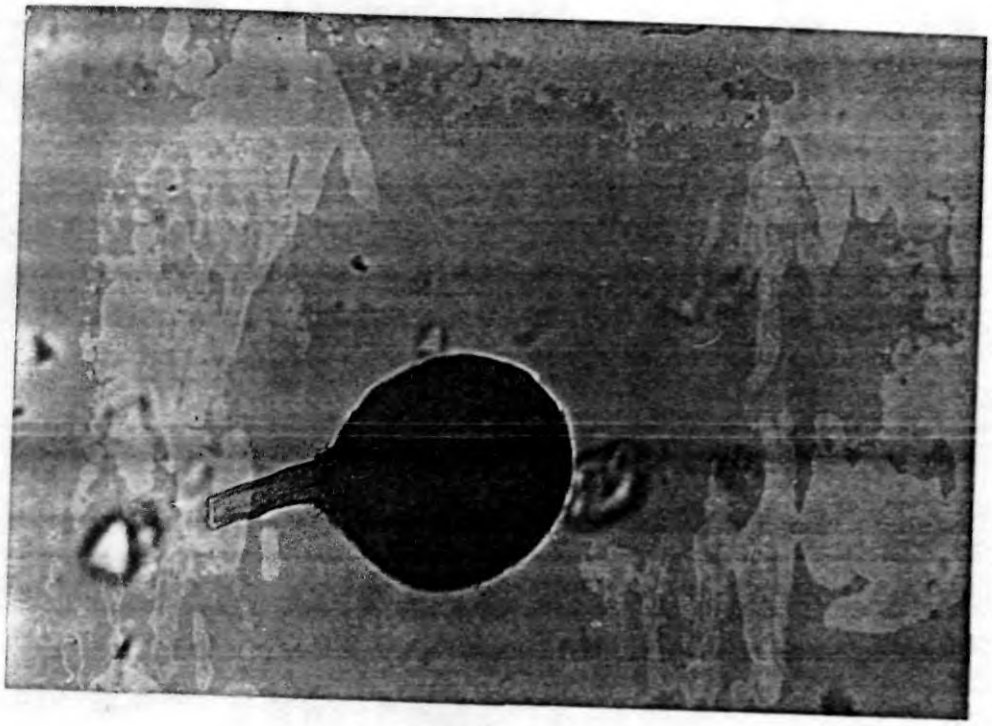

Plate 6 *G. macrocarpum*

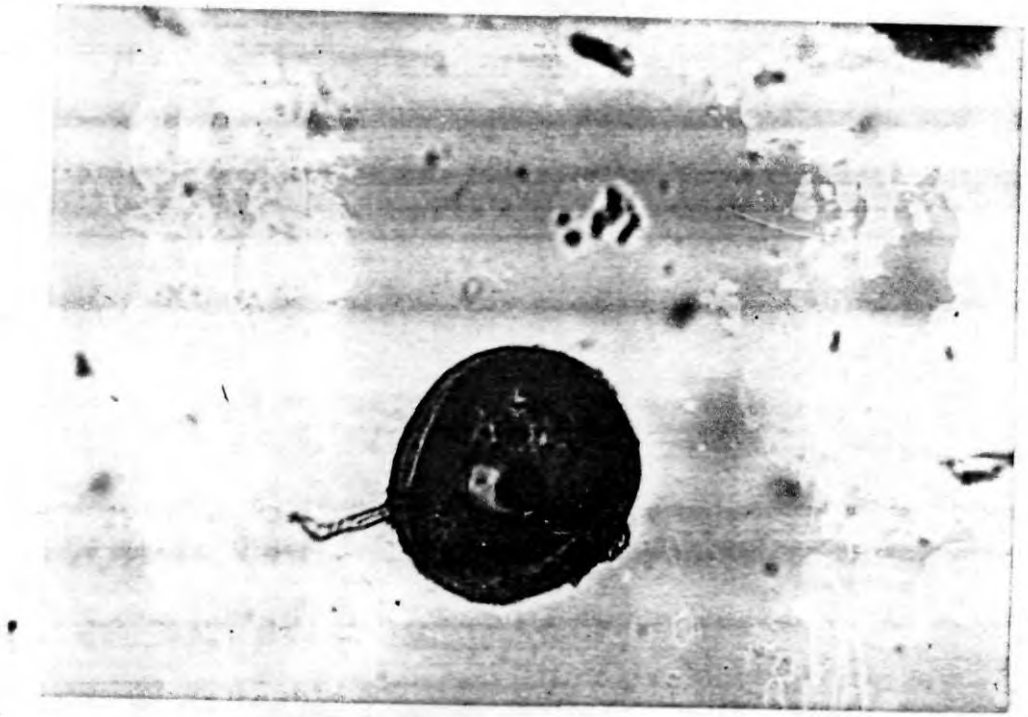

Plate 7 *G. epigaeum*

(vii) ***Glomus multicule*** (Plate 8)

Spores are globose to subglobose measuring 150-300 μm with single wall layer. Wall colour is yellowish brown with a thickness of 5-20 μm and 2-3 subtending hyphae, cylindrical at the point of attachment with a diameter of 13-20 μm. Hyphae is hyaline. The spore contents are separated from the attached hyphae by wall thickenings.

(viii) ***Glomus flavisporum*** (Plate 9)

Spores are ellipsoid with smooth surface, 150-225 μm, with single wall layer. Wall is yellow to brown, with a thickness of 9-20 μm. Single subtending hyphae is yellow to brown in colour and cylindrical towards the point of attachment. Hyphae is closed from the spore by spore wall.

(ix) ***Glomus fasciculatum*** (Plate 10)

Spores are globose mixed with subglobose and ellipsoid ones measuring 75-125 μm. They are smooth with single, yellowish brown wall layers. The wall thickness is 5-8 μm. The subtending hyphae is single, cylindric and yellow to brown in colour. The spore is separated by spore wall thickening. Sporocarp is formed of irregular aggregations of spores.

(x) ***Acaulospora scrobiculata*** (Plate 11)

Spores are globose, measuring 100-300 μm with pitted surface wall. They are having multiple wall layers with thick outer layer over one or more thinner layers. The wall thickness vary from 2-8 μm. Outer wall layer is yellow and the inner layers are hyaline.

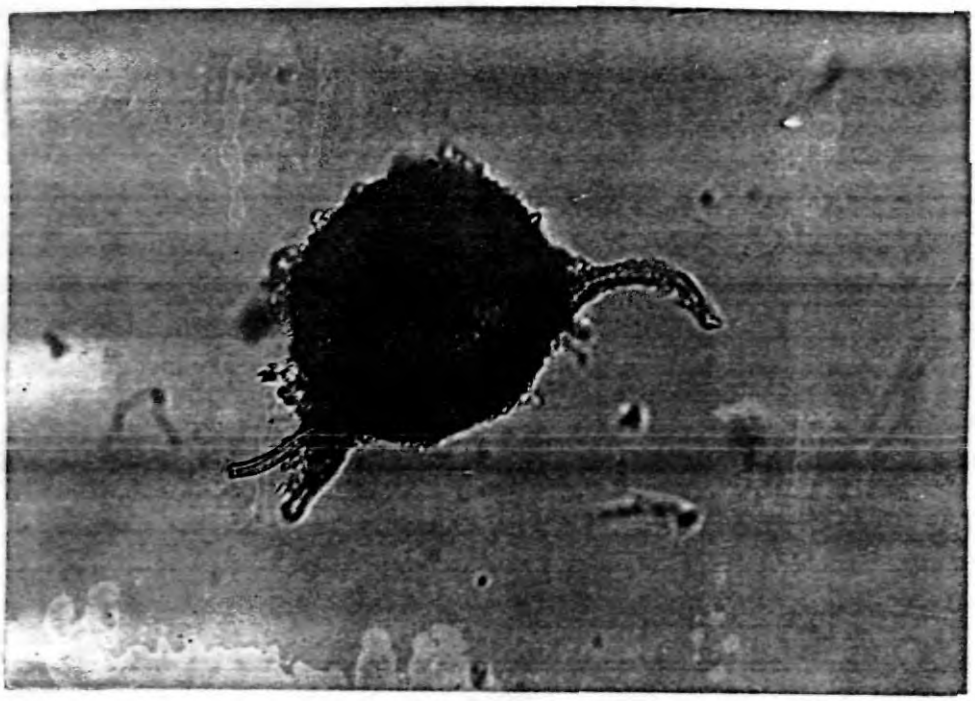

Plate 8 *G. multicule*

Plate 9 *G. flavisporum*

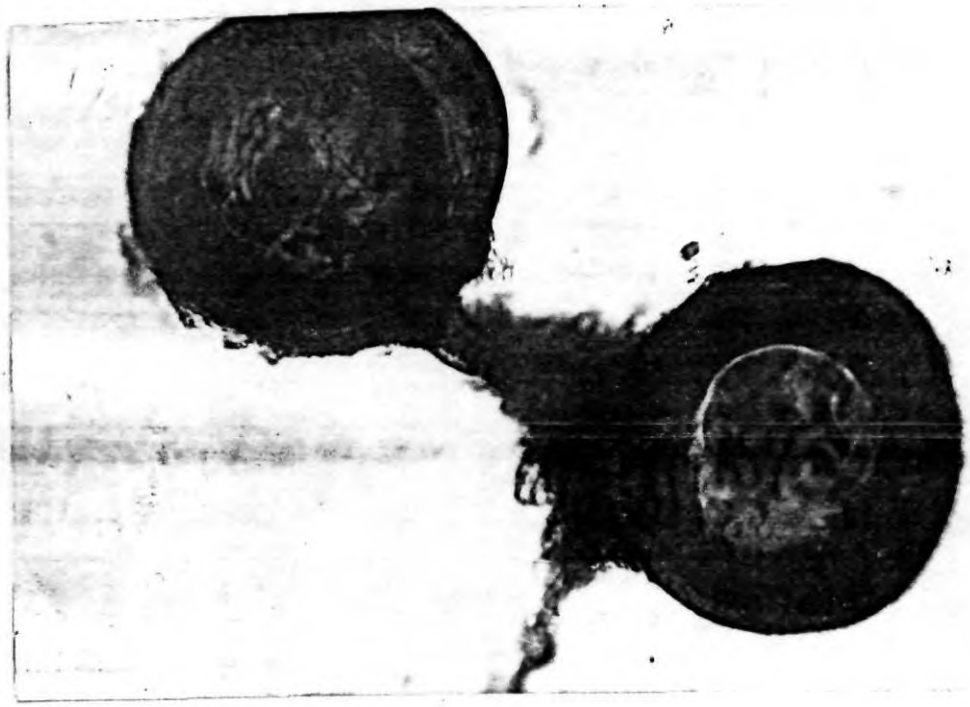

Plate 10 *G. fasciculatum*

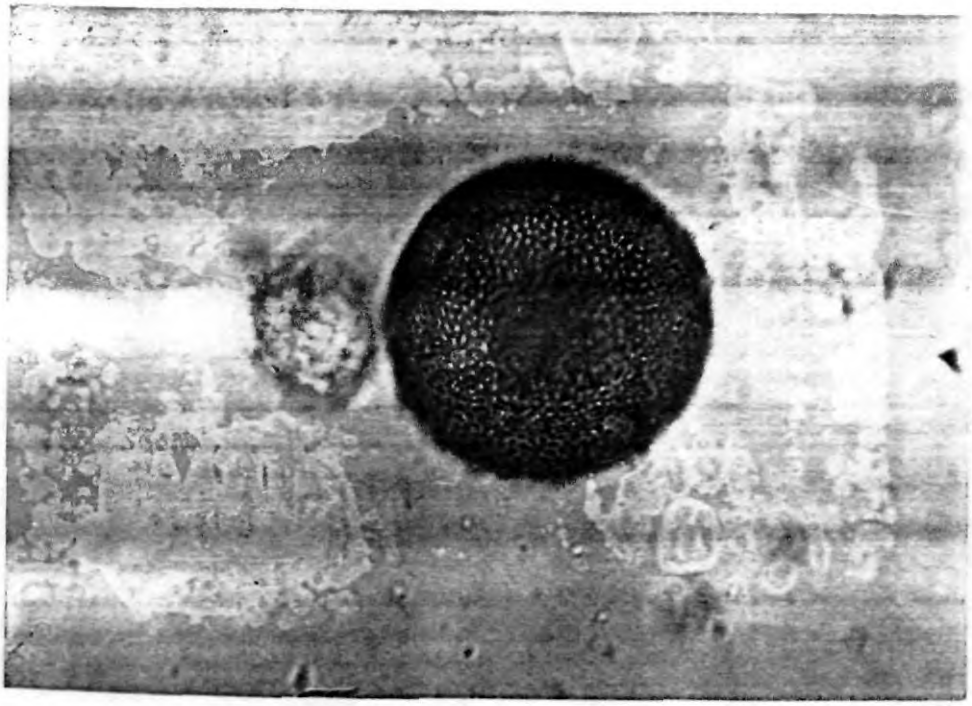

Plate 11 *A. scrobiculata*

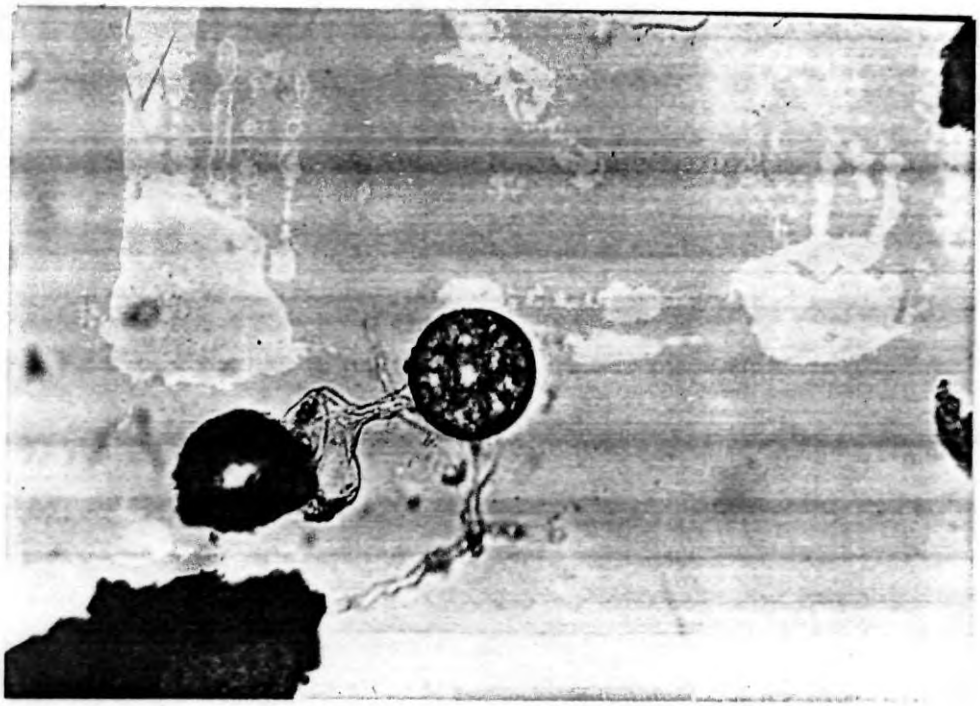

Plate 12 ***A. laevis***

(xi) Acaulospora laevis (Plate 12)

Single globose spore developed laterally on hyphae below epical swelling. Hyphal apex is inflated and with maturity of the spore the stalk cell collapses. Spores are with reticulate wall, mature ones measure 100-300 μm. Multiple wall layers are present, with outer thick layer over one or more thin layers. The wall colour is yellowish brown.

4.2 Effect of Inoculation of Different VAM Isolates on *P. phaseoloides*

4.2.1 Under sterile condition

4.2.1.1 Root colonisation and spore count in soil

All the isolates of VAM studied colonised the roots of *P. phaseoloides* seedlings and produced spores when inoculated under aseptic condition (Table 2). The extent of infection varied considerably among different isolates. The per cent of infection was maximum with *G. fasciculatum* followed by *A. laevis*. On the other hand, the spore count in soil was more in *A. laevis* treatment followed by *G. fasciculatum* treatment.

4.2.1.2 Shoot length and weight

All the isolates of VAM significantly augmented the growth of *P. phaseoloides*. The uninoculated control plants were stunted in growth with fewer leaves.

All the isolates of VAM enhanced the shoot growth as seen in Table 3. *G. fasciculatum* registered maximum length and weight of shoot followed by *A. laevis*. *Gi. calospora* and *Sclerocystis* sp. showed poor response on shoot length and shoot weight of *P. phaseoloides*, among different VAM inoculated plants. The uninoculated plants showed least shoot length and weight.

4.2.1.3 Root length and weight

Inoculation of spores of different VAM isolates significantly increased root length and weight of *P. phaseoloides* (Table 3). However *G. fasciculatum* and *A. laevis* recorded uniformly more effect on root length and weight than other isolates.

4.2.1.4 Nodulation and nitrogenase activity

Significant increase in both nodule number and weight was recorded upon inoculation with different isolates of VAM. *G. fasciculatum* and *A. laevis* were more effective in increasing the nodule number and weight (Table 3).

Inoculation of *P. phaseoloides* with different VAM species significantly augmented nitrogenase activity (Table 3). *G. fasciculatum* registered maximum nitrogenase activity followed by *A. laevis*. The control plant which did not receive VAM inoculum registered least nitrogenase activity.

Table 2

Root colonising ability of different VAM fungi in *P. phaseoloides* and spore population in soil under aseptic condition

Treatments	Root colonisation (per cent)	Spore count (ml^{-50})
Gi. calospora	26	72
Sclerocystis sp.	25	69
G. monosporum	35	79
G. boreale	28	74
G. macrocarpum	56	83
G. epigaeum	31	74
G. multicule	58	83
G. flavisporum	33	74
G. fasciculatum	73	91
A. scrobiculata	40	82
A. laevis	67	106
CD (P = 0.05)	5	7

Table 3

Effect of inoculation of different VAM fungi on *P. phaseoloides* under sterile condition

Treatments	Shoot length (cm)*	Shoot weight (g)**	Root length (cm)*	Root weight (g)**	Nodule number**	Nodule weight (g)**	Nitrogenase activity (n moles ethylene produced hr^{-1} $plant^{-1}$)
Gl. calospora	65	6.4	22	2.8	85	0.4	420
Sclerocystis sp.	64	6.4	20	2.8	82	0.4	418
G. monosporum	80	9.6	28	2.9	101	0.5	442
G. boreale	74	7.7	25	2.9	85	0.4	426
G. macrocarpum	97	13.2	28	3.4	121	0.7	461
G. epigaeum	78	9.0	26	3.0	94	0.6	436
G. multicule	109	13.5	34	4.0	128	0.9	476
G. flavisporum	81	8.1	26	3.0	92	0.5	436
G. fasciculatum	129	16.2	39	6.1	140	1.4	500
A. scrobiculata	82	10.6	28	3.2	108	0.7	455
A. laevis	119	14.8	37	5.5	139	1.2	488
Control	10	0.5	7	0.3	70	0.2	224
CD (P = 0.05)	8	1.2	8	0.6	9	0.2	10

* Mean of 5 plants
** Total of 5 plants

4.2.2 Under unsterile condition

4.2.2.1 Root colonisation and spore count in soil

Under unsterile condition, all the selected isolates of VAM significantly augmented root colonisation and it varied in different isolates (Table 4). *G. fasciculatum* recorded maximum per cent of root colonisation and this is on par with *A. laevis*.

Soil inoculated with *G. fasciculatum* and *A. laevis* showed maximum counts of VAM spore. Among other VAM fungi inoculated soil, there was not much variation in spore count.

4.2.2.2 Shoot length and weight

Inoculation with different VAM species increased the shoot length and weight of *P. phaseoloides* (Table 5 and Plates 13-23). Though all the isolates of VAM augmented the shoot length and weight, *G. fasciculatum* was significantly superior to other species followed by *A. laevis*.

4.2.2.3 Root length and weight

Different species of VAM fungi significantly increased both length and weight of *P. phaseoloides* roots (Table 5). However, there were considerable differences among different isolates. Inoculation with *G. fasciculatum* and *A. laevis* registered maximum length and weight of roots. The rate of increase due to VAM inoculation was more pronounced in root weight than root length.

Table 4

Effect of different VAM fungi on root colonisation in *P. phaseoloides* and VAM spore population in soil under unsterile condition

Treatments	Root colonisation (per cent)	Spore count (ml^{-50})
Gi. calospora	61	334
Sclerocystis sp.	56	324
G. monosporum	70	353
G. boreale	62	347
G. macrocarpum	72	361
G. epigaeum	68	352
G. multicule	70	366
G. flavisporum	64	349
G. fasciculatum	85	383
A. scrobiculata	71	283
A. laevis	80	378
Control	45	16
CD (P = 0.05)	5	9

Table 5

Effect of inoculation of different VAM fungi on *P. phaseoloides* under unsterile condition

Treatments	Shoot length (cm)*	Shoot weight (g)**	Root length (cm)*	Root weight (g)**	Nodule number**	Nodule weight (g)**	Nitrogenase activity (n moles ethylene produced hr^{-1} $plant^{-1}$)
Gi. calospora	87	10.2	28	5.7	117	0.68	533
Sclerocystis sp.	74	9.5	28	5.4	111	0.60	525
G. monosporum	92	12.7	31	6.1	125	0.83	564
G. boreale	86	10.2	29	5.8	118	0.71	543
G. macrocarpum	106	15.5	32	7.0	129	0.92	575
G. epigaeum	93	11.3	30	6.2	120	0.82	548
G. multicule	117	17.5	34	7.5	135	1.02	576
G. flavisporum	88	10.6	29	5.8	117	0.72	548
G. fasciculatum	137	24.5	43	9.7	160	1.43	611
A. scrobiculata	99	12.8	31	7.1	128	0.88	566
A. laevis	128	21.2	42	8.8	157	1.28	590
Control	31	6.6	24	2.8	89	0.20	486
CD (P = 0.05)	8	1.8	5	0.9	9	0.07	10

* Mean of 5 plants
** Total of 5 plants

Plate 13

1. ***Gi. calospora*** **treatment**

Plate 14

2. ***Sclerocystis*** **sp. treatment**

Plate 15

3. *G. monosporum* treatment

Plate 16

4. *G. boreale* treatment

Plate 17

5. *G. macrocarpum* treatment

Plate 18

6. *G. epigaeum* treatment

Plate 19

7. ***G. multicule*** **treatment**

Plate 20

8. ***G. flavisporum*** **treatment**

Plate 23 11 ***A. laevis*** **treatment**

4.2.2.4 Nodulation and nitrogenase activity

The effect of VAM inoculation on nodule number and weight is given in Table 5. VAM inoculation in general promoted the nodule formation and increased nodule weight. Among different isolates, *G. fasciculatum* and *A. laevis* were equally superior to other species of VAM fungi with respect to nodulation. However, plants inoculated with *G. fasciculatum* were significantly superior in increasing the weight of nodules in *P. phaseoloides* followed by *A. laevis*. *Sclerocystis* sp. was found to be the poor promoter of nodule weight.

Nitrogenase activity of root nodules also increased upon inoculation with different species of VAM (Table 5). But *G. fasciculatum* showed more nitrogenase activity followed by *A. laevis*.

4.2.2.5 Nutrient content in plants

The results of the analysis of both shoot and root portions of *P. phaseoloides* inoculated with different isolates of VAM fungi for N, P and K are given in Table 6. VAM inoculation in general, registered a higher level of nutrients than the uninoculated plants. Among the 11 species of VAM, *G. fasciculatum* showed maximum level of N, P and K in both shoot and root tissues followed by *A. laevis*. There was not appreciable differences among plants inoculated with other species of VAM in respect of nutrient content.

Table 6

Effect of inoculation of different VAM fungi on nutrient content in *P. phaseoloides* under unsterile condition (mg plant^{-5})

Treatments	Shoot			Root		
	N	P	K	N	P	K
Gi. calospora	336	6.21	232	64	2.13	61
Sclerocystis sp.	333	6.19	211	61	2.09	55
G. monosporum	345	7.23	241	65	2.53	65
G. boreale	335	6.61	243	65	2.29	63
G. macrocarpum	347	8.14	261	72	2.74	69
G. epigaeum	345	7.04	251	68	2.36	61
G. multicule	353	8.72	264	76	3.01	73
G. flavisporum	345	6.61	245	66	2.26	65
G. fasciculatum	396	11.43	313	108	4.72	107
A. scrobiculata	347	8.08	247	71	2.71	68
A. laevis	374	9.75	284	93	4.07	91
Control	172	2.98	106	29	1.06	42
CD (P = 0.05)	12	0.90	18	8.0	0.47	10.0

Table 7

Effect of inoculation of different VAM fungi on certain biochemical constituents of *P. phaseoloides* under unsterile condition (mg g^{-100})

Treatments	Total phenols	OD phenol	Amino nitrogen	Reducing sugars	Non reducing sugars	Star
Gi. calospora	369	285	313	489	665	112
Sclerocystis sp.	366	279	315	453	654	115
G. monosporum	393	300	360	527	690	116
G. boreale	372	293	326	493	660	118
G. macrocarpum	463	318	366	532	703	130
G. epigaeum	379	297	353	505	685	120
G. multicule	488	323	406	563	704	136
G. flavisporum	373	299	363	514	683	118
G. fasciculatum	574	417	474	631	767	156
A. scrobiculata	404	316	363	532	694	128
A. laevis	532	383	455	595	736	142
Control	297	196	268	470	583	104
CD (P = 0.05)	19	32	18	17	30	11

Table 8

Effect of inoculation of different VAM fungi on total chlorophyll content and photosynthetic activity of *P. phaseoloides*

Treatments	Total chlorophyll (mg g^{-1} tissue on fresh weight basis)	Photosynthetic rate (rate of CO_2 assimilated μ mol/m^2/sec^{-1})
Gi. calospora	1.125	9.82
Sclerocystis sp.	1.136	10.00
G. monosporum	1.186	10.24
G. boreale	1.209	10,32
G. macrocarpum	1.312	11.77
G. epigaeum	1.256	10.84
G. multicule	1.396	14.69
G. flavisporum	1.238	11.58
G. fasciculatum	1.652	16.81
A. scrobiculata	1.286	11.08
A. laevis	1.489	15.24
Control	1.105	5.67
CD (P = 0.05)	0.082	1.52

4.2.2.6 Biochemical constituents

The results of the analysis of biochemical constituents are given in Table 7. VAM inoculation, in general, recorded a higher value of the biochemical constituents than the uninoculated control. Inoculation with *G. fasciculatum* however, recorded significantly higher levels of phenols, amino nitrogen, sugars and starch, and this is followed by *A. laevis* treatment.

4.2.2.7 Chlorophyll content and photosynthetic activity

Under unsterile condition, VAM inoculation in general, significantly increased chlorophyll content of plants. However, there was wide variation in chlorophyll of plants inoculated with different species of VAM fungi and the maximum was observed with *G. fasciculatum* inoculated plants followed by *A. laevis*. Corresponding to the enhanced chlorophyll content, there was an increase in photosynthetic activity upon VAM inoculation (Table 8). The photosynthetic activity is positively correlated with chlorophyll content (R = 0.92 at 1 per cent level). The photosynthetic activity ranged from 5.67-16.81 μ mol $CO_2/m^2/sec$. Photosynthetic rate was the highest in *P. phaseoloides* plants inoculated with *G. fasciculatum* followed by *A. laevis*.

4.3 Effect of Inoculation of *G. fasciculatum* and *A. laevis* on Root Colonisation, Growth and Some Biochemical Constituents of *P. phaseoloides* at Different Intervals after Planting

4.3.1 Root colonisation

Root colonisation by VAM fungi was observed on 10th day of planting which increased progressively up to 50th day in all the three treatments. The rate of increase in root colonisation in *G. fasciculatum* and *A. laevis* inoculated plants was

more upto 40th day (Fig. 2). Root colonisation was more in *G. fasciculatum* inoculated plants till the end of the experiment. However, in control plants the ascending trend was noticed upto 50th day.

4.3.2 Spore count in soil

VAM spore count in control and inoculated soil gradually increased from 10th to 30th day and thereafter the increase in spore count was more pronounced. The spore count was more in soil inoculated with *G. fasciculatum* followed by *A. laevis* and uninoculated control plants throughout the experiment (Fig. 3).

4.3.3 Shoot weight

The inoculation of *G. fasciculatum* and *A. laevis* did not cause much variation in shoot weight upto 20th day when compared to control. But on 30th day, these treated plants uniformly recorded more of shoot weight (Fig. 4). From 30th day, *G. fasciculatum* inoculated plants registered more shoot weight than *A. laevis* treated plants.

4.3.4 Root weight

Root weight of all the plants increased upto 50th day irrespective of VAM inoculation. VAM inoculation did not show much difference among the three treatments (Fig. 5) in root weight on 10th day. But from 20th day, both *G. fasciculatum* and *A. laevis* treated plants uniformly registered more root weight till the end of the experiment.

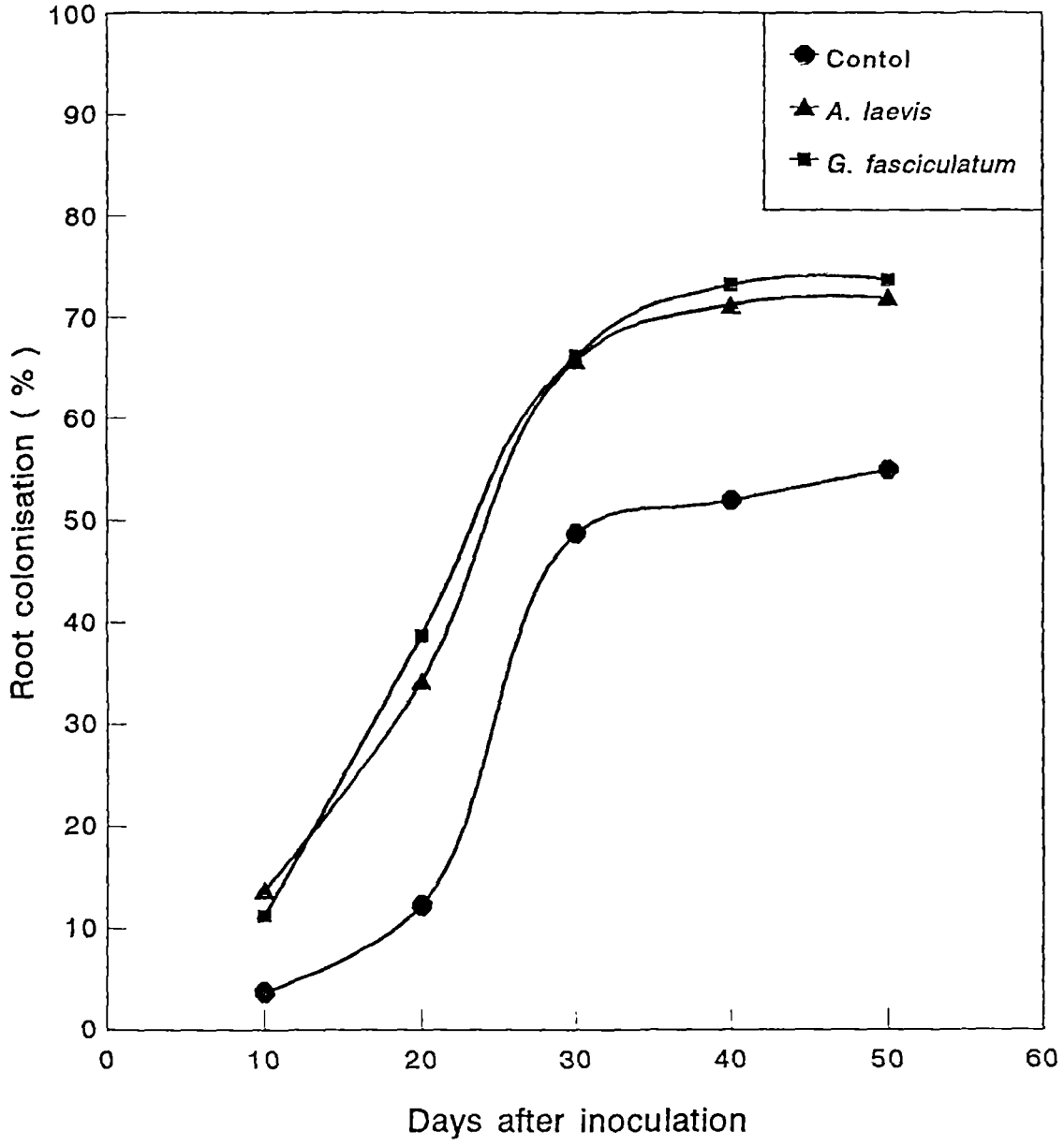

Figure 2. Root colonisation by VAM fungi in *P. phaseoloides*

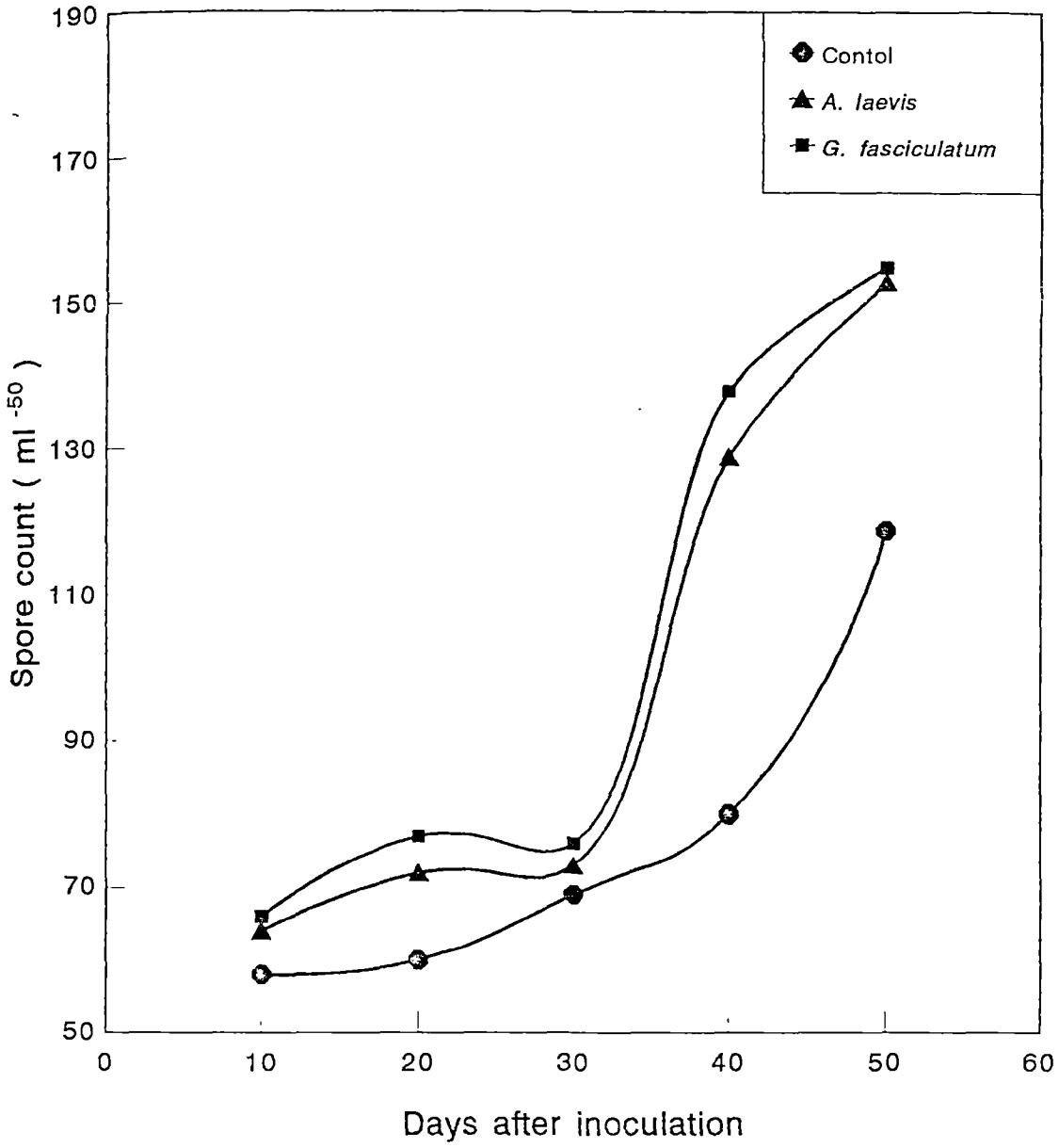

Figure 3. Spore count in soil inoculated wih VAM fungi

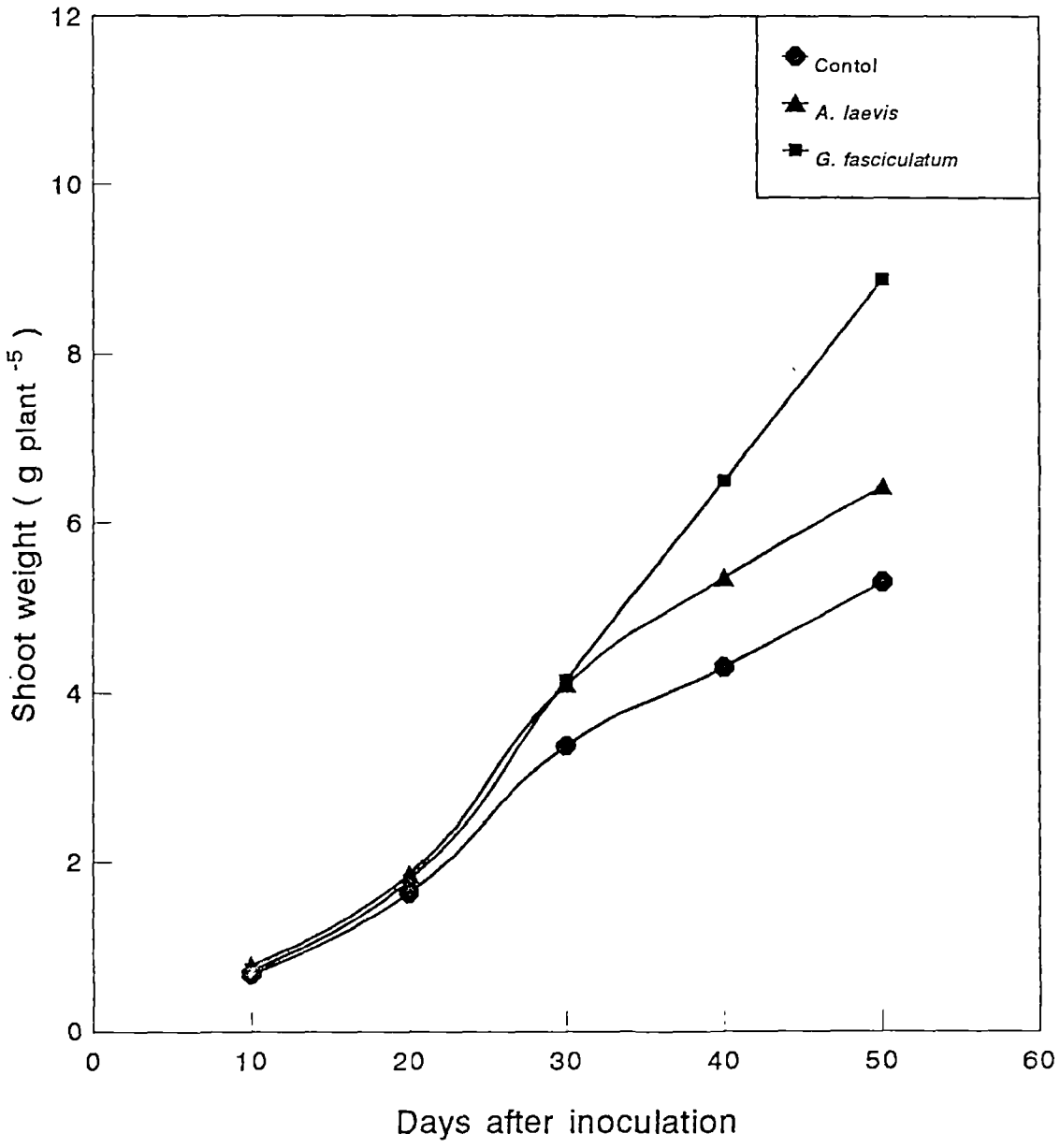

Figure 4. Shoot weight of *P. phaseoloides* inoculated with VAM fungi

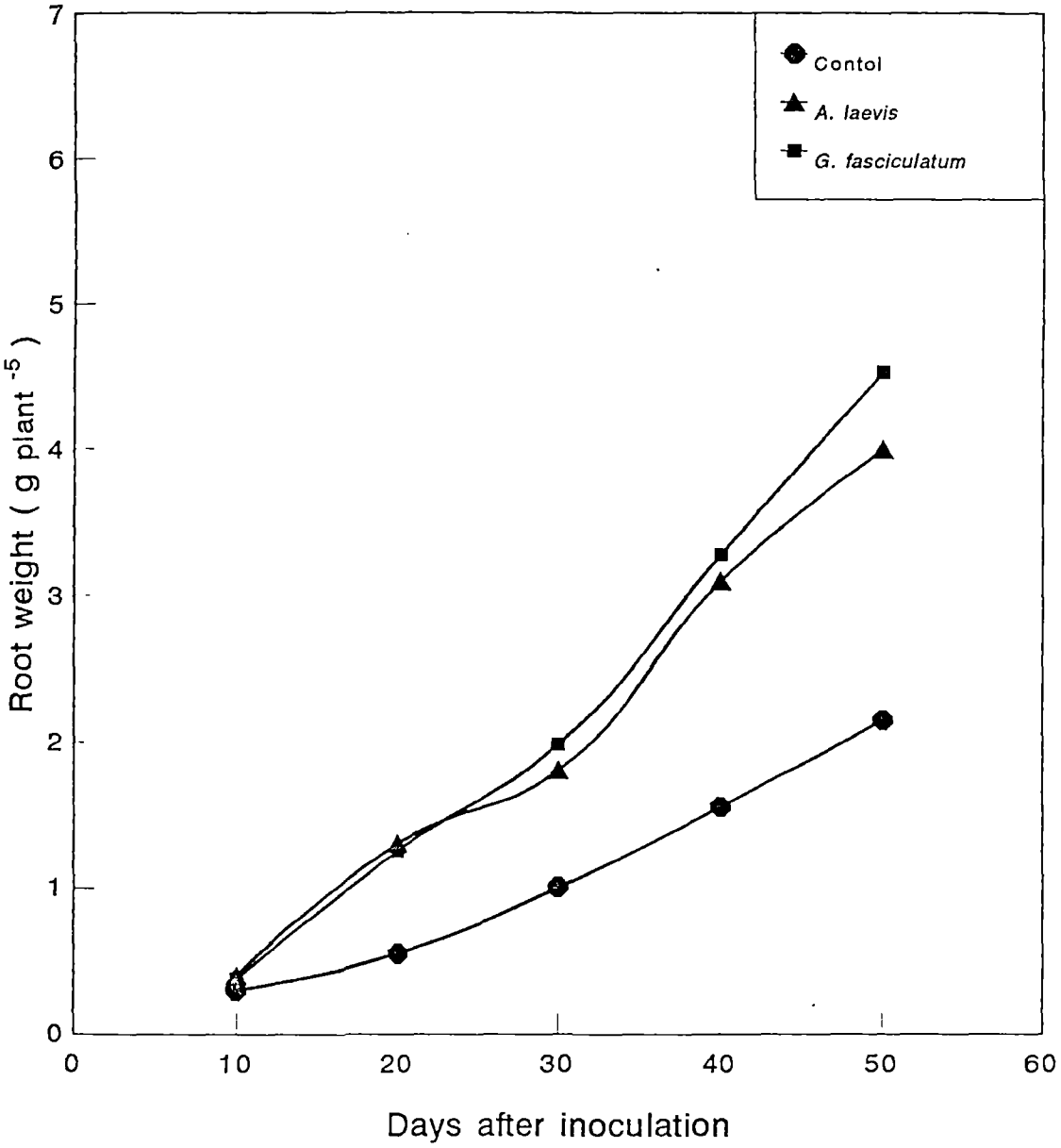

Figure 5. Root weight of *P. phaseoloides* inoculated with VAM fungi

4.3.5 Nodule number and weight

Root nodulation appeared after 20 days of plant growth and the number and weight of nodules increased with increase in age upto 50th day (Figs. 6 & 7). From 20th to 50th day, the nodule number and nodule weight of plants inoculated with *G. fasciculatum* and *A. laevis* were uniformly more than the control.

4.3.6 Nitrogenase activity

Both *A. laevis* and *G. fasciculatum* considerably augmented nitrogenase activity of *P. phaseoloides* from 20th day and the same trend was maintained upto the end of the experiment (Fig. 8). *G. fasciculatum* inoculated plants recorded more nitrogenase activity than plants inoculated with *A. laevis*. The enhanced nitrogenase activity is directly proportional to nodule number and weight.

4.3.7 Biochemical changes

VAM inoculation augmented both reducing and non reducing sugars (Figs. 9 & 10). *P. phaseoloides* irrespective of VAM inoculation contained more of non reducing sugars than reducing sugars. Irrespective of treatments increased reducing and non-reducing sugars were recorded from 30th day till the end of the experiment. Among the two VAM fungi treatments *G. fasciculatum* treated plants registered higher levels of these sugars.

Starch content of VAM inoculated plants was higher than the uninoculated plants (Fig. 11) and its level decreased on 20th day and thereafter increased. *G. fasciculatum* inoculated plants contained comparatively more starch than *A. laevis* inoculated plants.

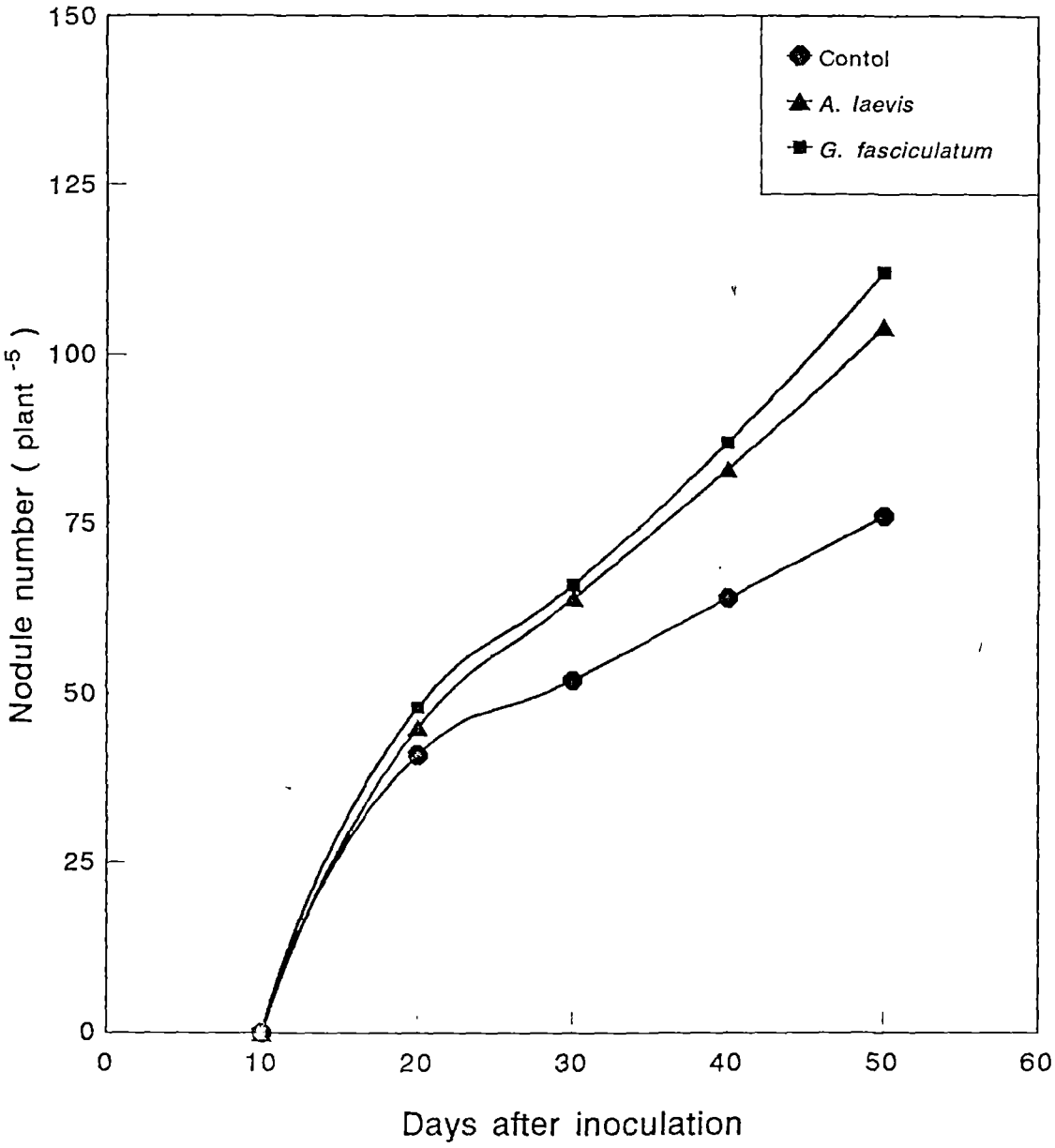

Figure 6. Nodulation in *P. phaseoloides* inoculated with VAM fungi

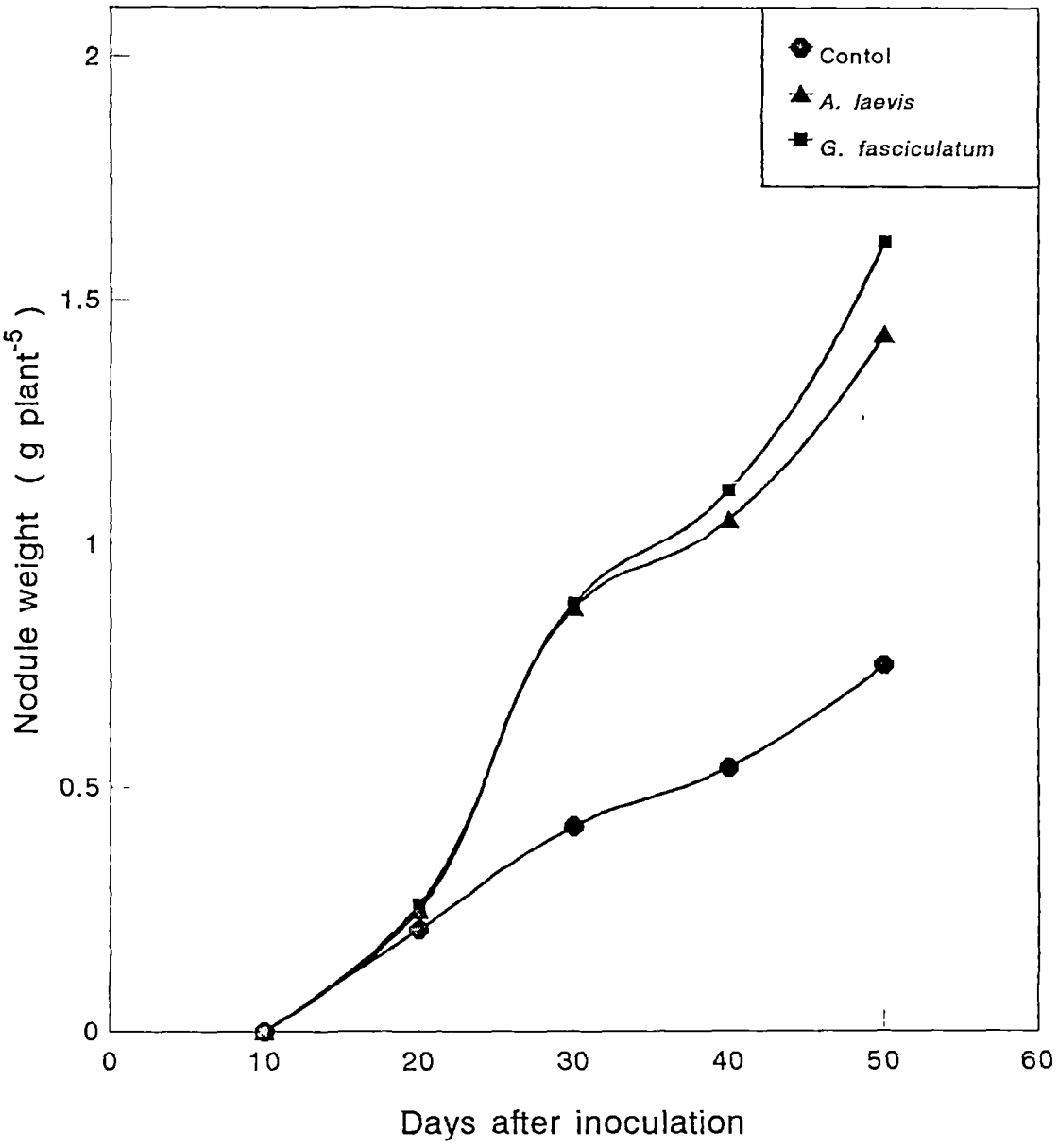

Figure 7. Nodule weight of *P. phaseoloides* inoculated with VAM fungi

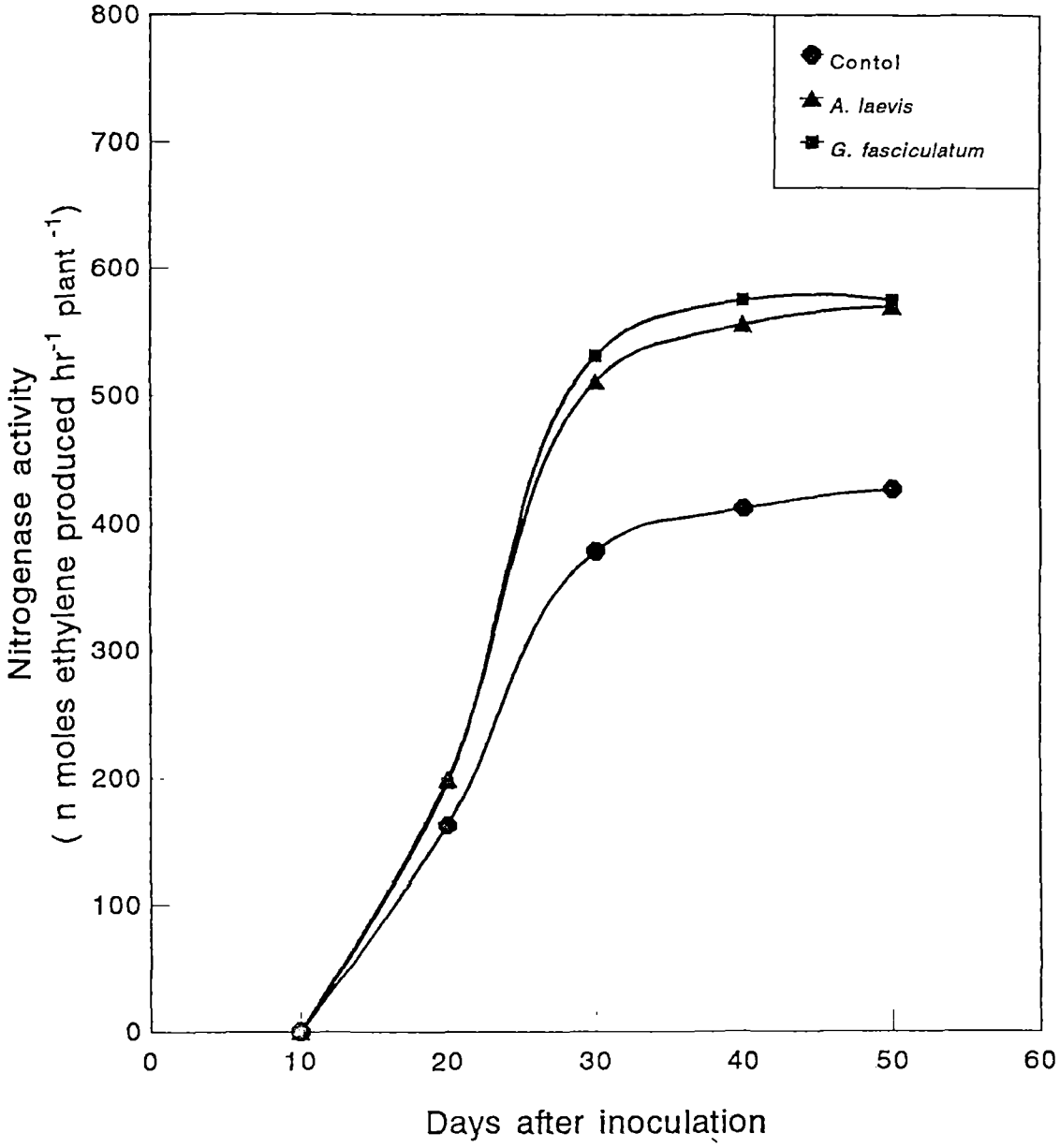

Figure 8. Nitrogenase activity of *P. phaseoloides* inoculated with VAM fungi

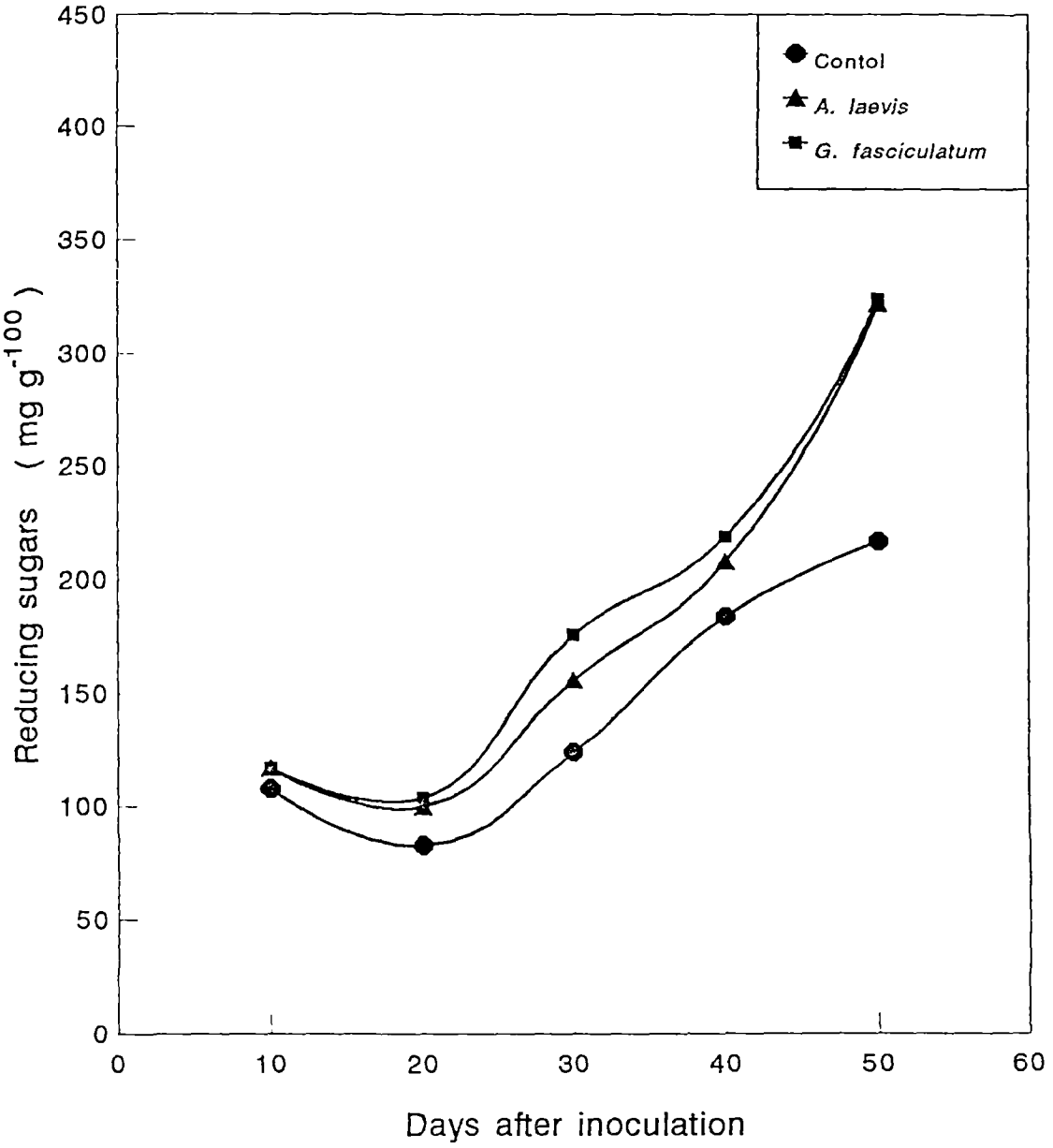

Figure 9. Reducing sugars content of *P. phaseoloides* inoculated with VAM fungi

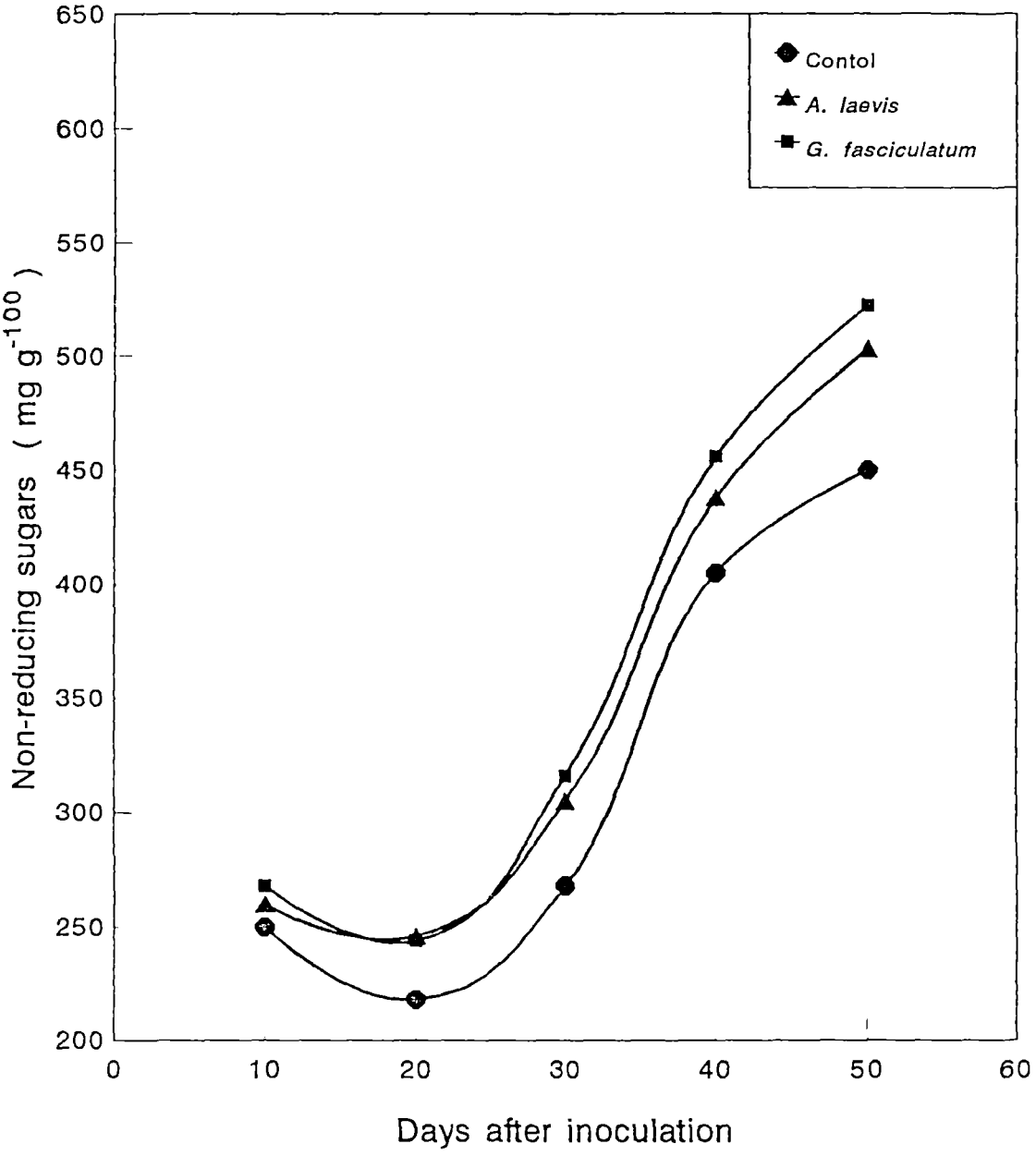

Figure 10. Non-reducing sugars content of *P. phaseoloides* inoculated with VAM fungi

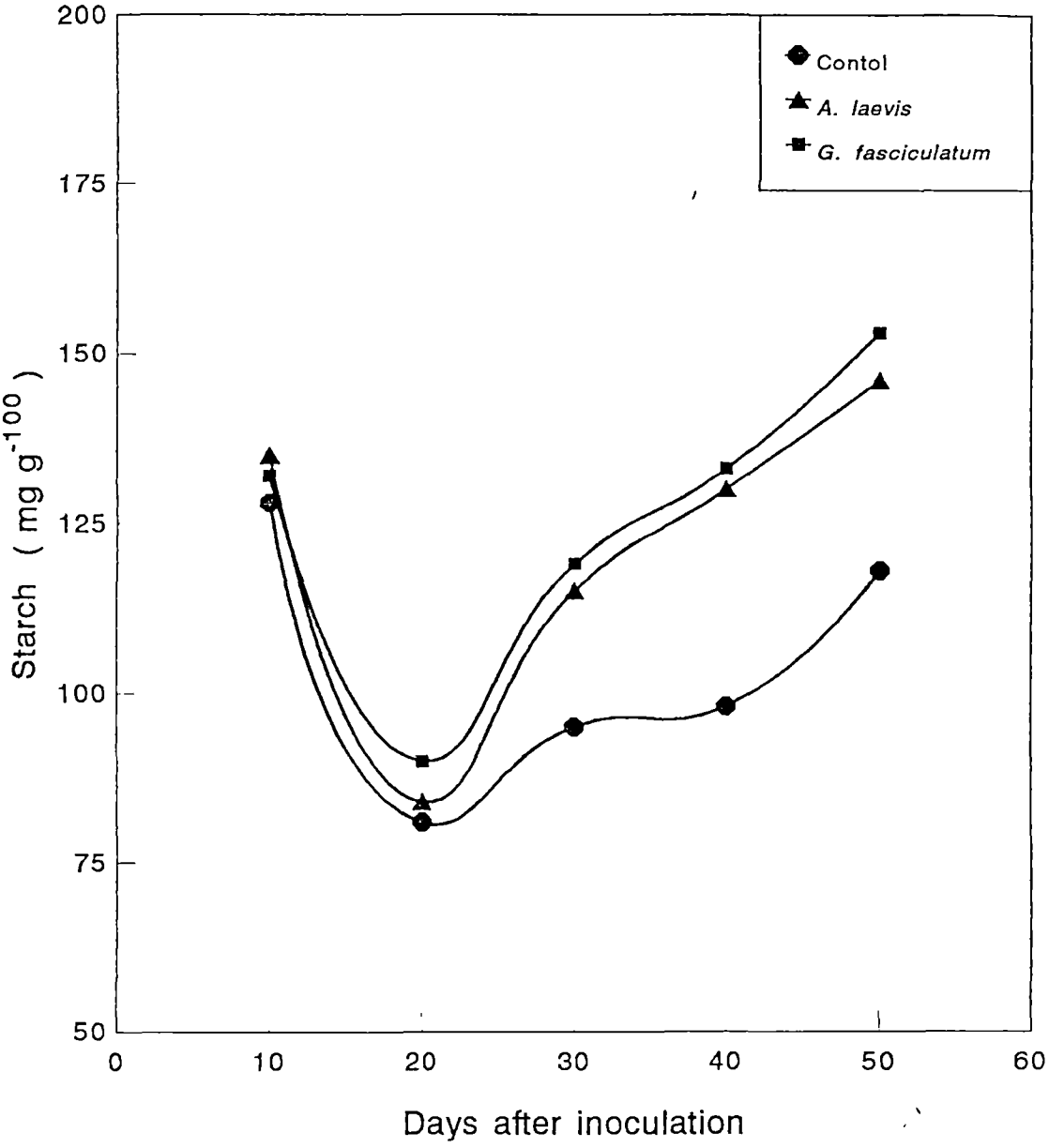

Figure 11. Starch content of *P. phaseoloides* inoculated with VAM fungi

Amino nitrogen content of both VAM inoculated and control plants tended to decrease significantly from 10th day to 20th day (Fig. 12). On an average VAM inoculation augmented amino nitrogen content in *P. phaseoloides*. But among the two VAM isolates, *G. fasciculatum* led to increased level of amino nitrogen in plants from 20th to 50th day.

VAM inoculated plants recorded higher level of both *ortho* dihydroxy and total phenols. However, *G. fasciculatum* inoculated plants contained more of *ortho* dihydroxy and total phenols than plants inoculated with *A. laevis* (Figs. 13 & 14). In general levels of phenols increased with the age of the plant.

4.3.8 Nutrient content in plants

All the three major plant nutrients, N, P and K increased with the age of the plant (Figs. 15-17). When compared to control VAM inoculated plants registered higher levels of N, P and K. The values of N, P and K increased marginally upto 30th day and thereafter there was an increase at a logarithmic rate in VAM inoculated plants. *G. fasciculatum* inoculated *P. phaseoloides* contained more of N, P and K from 30th to 50th day of inoculation.

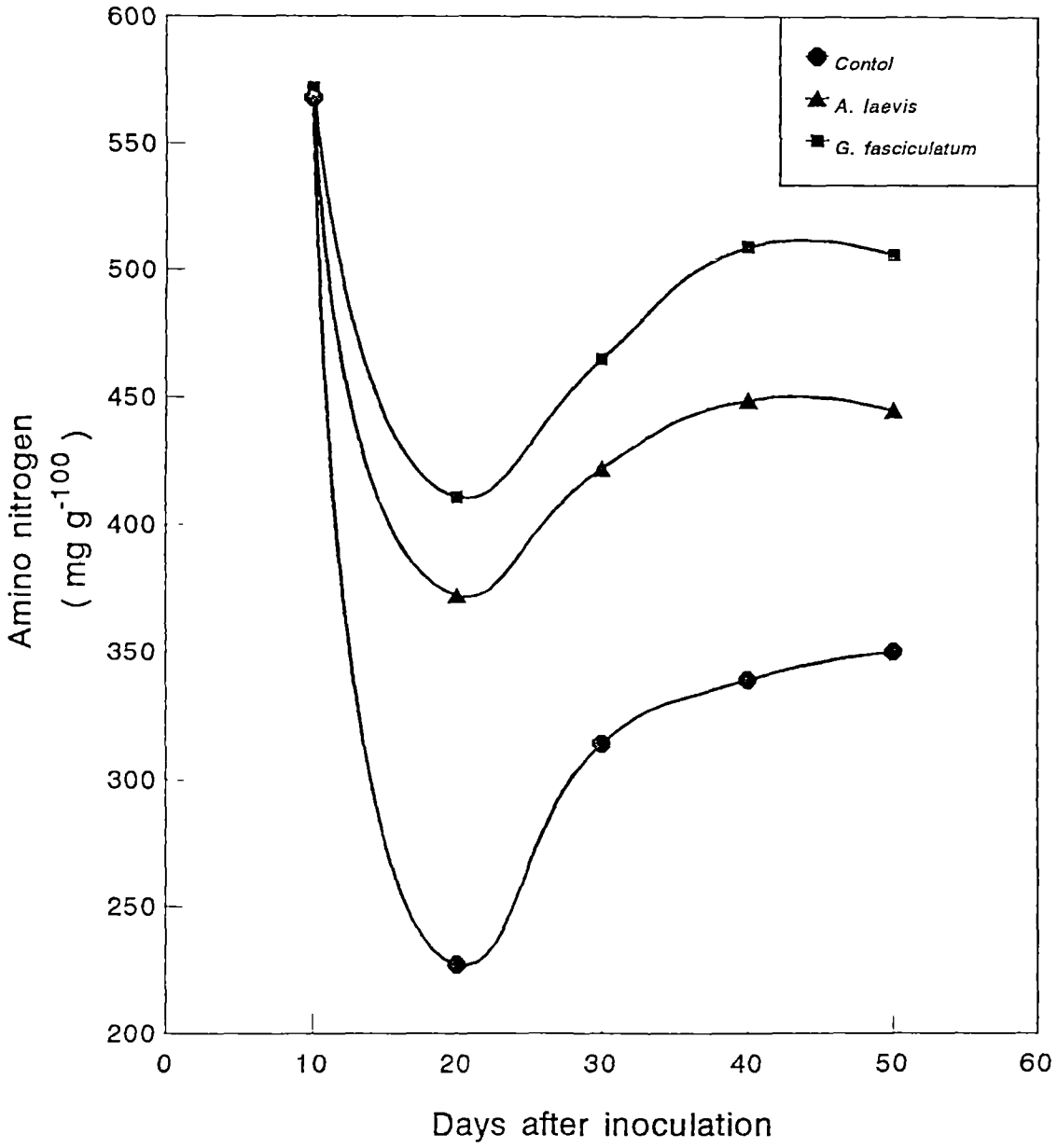

Figure 12. Amino nitrogen content of *P. phaseoloides* inoculated with VAM fungi

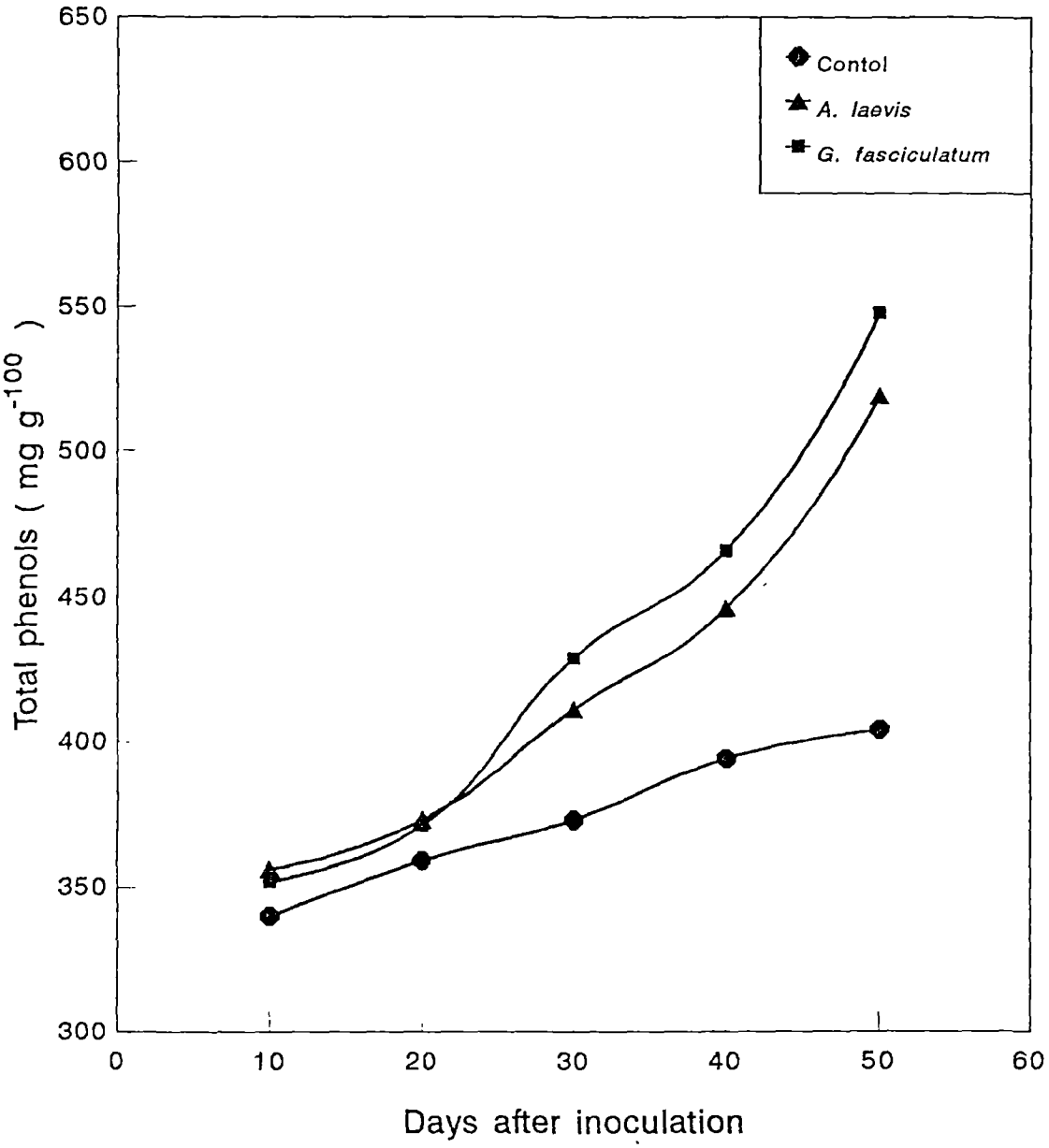

Figure 13. Total phenols content of *P. phaseoloides* inoculated with VAM fungi

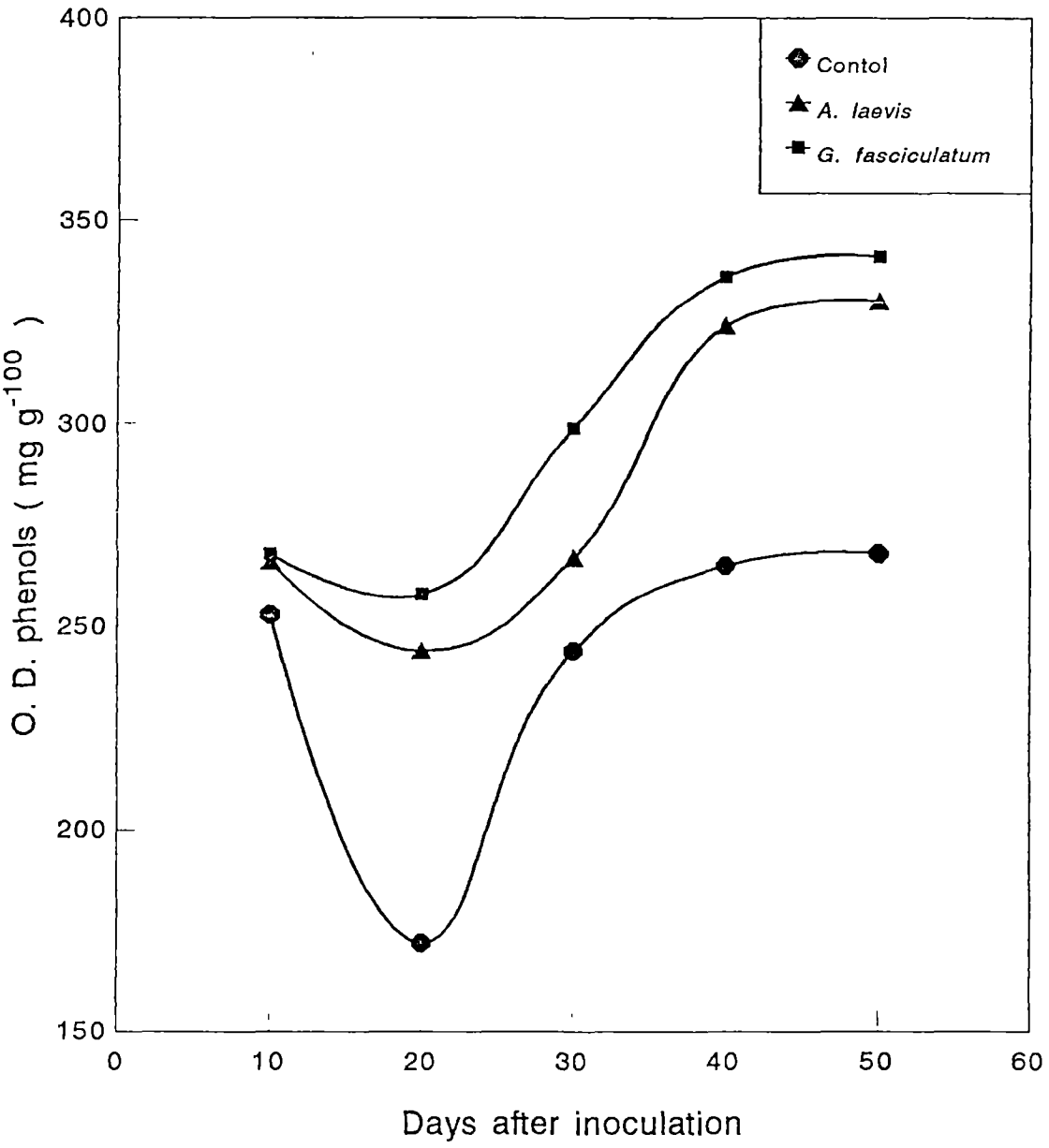

Figure 14. O. D. phenols content of *P. phaseoloides* inoculated with VAM fungi

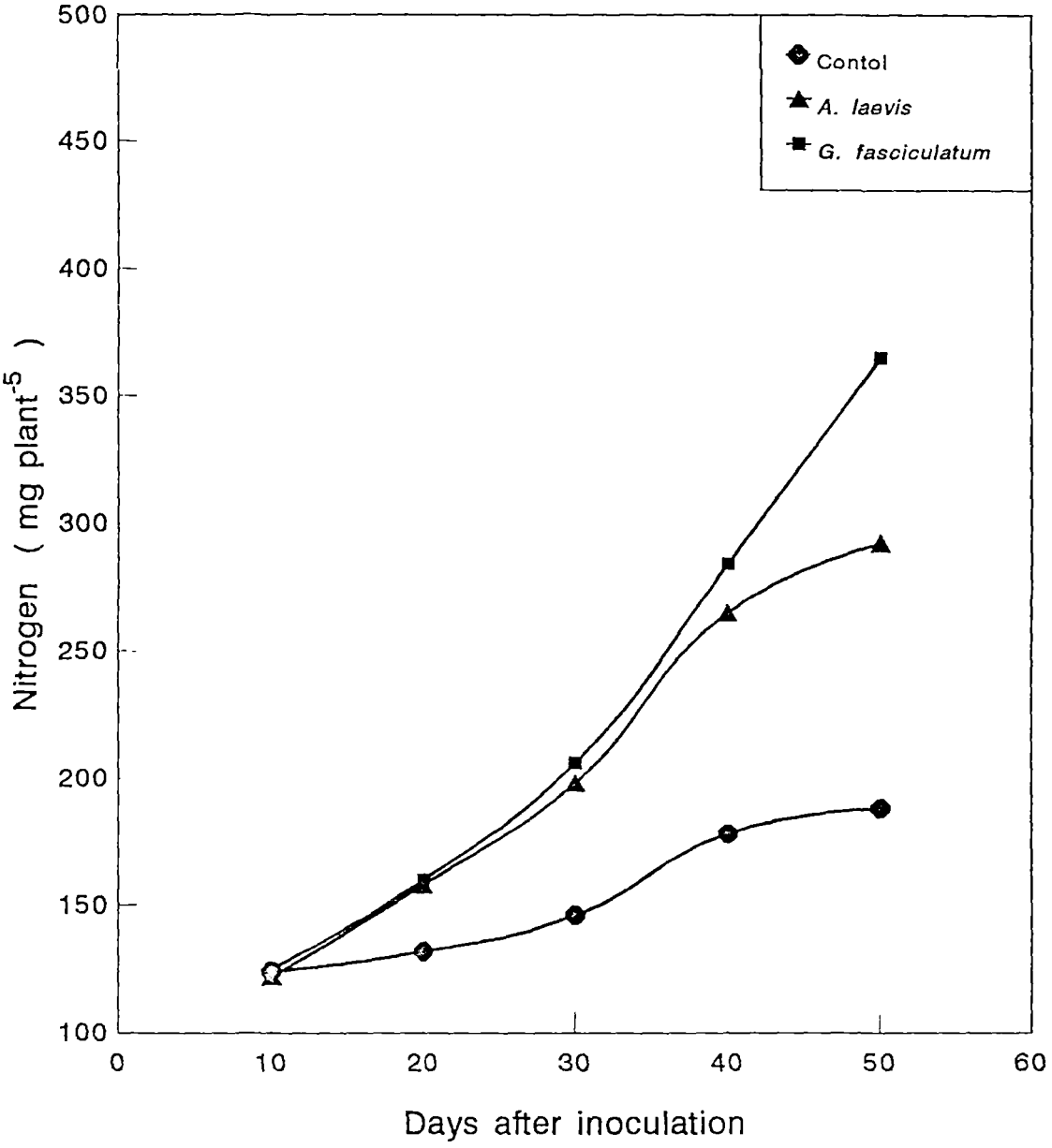

Figure 15. Nitrogen content of *P. phaseoloides* inoculated with VAM fungi

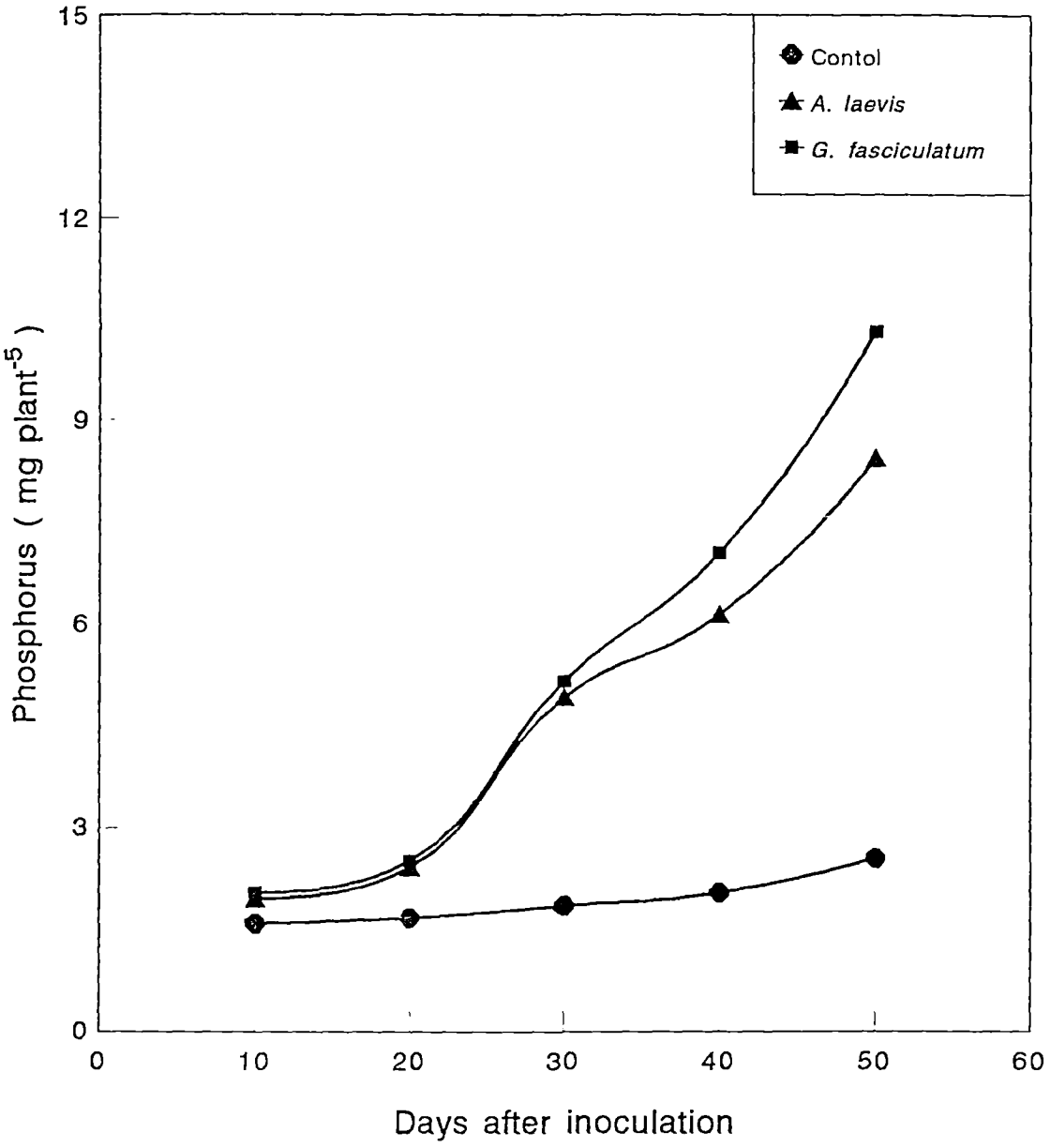

Figure 16. Phosphorus content of *P. phaseoloides* inoculated with VAM fungi

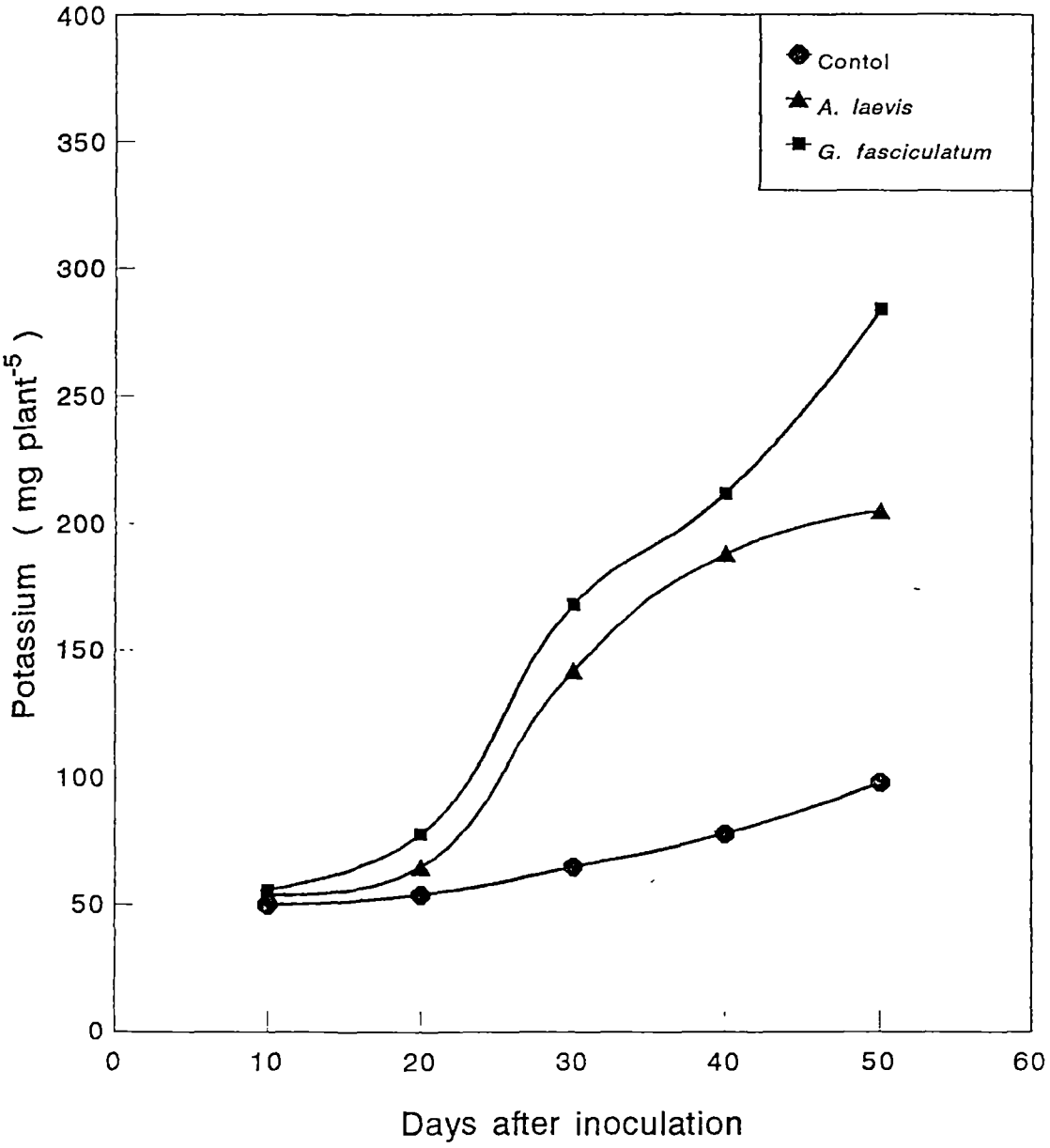

Figure 17. Potassium content of *P. phaseoloides* inoculated with VAM fungi

4.4 Effect of Different Levels of Rock Phosphate Application and Inoculation of *G. fasciculatum* and *A. laevis* on Root Colonisation, Nutrient Content and Growth of *P. phaseoloides*

4.4.1 Root colonisation and spore count in soil

Results show that rock phosphate application at 50 per cent recommended level increased root colonisation by *G. fasciculatum*, *A. laevis* and native VAM fungi (Fig. 18). But at 100 per cent rock phosphate level it got reduced in the treatments with and without *G. fasciculatum* and *A. laevis*. The reduction in root colonisation upon increased rock phosphate application was much pronounced in control plants. The root colonisation was more in *G. fasciculatum* than *A. laevis* at all the P treatments.

VAM spore count in soil also increased upon application of 50 per cent rock phosphate (Fig. 19). At 100 per cent rock phosphate level, a reduced spore population was recorded in all treatments. VAM spore count in *G. fasciculatum* treated soil was more when compared to *A. laevis* treated soil and control.

4.4.2 Growth, nodulation and nitrogenase activity

Figures 20-26 show the effect of different levels of rock phosphate application on growth, nodulation and nitrogenase activity of *P. phaseoloides*. Application of 50 per cent recommended dose of rock phosphate significantly increased length and weight of both shoot and root, nodule number and weight as well as nitrogenase activity. Further increase of P to 100 per cent of recommended dose did not cause much change in plants inoculated with VAM fungi except root length and nodule number which were reduced slightly. However, therewas gradual increase with increased rock phosphate application in all the above parameters of uninoculated plants.

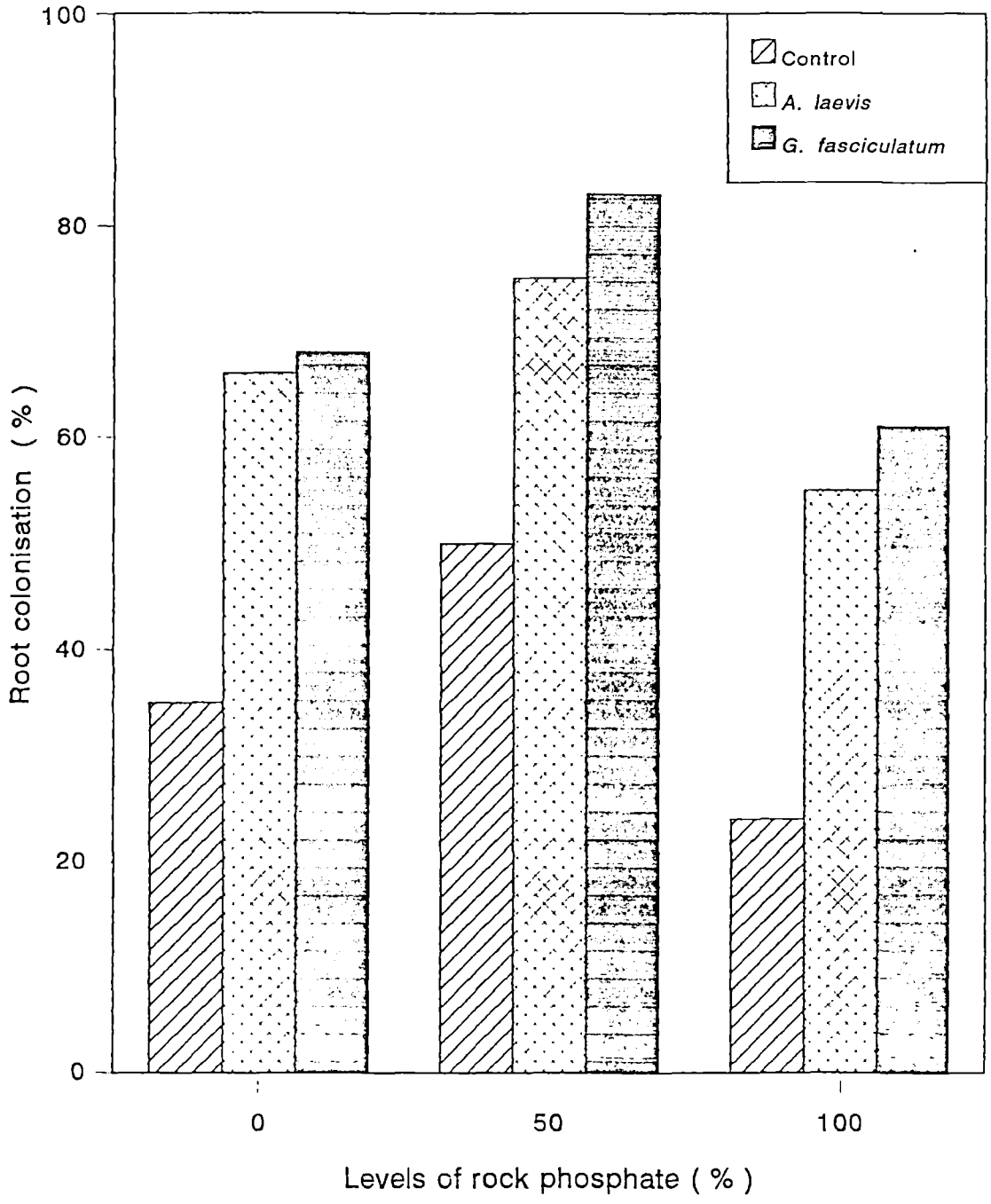

Figure 18. Effect of VAM inoculation at 3 levels of P on root colonisation of *P. phaseoloides*

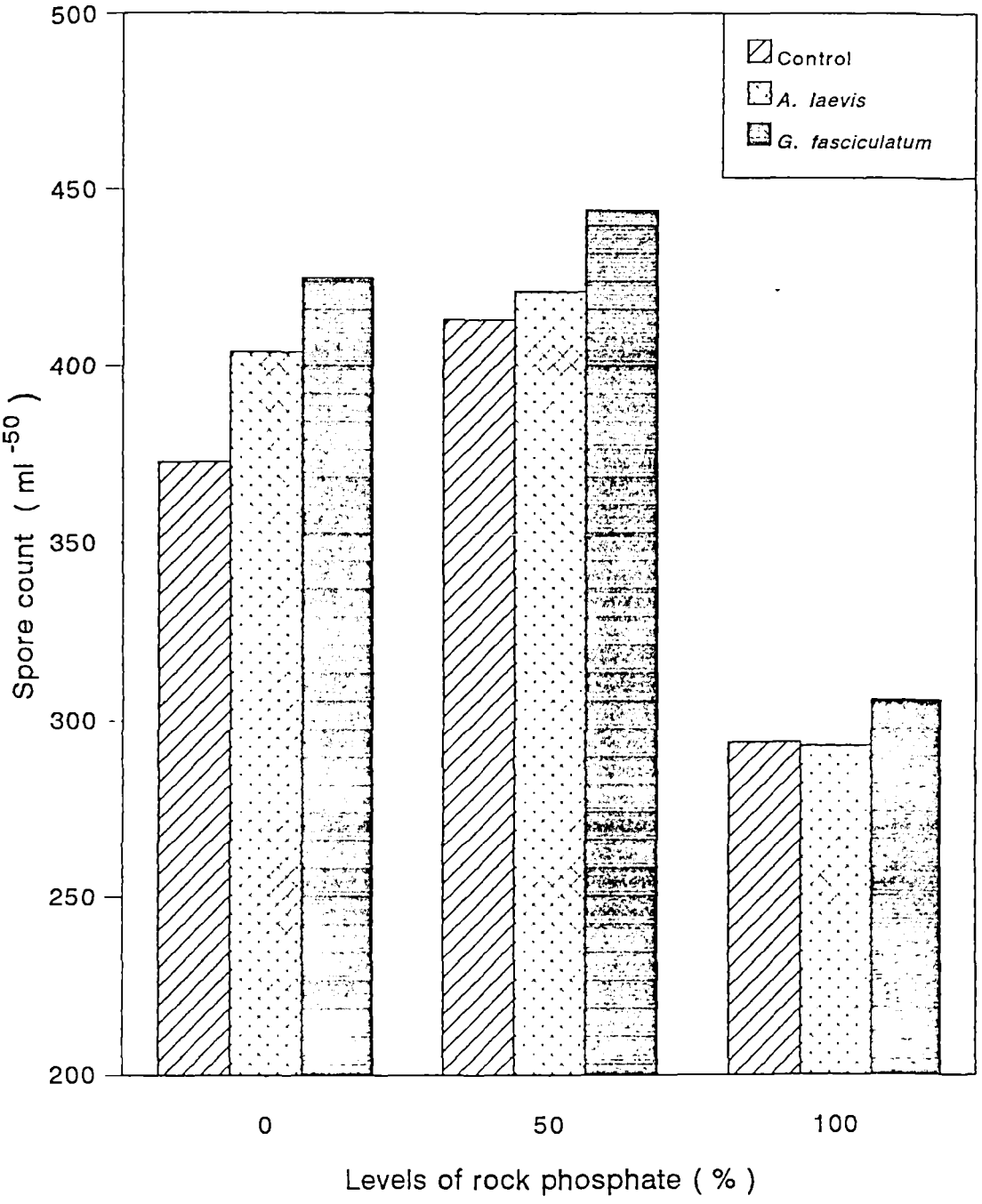

Figure 19. Effect of VAM inoculation at 3 levels of P on spore count in soil of *P. phaseoloides*

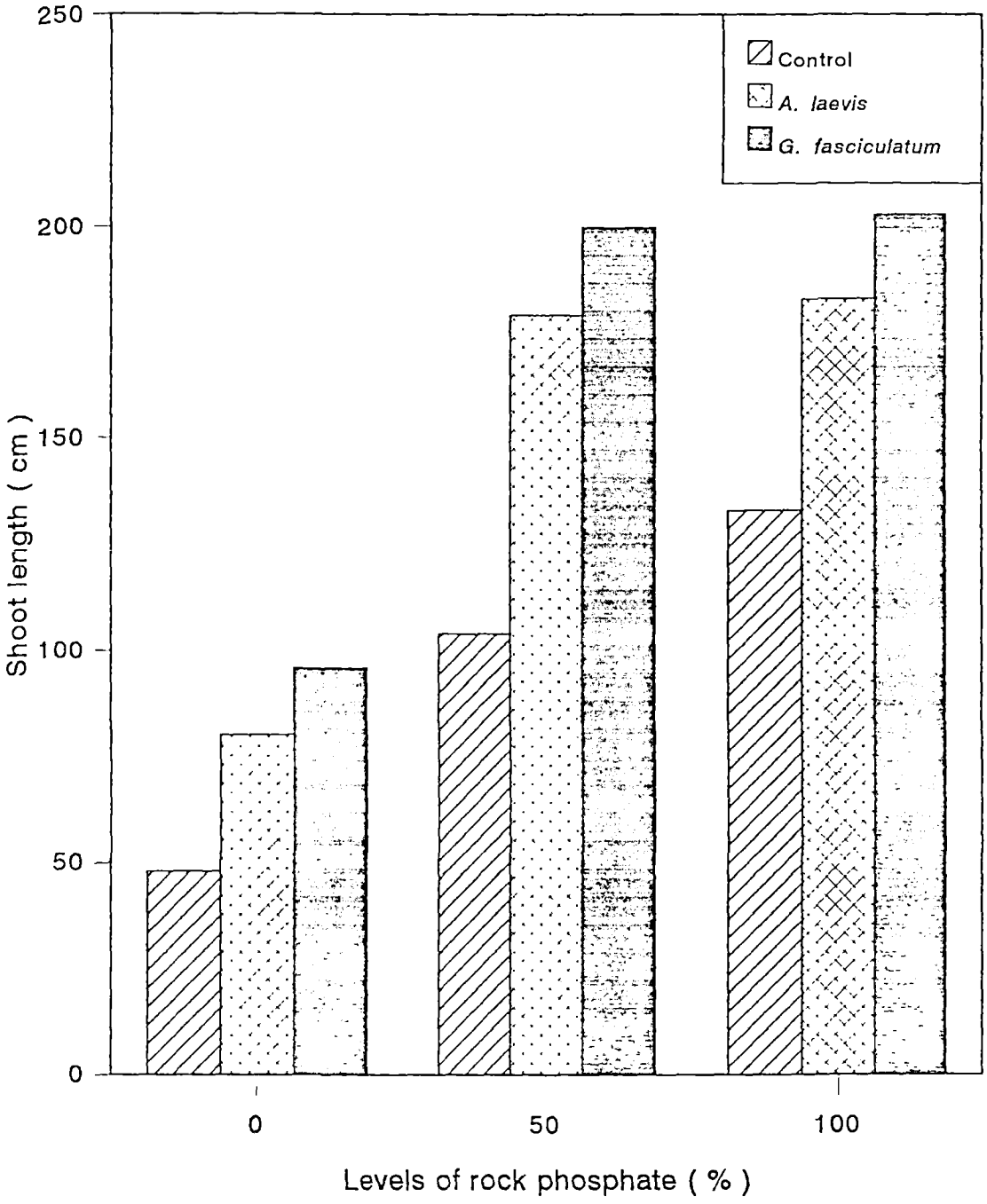

Figure 20. Effect of VAM inoculation at 3 levels of P on shoot length of *P. phaseoloides*

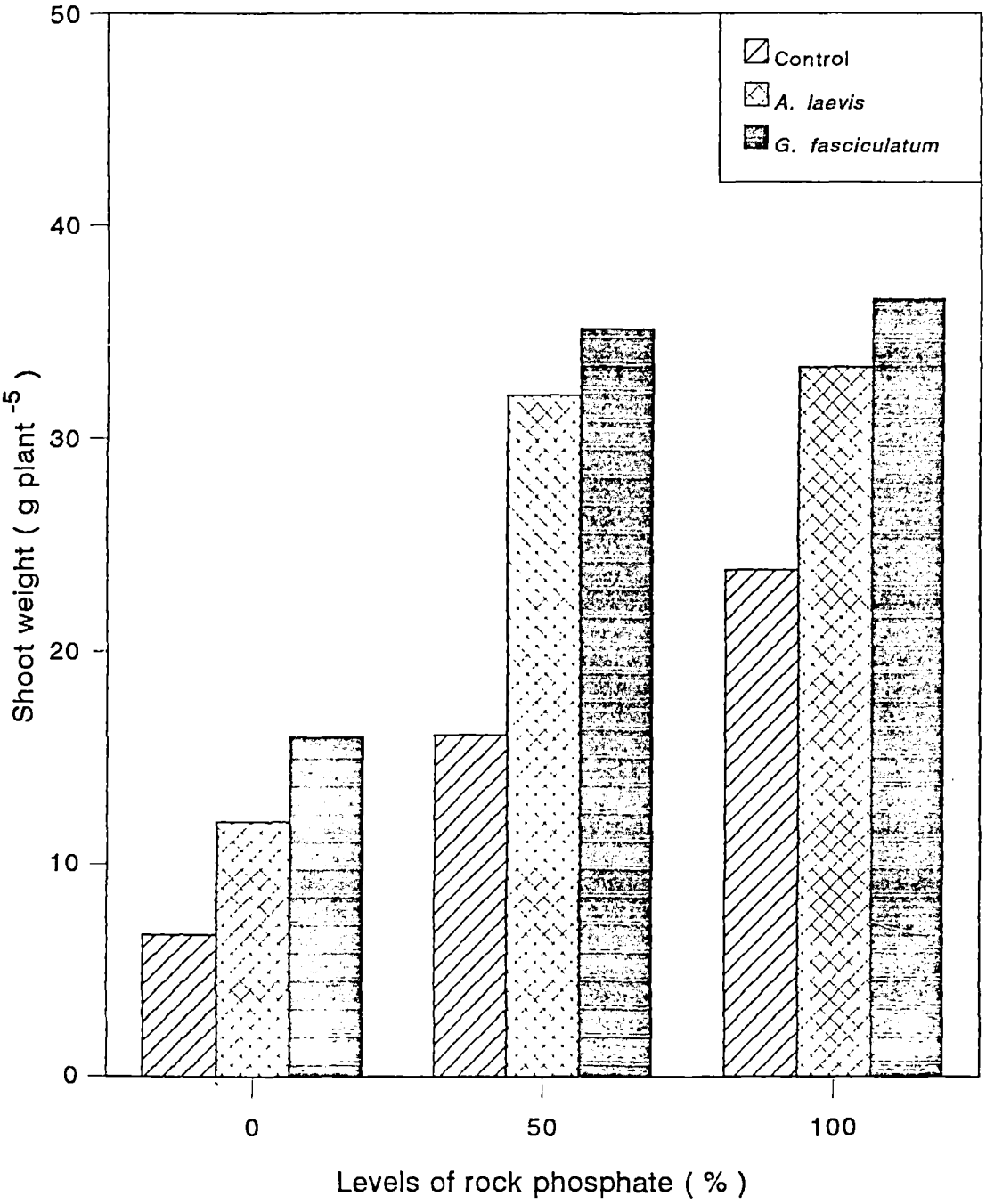

Figure 21. Effect of VAM inoculation at 3 levels of P on shoot weight of *P. phaseoloides*

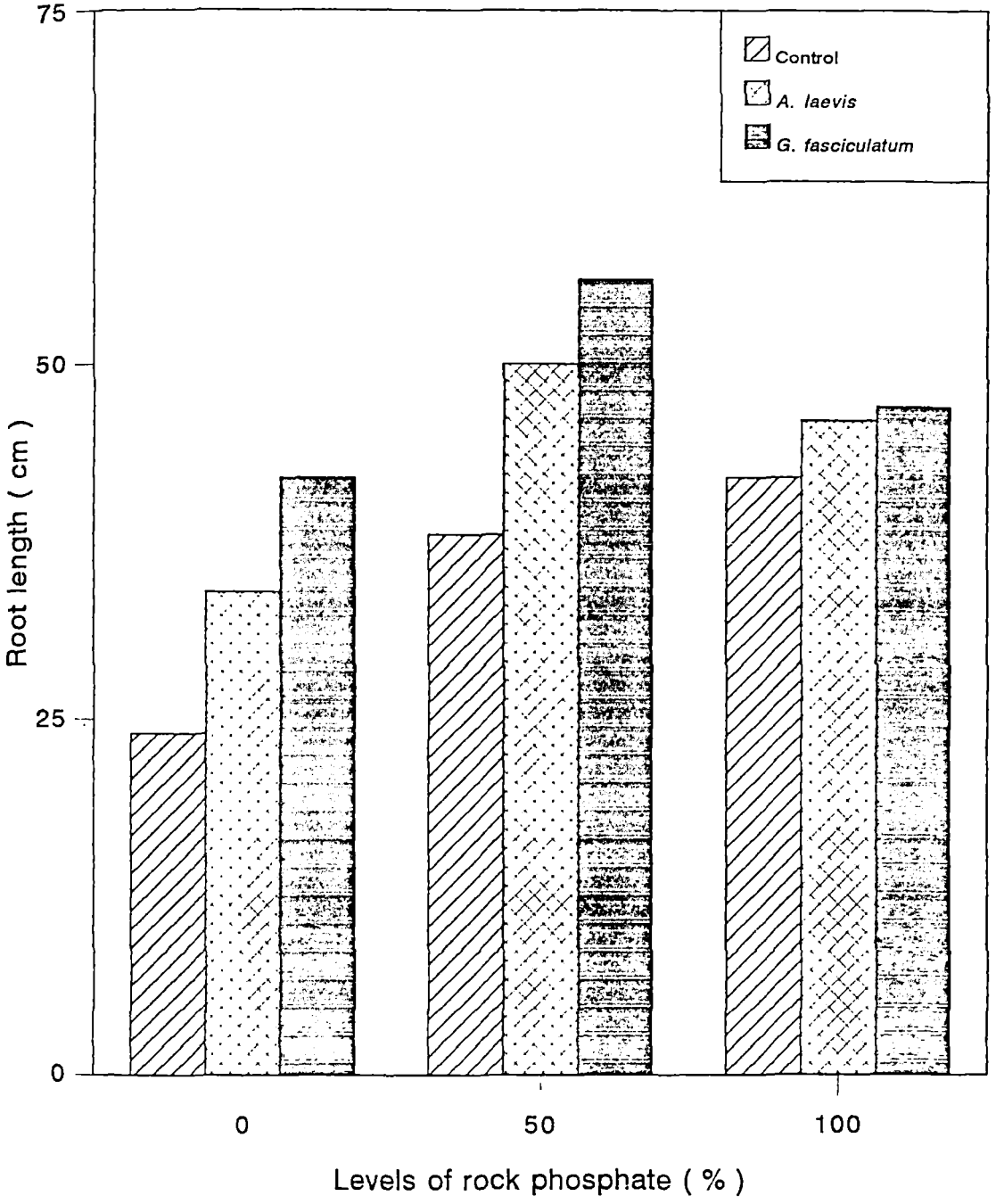

Figure 22. Effect of VAM inoculation at 3 levels of P on root length of *P. phaseoloides*

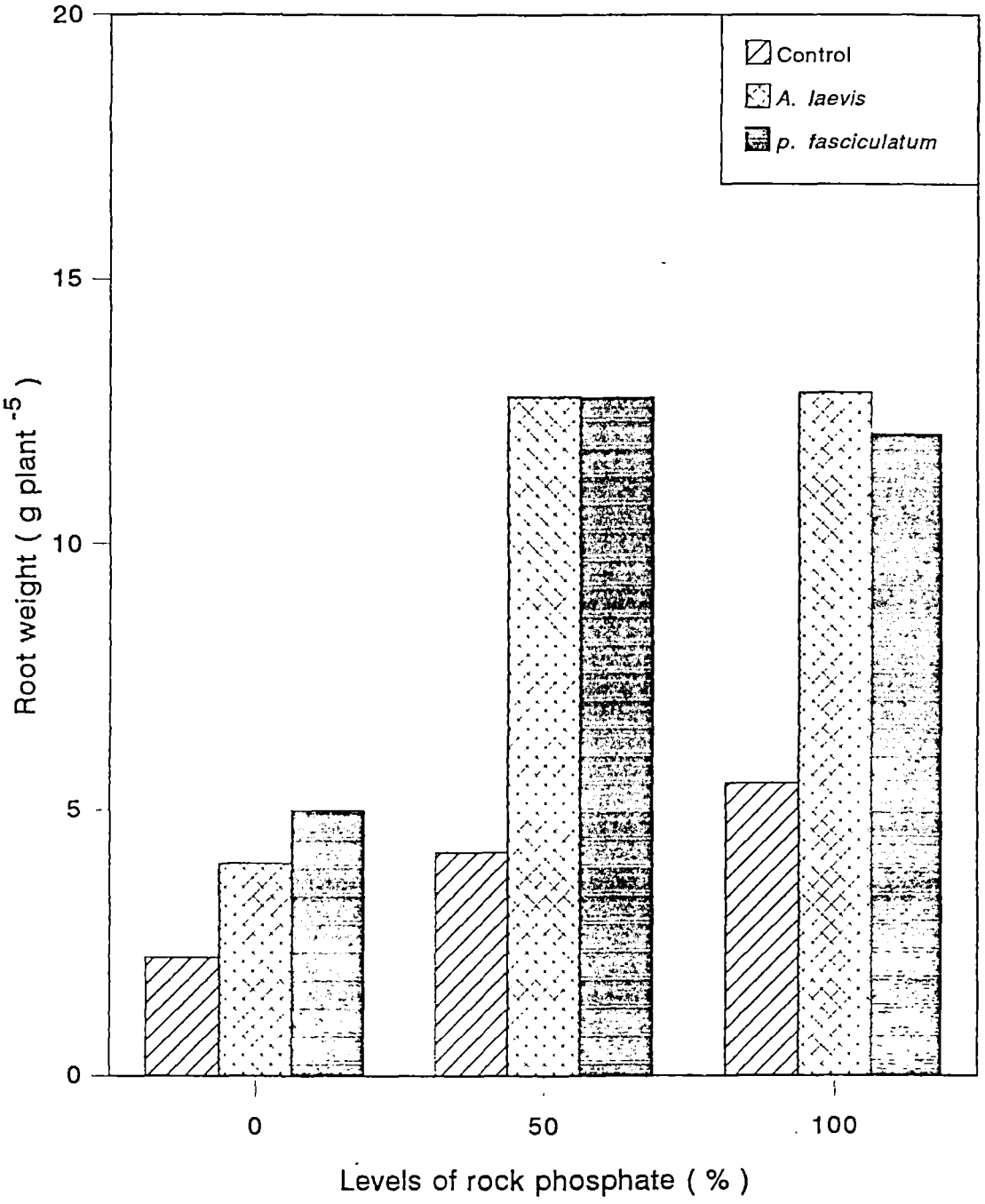

Figure 23. Effect of VAM inoculation at 3 levels of P on root weight of *P. phaseoloides*

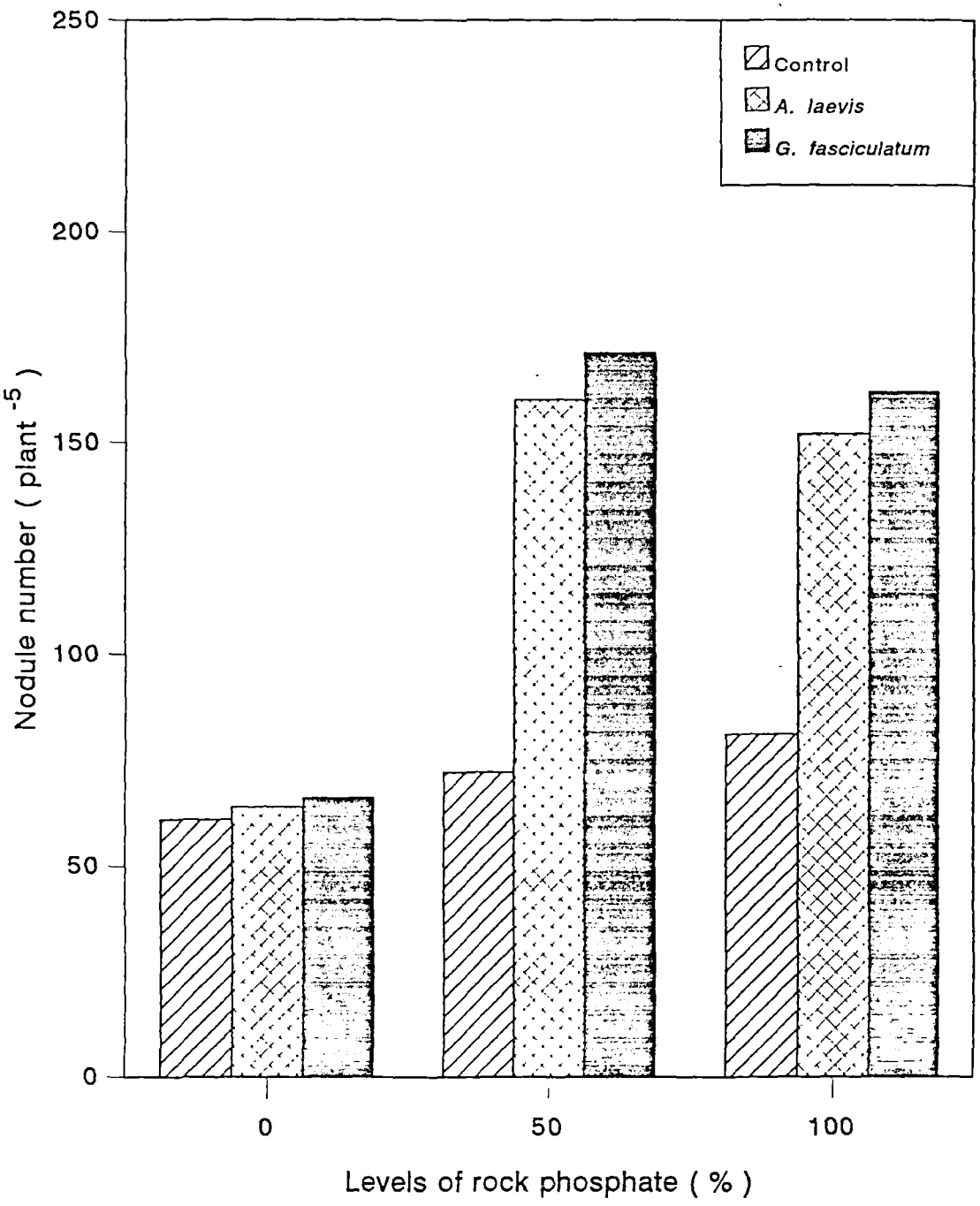

Figure 24. Effect of VAM inoculation at 3 levels of P on nodule number of *P. phaseoloides*

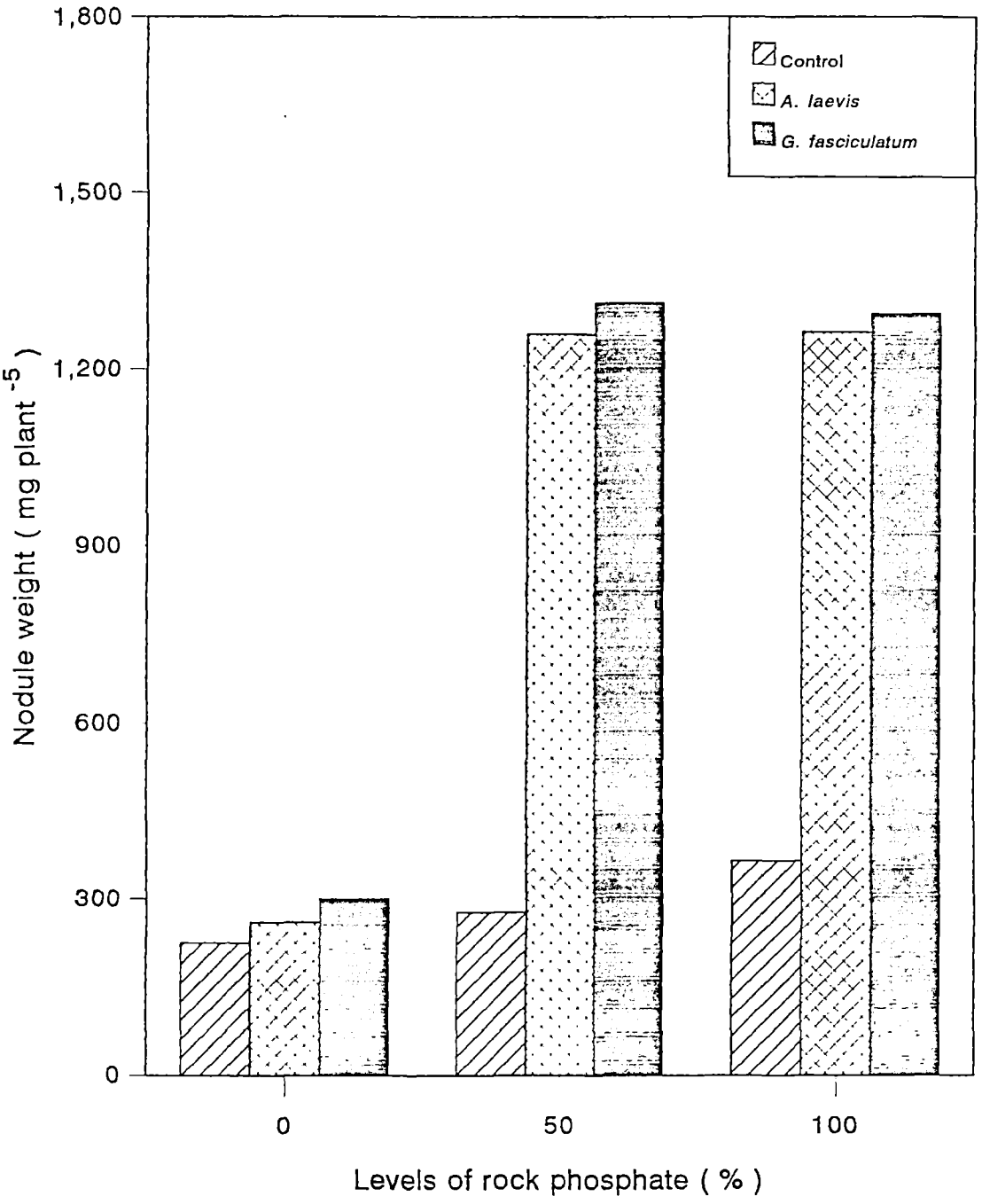

Figure 25. Effect of VAM inoculation at 3 levels of P on nodule weight of *P. phaseoloides*

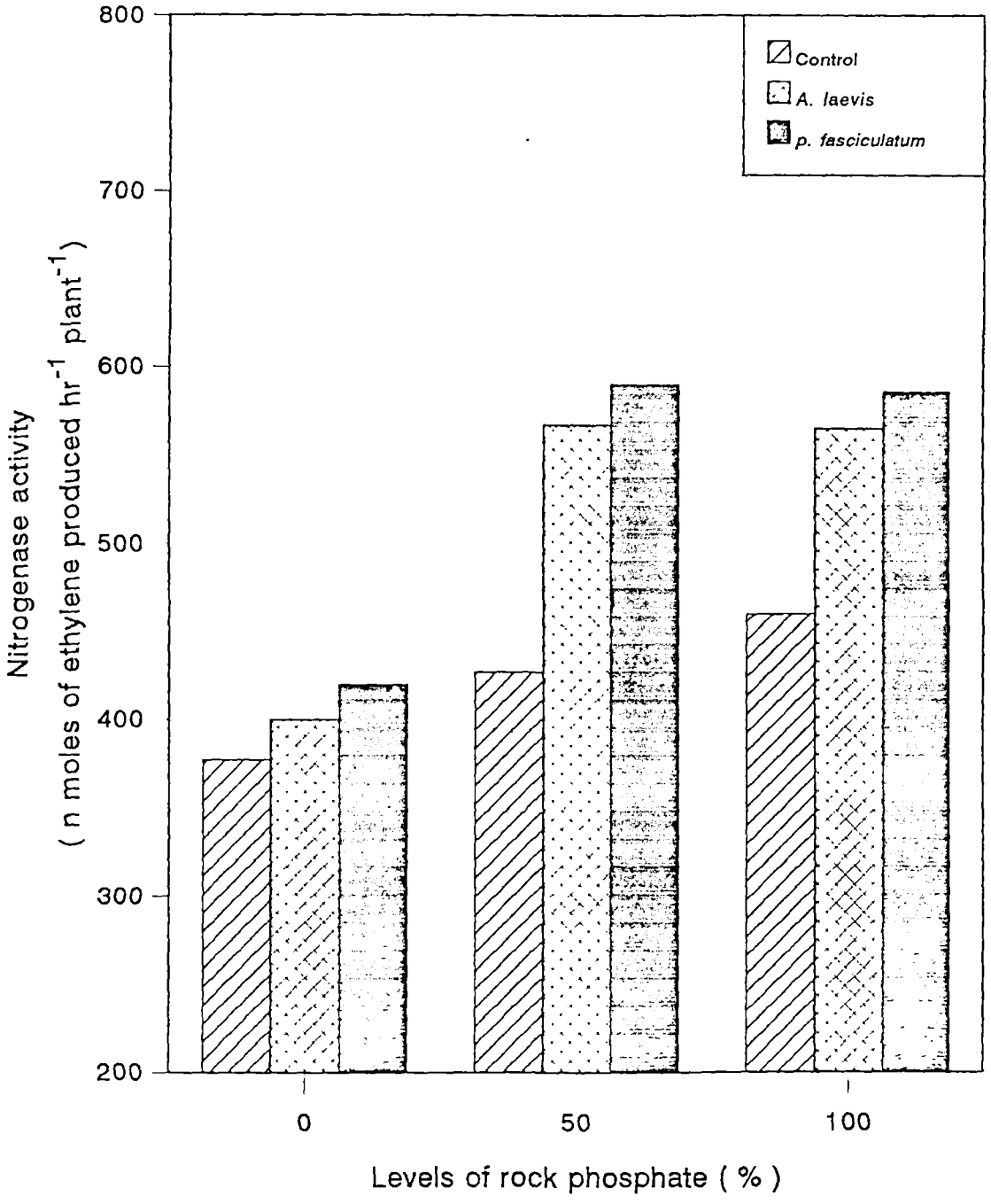

Figure 26. Effect of VAM inoculation at 3 levels of P on nitrogenase activity of *P. phaseoloides*

Both *G. fasciculatum* and *A. laevis* significantly increased all the parameters when 50 per cent P was applied as rock phosphate. But the increase in shoot length, shoot weight, root weight, nodule weight and nitrogenase activity upon VAM application was same in 50 per cent and 100 per cent rock phosphate levels.

G. fasciculatum was more efficient than *A. laevis* in promoting growth, nodulation and nitrogenase activity in *P. phaseoloides* at different levels of rock phosphate application.

4.4.3 Nutrient content in plants

The results of the study on the effect of application of different levels of rock phosphate on N, P and K in shoots and roots of *P. phaseoloides* are represented in the Figures 27-32. Fifty per cent of recommended dose of rock phosphate application augmented the levels of major nutrients in shoots as well as the roots of *P. phaseoloides*. As the level of applied phosphate increased to 100 per cent there was a corresponding increase in the N, P and K in both shoot and root tissues of control plants. However, there was not much pronounced increase in nutrient content due to VAM inoculation at 100 per cent recommended level of rock phosphate application.

Inoculation of *G. fasciculatum* and *A. laevis* significantly increased the N, P and K content in *P. phaseoloides* raised with two levels of rock phosphate and control plants receiving no phosphate. Among the two VAM species *G. fasciculatum* was superior to *A. laevis* in increasing the nutrients. The rate of increase of N, P and K due to VAM inoculation was more in plants receiving no phosphate than those receiving 50 and 100 per cent rock phosphate. Compared to other nutrients the increase in N levels in VAM inoculated plants was much pronounced.

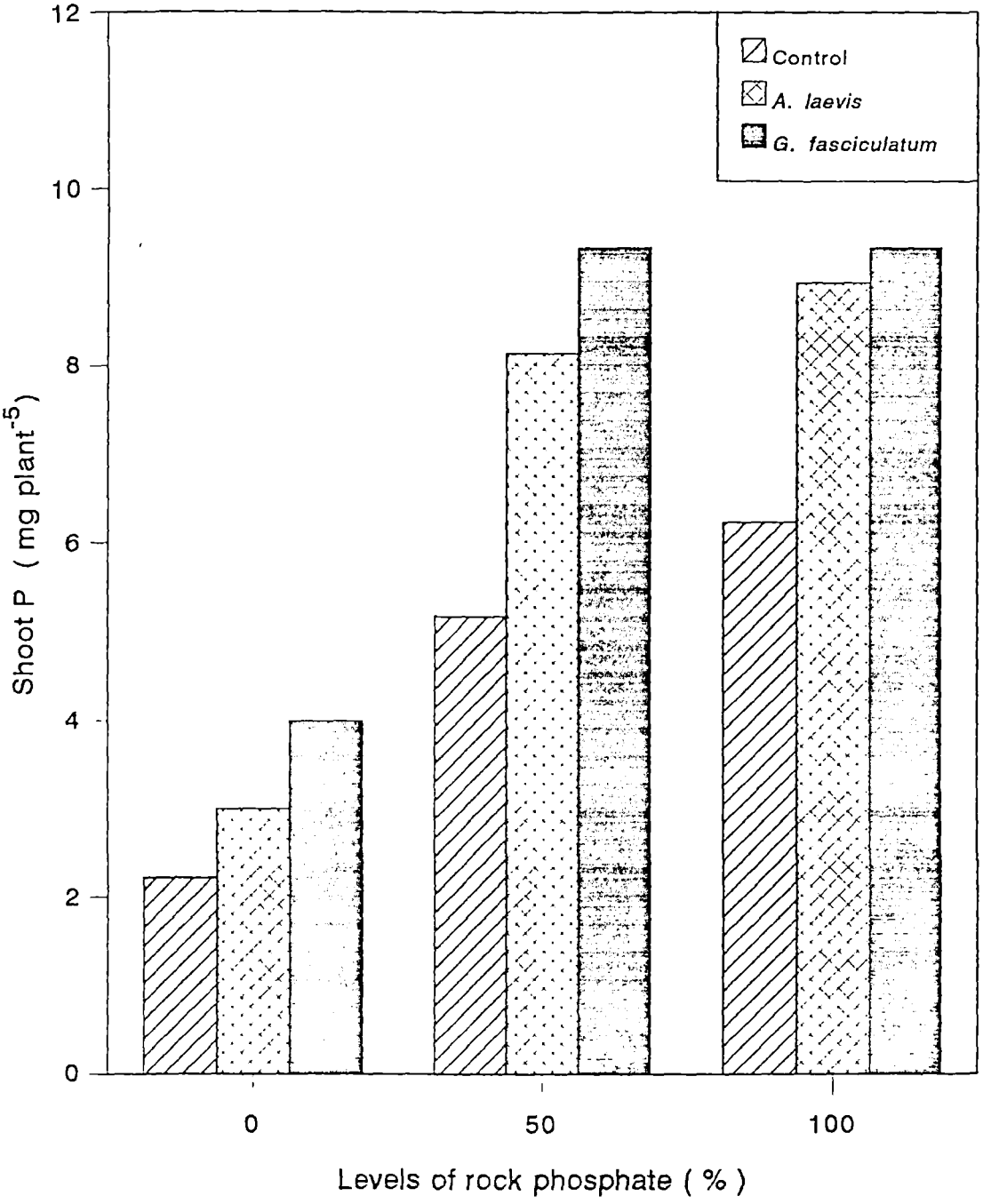

Figure 27. Effect of VAM inoculation at 3 levels of P on shoot P content of *P. phaseoloides*

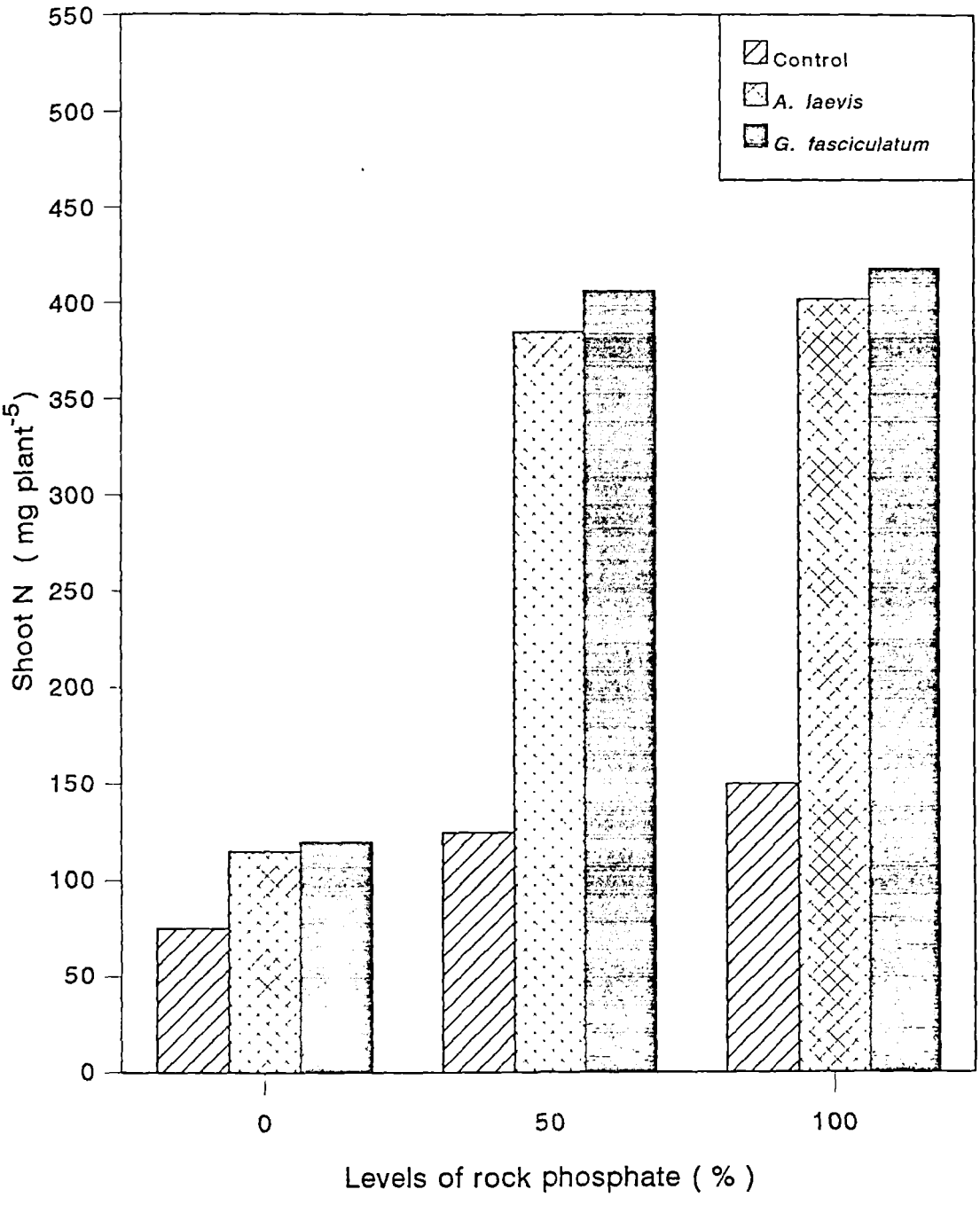

Figure 28. Effect of VAM inoculation at 3 levels of P on shoot N content of *P. phaseoloides*

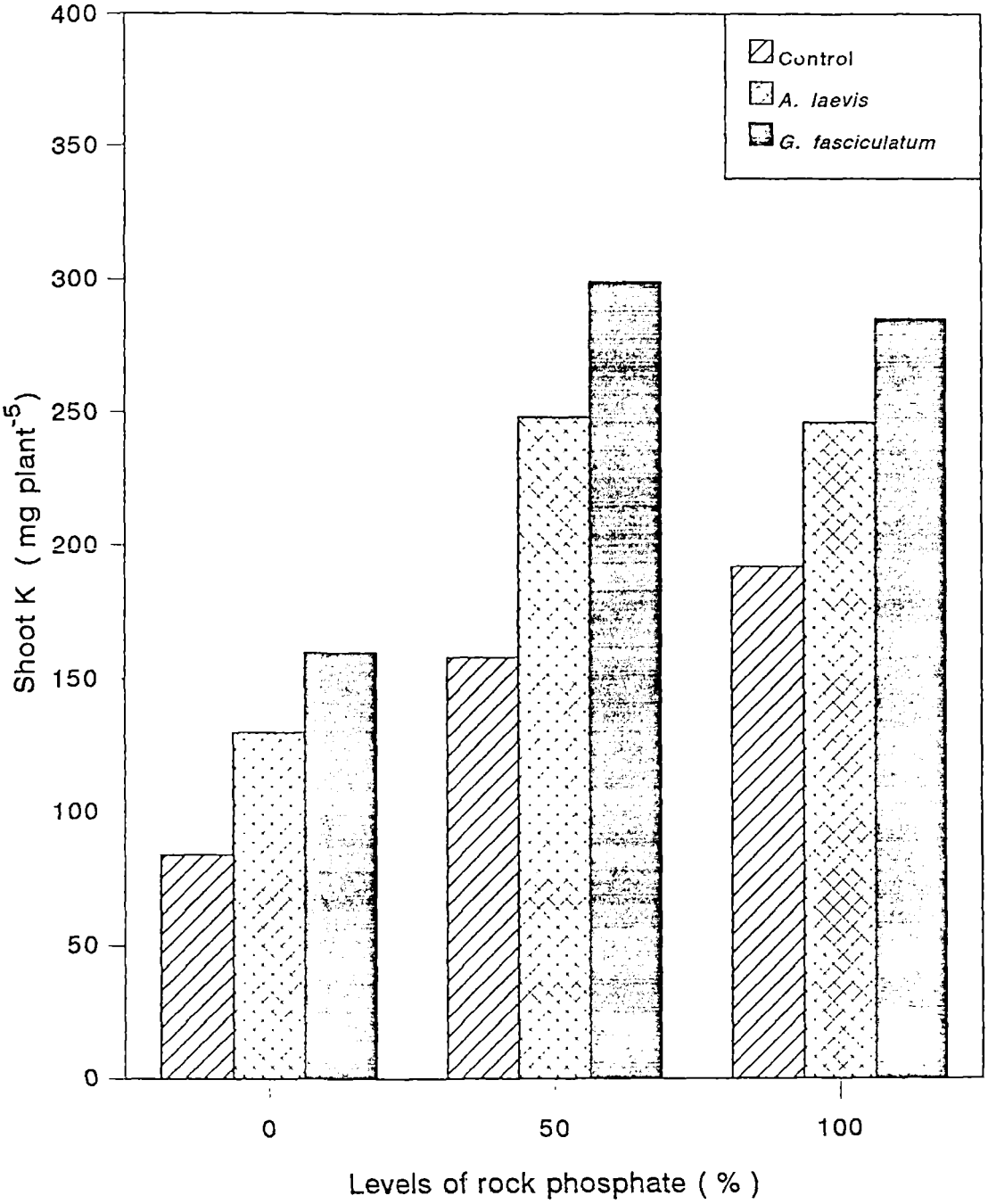

Figure 29. Effect of VAM inoculation at 3 levels of P on shoot K content of *P. phaseoloides*

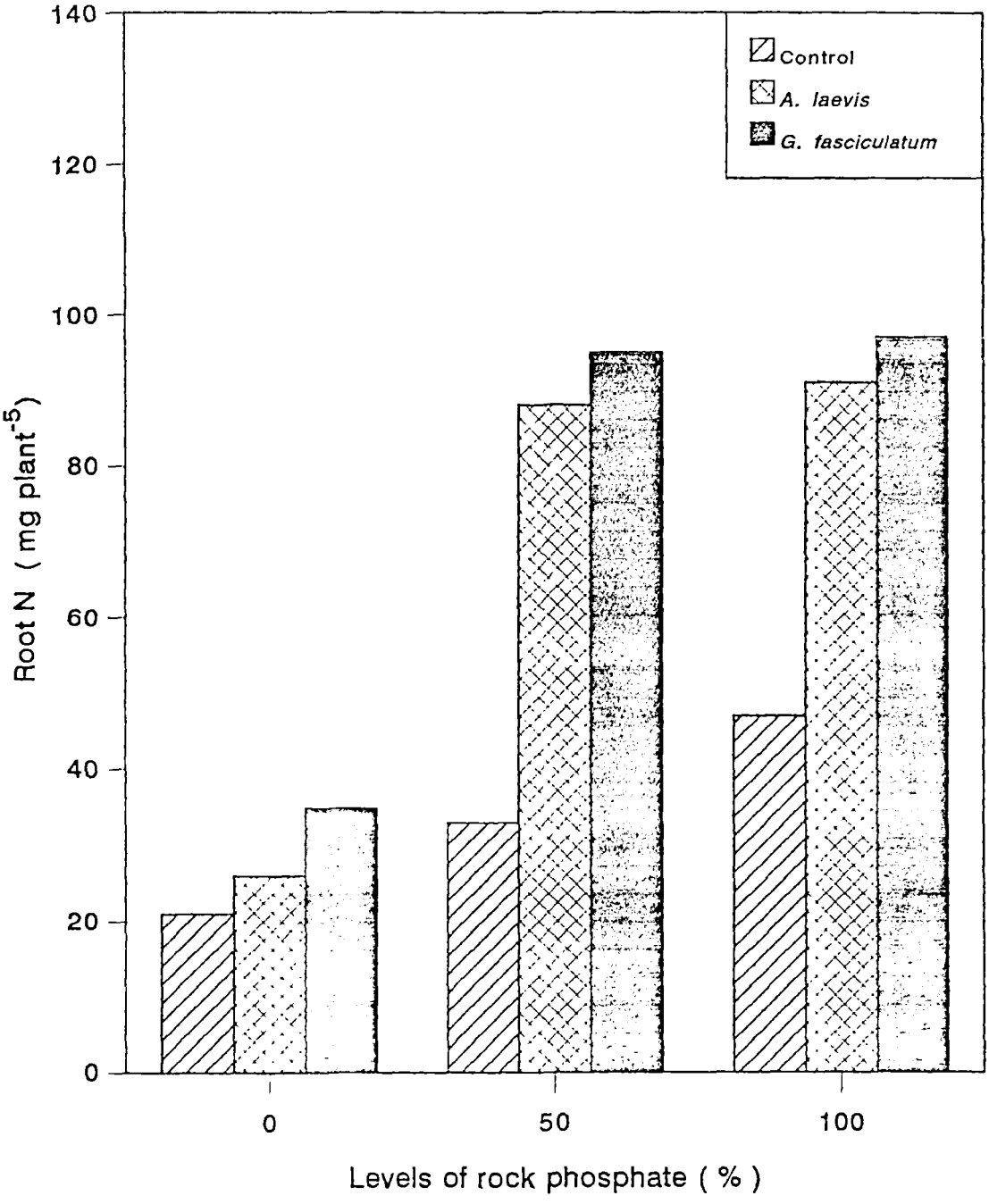

Figure 30. Effect of VAM inoculation at 3 levels of P on root N content of *P. phaseoloides*

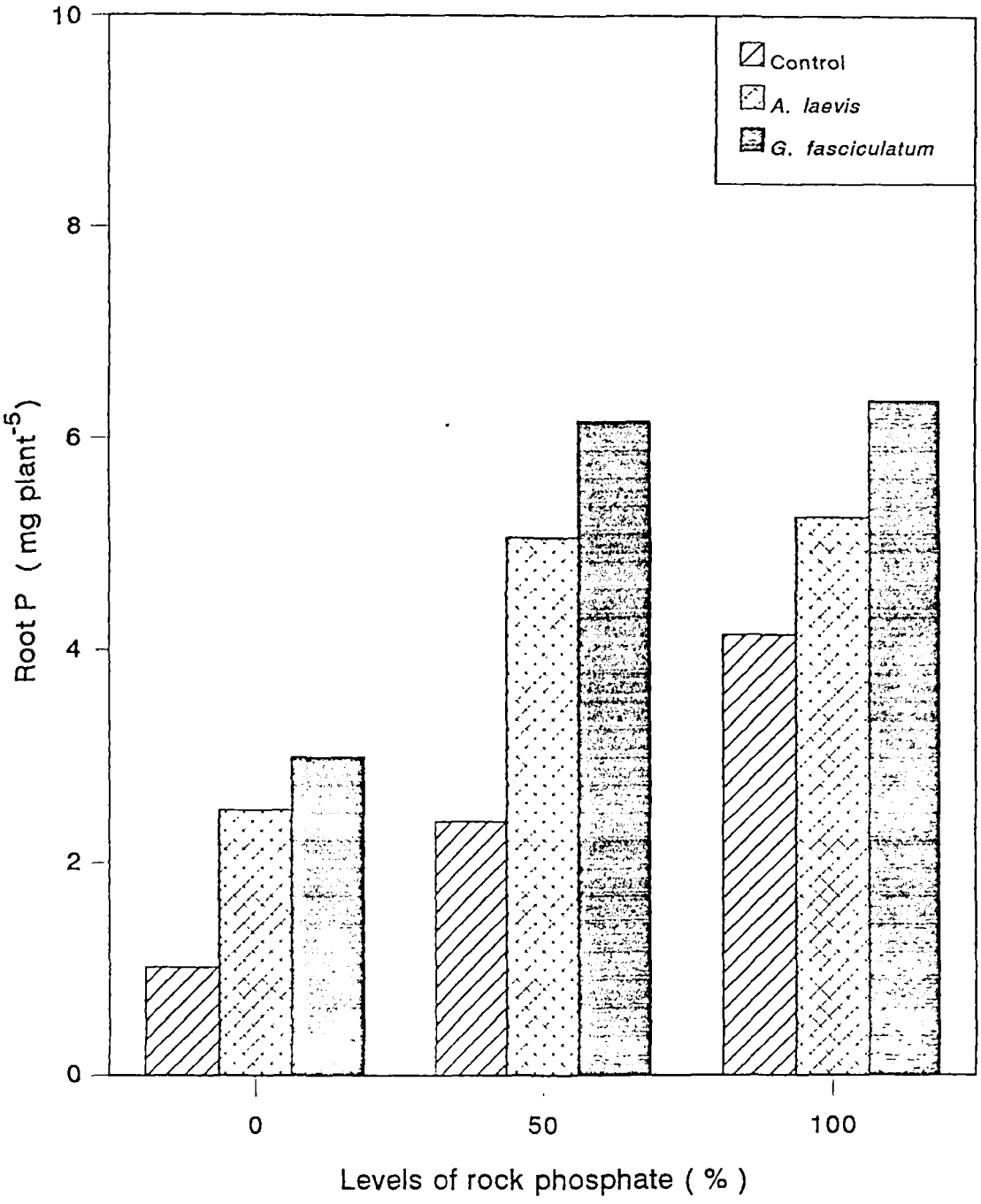

Figure 31. Effect of VAM inoculation at 3 levels of P on root P content of *P. phaseoloides*

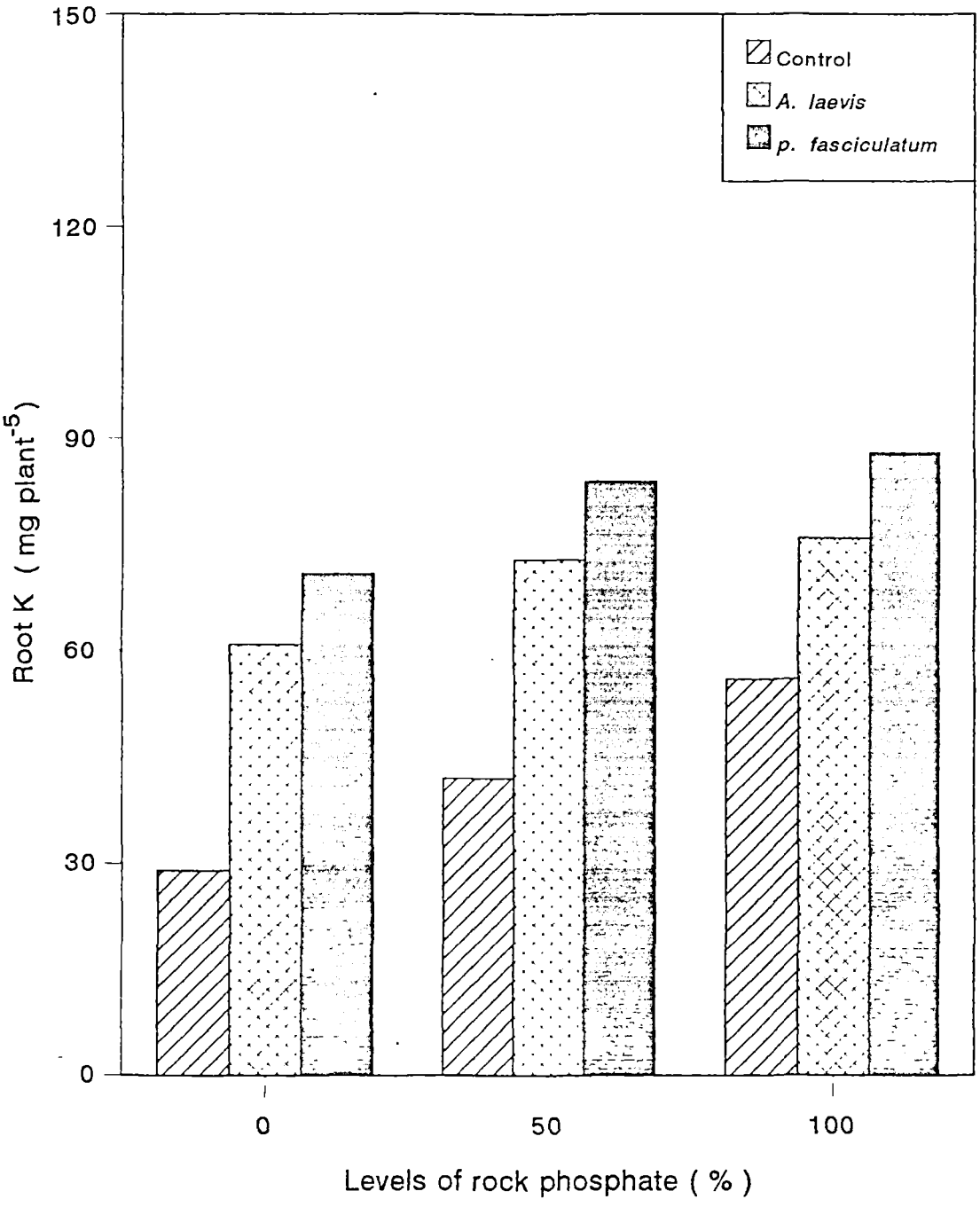

Figure 32. Effect of VAM inoculation at 3 levels of P on root K content of *P. phaseoloides*

The level of nutrients in VAM inoculated *P. phaseoloides* at 50 per cent and 100 per cent of recommended dose of rock phosphate was same. However, the degree of response upon VAM inoculation got reduced beyond 50 per cent rock phosphate application.

4.5 Impact of *Azotobacter* sp., *Beijerinckia* sp. and *B. circulans* on Root Colonisation by *G. fasciculatum* and Growth, Nutrient Content and Rhizosphere Microbial Population of *P. phaseoloides*

4.5.1 Root colonisation and spore count in soil

P. phaseoloides inoculated separately with *G. fasciculatum*, *Azotobacter* sp., *Beijerinckia* sp. and *B. circulans* (phosphobacteria) registered more VAM colonisation compared to uninoculated plants raised under unsterile condition (Table 9). Compared to different bacteria *G. fasciculatum* recorded more root colonisation. Among the three bacteria, there was not much difference in root colonisation by VAM when inoculated without *G. fasciculatum*. Dual inoculation of *P. phaseoloides* with *G. fasciculatum* and any of the bacteria further intensified root colonisation. However, maximum root colonisation was recorded in plants coinoculated with *G. fasciculatum* and phosphobacteria followed by *G. fasciculatum—Azotobacter* sp. and *G. fasciculatum—Beijerinckia* sp. treatments.

Spore count of VAM in soil was more when *G. fasciculatum* was inoculated with and without beneficial bacteria (Table 9). Bacterial inoculation alone did not show significant increase in spore population over the uninoculated control.

Table 9

Effect of *G. fasciculatum* and beneficial bacteria on *P. phaseoloides* root colonisation and spore count in soils

Treatments	Root colonisation per cent	Spore count (ml^{-50})
G. fasciculatum alone	63.67	324
Azotobacter sp. alone	50.33	305
Beijerinckia sp. alone	50.33	304
Phosphobacteria alone	48.18	310
G. fasciculatum + *Azotobacter* sp.	72.67	335
G. fasciculatum + *Beijerinckia* sp.	68.67	330
G fasciculatum + Phosphobacteria	79.68	333
Control	35.24	283
CD (P = 0.05)	3.34	32

4.5.2 Growth, nodulation and nitrogenase activity

Inoculation of *P. phaseoloides* with either *G. fasciculatum*, *Azotobacter* sp., *Beijerinckia* sp. or phosphobacteria significantly increased shoot length, shoot weight, root length, root weight, nodule number and nodule weight (Table 10). Coinoculation of *P. phaseoloides* with VAM and any one of the bacteria mentioned above recorded more biomass and nodule characters than those inoculated with any one of these isolates alone. Inoculation of phosphobacteria with *G. fasciculatum* recorded maximum shoot length, shoot weight, root length and root weight.

Inoculation of *P. phaseoloides* with *G. fasciculatum*, *Azotobacter* sp., *Beijerinckia* sp. and phosphobacteria separately augmented nitrogenase activity (Table 10). Out of the four microorganisms studied maximum nitrogenase activity was recorded in the treatment of *Azotobacter* sp. followed by *Beijerinckia* sp., *G. fasciculatum* and phosphobacteria. Combined inoculation of *G. fasciculatum* with one of the bacteria used in this study further increased the nitrogenase activity and the maximum being with *Azotobacter* sp. combination followed by *G. fasciculatum*—*Beijerinckia* sp. and *G. fasciculatum*—phosphobacteria combinations.

4.5.3 Rhizosphere microflora

Inoculation of soil having *P. phaseoloides* separately with *G. fasciculatum*, *Azotobacter* sp., *Beijerinckia* sp. and phosphobacteria significantly increased the saprophytic bacteria and fungi (Table 11). The population of actinomycetes was not altered. Saprophytic bacterial population was more in soil inoculated with phosphobacteria followed by *G. fasciculatum*, *Azotobacter* sp. and *Beijerinckia* sp. With regard to fungi the increase was minimum in soil inoculated with *Beijerinckia* sp. and maximum was in *G. fasciculatum*, phosphobacteria and *Azotobacter* sp. inoculated soil.

Table 10

Effect of *G. fasciculatum* and beneficial bacteria on growth, nodulation and nitrogenase activity of *P. phaseoloides*

Treatments	Shoot length (cm)*	Shoot weight (g)**	Root length (cm)*	Root weight (g)**	Nodule number**	Nodule weight (g)**	Nitrogenase activity (n moles ethylene produced hr^{-1} $plant^{-1}$)
G. fasciculatum alone	111	22.30	22	8.90	115	1.42	471
Azotobacter sp. alone	80	15.30	20	7.90	126	1.62	504
Beijerinckia sp. alone	88	18.00	23	9.00	134	1.68	486
Phosphobacteria alone	124	24.70	24	10.60	130	1.67	435
G. fasciculatum + *Azotobacter* sp.	118	26.10	26	12.07	150	1.84	568
G. fasciculatum + *Beijerinckia* sp.	133	28.20	26	11.63	158	1.80	552
G. fasciculatum + Phosphobacteria	159	32.90	30	15.20	165	1.92	506
Control	42	7.20	17	4.00	64	0.35	386
CD (P = 0.05)	6	0.78	2	0.65	5	0.11	14

* Mean of 5 plants
** Total of 5 plants

Table 11

Effect of *G. fasciculatum* and beneficial bacteria on the rhizosphere microflora population per g of rhizosphere soil (on dry weight basis)

Treatments	Total saprophytic bacteria x 10^6	Fungi x 10^4	*Actinomycetes* x 10^3	*Azotobacter* spp. x 10^3	*Beijerinckia* spp. x 10^2	Phosphobacteria x 10^3
G. fasciculatum alone	86	51	20	ND	8	53
Azotobacter sp. alone	74	46	22	86	5	71
Beijerinckia sp. alone	64	40	21	ND	145	54
Phosphobacteria alone	95	49	19	ND	14	120
G. fasciculatum + *Azotobacter* sp.	112	68	22	106	9	82
G. fasciculatum + *Beijerinckia* sp.	95	63	23	ND	171	64
G. fasciculatum + Phosphobacteria	125	75	20	ND	19	143
Control	54	31	21	ND	4	42
CD (P = 0.05)	7	5	6		4	4

ND - Not detected

Dual inoculation comprising of *G. fasciculatum* with one of the bacteria used increased bacterial and fungal count over the corresponding bacterial inoculation alone. Bacterial count was maximum in the combination of *G. fasciculatum* and phosphobacteria followed by *G. fasciculatum* with *Azotobacter* sp. and *G. fasciculatum* with *Beijerinckia* sp. The fungal population was also more in *G. fasciculatum*—phosphobacteria combination closely followed by *G. fasciculatum—Azotobacter* sp. and *G. fasciculatum—Beijerinckia* sp. Dual inoculation did not alter the actinomycetes population. Soils inoculated with *Azotobacter* sp. with and without *G. fasciculatum* only contained *Azotobacter* sp. and the maximum was in dual inoculation treatment.

Soil used in this study contained a few counts of *Beijerinckia* sp. But inoculation with *Beijerinckia* sp. considerably increased its population. Phosphobacterial inoculation and *G. fasciculatum* inoculation enhanced the population of *Beijerinckia* sp. But *Azotobacter* sp. inoculation did not influence *Beijerinckia* sp. population.

Dual inoculation of *G. fasciculatum* and *Beijerinckia* sp. recorded more *Beijerinckia* sp. population when compared to treatment with *Beijerinckia* sp. alone. Similarly combined treatment of *G. fasciculatum* and phosphobacteria increased the count of *Beijerinckia* sp than phosphobacteria alone. However *Azotobacter* sp. with *G. fasciculatum* treatment did not change the count of *Beijerinckia* sp when compared to *Azotobacter* sp. treatment alone.

Separate inoculation of *G. fasciculatum*, *Azotobacter* sp., *Beijerinckia* sp. and phosphobacteria as well as dual inoculation of *G.fasciculatum* and either *Azotobacter* sp., *Beijerinckia* sp. or phosphobacteria significantly increased soil

phosphobacteria population. The maximum count of phosphobacteria was recorded in *G. fasciculatum*—phosphobacteria treatment followed by inoculation of phosphobacteria alone. Both *Azotobacter* sp. and *Beijerinckia* sp. with and without *G. fasciculatum* stimulated phosphobacteria population in the rhizosphere of *P. phaseoloides*.

4.5.4 Nutrient content

Inoculation of *P. phaseoloides* with *G. fasciculatum*, *Azotobacter* sp., *Beijerinckia* sp. and phosphobacteria alone and *G. fasciculatum* in combination with *Azotobacter* sp., *Beijerinckia* sp. and phosphobacteria separately increased N, P and K in shoot and root portions (Table 12). In the case of individual microbial treatments phosphobacteria recorded more of NPK in both shoot and root followed by *G. fasciculatum* treatments. Among the two non-symbiotic nitrogen fixing bacteria, inoculation with *Azotobacter* sp. showed enhanced level of N and P of shoot and P and K of root in *P. phaseoloides*. Inoculation of either *Azotobacter* sp., *Beijerinckia* sp. or phosphobacteria with *G. fasciculatum* caused substantial improvement of NPK content of shoot and root compared to the corresponding bacterial treatment without *G. fasciculatum*. Dual inoculation of *P. phaseoloides* with *G. fasciculatum* and phosphobacteria caused maximum NPK content in tissues followed by the treatment consisting of *G. fasciculatum* and *Azotobacter* sp. However, P content of roots of plants treated with phosphobacteria alone, *G. fasciculatum* with *Azotobacter* sp. and *G. fasciculatum* with *Beijerinckia* sp. was same.

Table 12

Effect of *G. fasciculatum* and beneficial bacteria on NPK content (mg plant^{-5}) of *P. phaseoloides*

Treatments	Shoot			Root		
	N	P	K	N	P	K
G. fasciculatum alone	256	10.78	232	96	3.84	97
Azotobacter sp. alone	238	6.24	220	84	2.98	88
Beijerinckia sp. alone	225	5.16	218	80	2.24	80
Phosphobacteria alone	302	11.84	245	108	4.10	106
G. fasciculatum + *Azotobacter* sp.	368	14.38	320	126	4.12	119
G. fasciculatum + *Beijerinckia* sp.	343	13.06	305	108	4.10	112
G fasciculatum + Phosphobacteria	416	15.84	340	138	5.14	128
Control	65	2.76	98	22	0.92	38
CD (P = 0.05)	10	0.51	11	10	0.14	7

4.6 Screening of Plants for Mass Multiplication of *G. fasciculatum*

After 40 days of growth of *P. phaseoloides*, *S. bicolor*, *Z. mays* and *P. polystygon* (Plate 24) were examined for root colonisation and the results are given in Table 13. Maximum root colonisation by *G. fasciculatum* was in *S. bicolor*, followed by *Z. mays*, *P. polystygon* and *P. phaseoloides*. External hyphae, spore formation and development of vesicles and arbuscules were also more in *S. bicolor* treatment. Corresponding to root colonisation, there was more VAM spore population in soils supporting *S. bicolor*. Least spore count was in *P. phaseoloides* grown soils.

The vesicles, arbuscules and external spores with mycelia of *G. fasciculatum* are shown in Plates 25-27.

Plate 24 **Mass multiplication of *G. fasciculatum* in different host plants**

1. *P. phaseoloides*
2. *S. bicolor*
3. *Z. mays*
4. *P. polystygon*

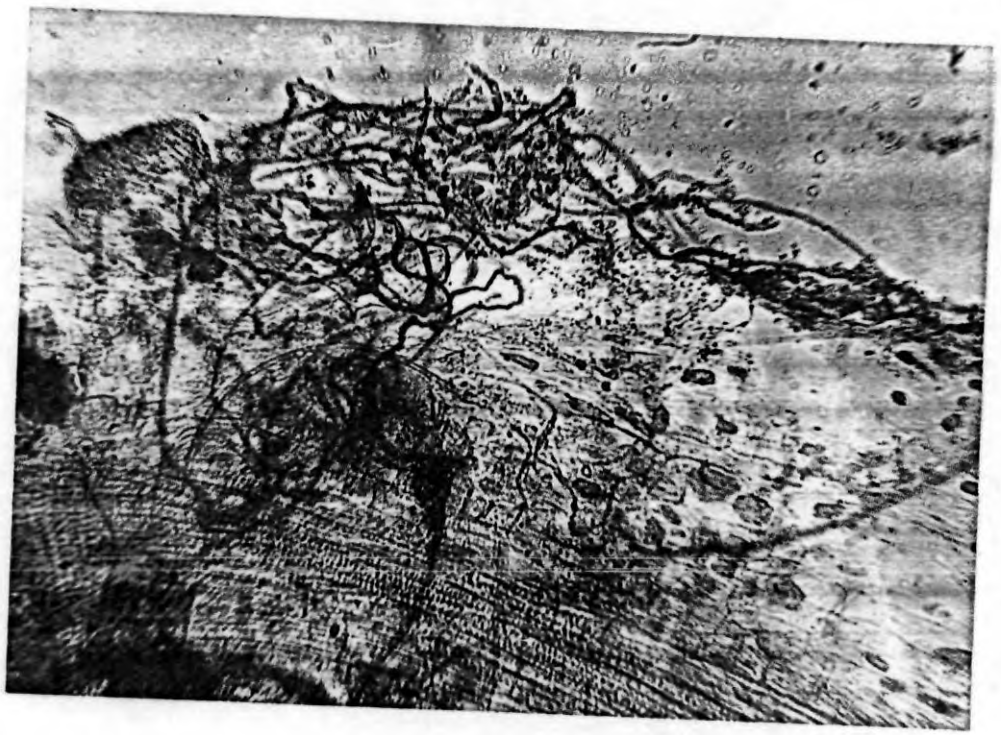

Plate 25 **Arbuscules and external mycelia of *G. fasciculatum***

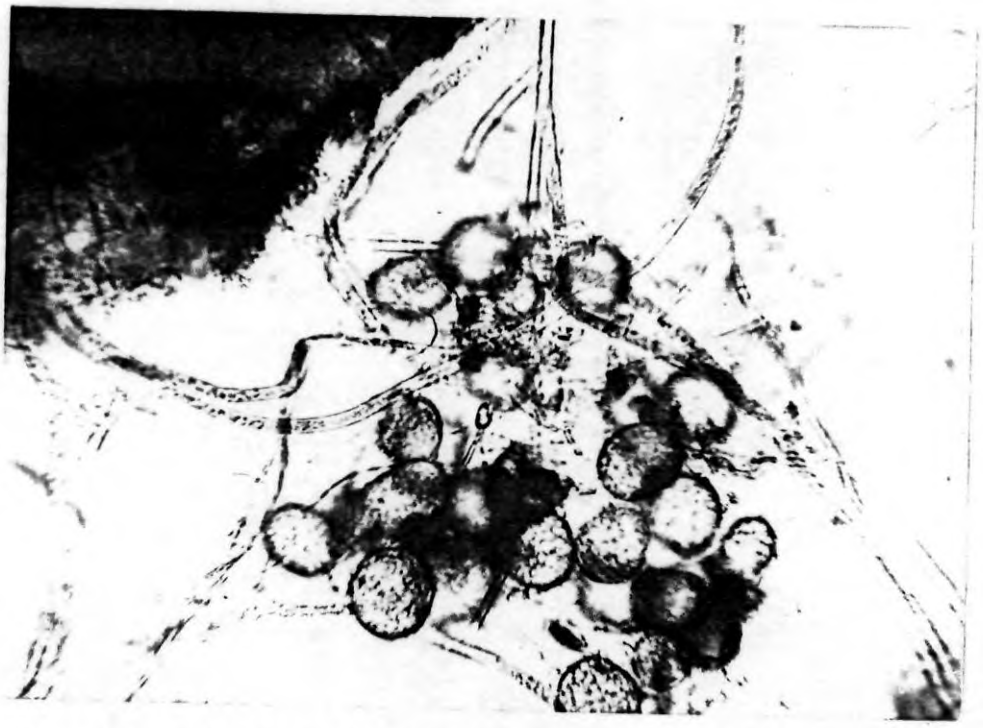

Plate 26 **Extramatrical spores of *G. fasciculatum***

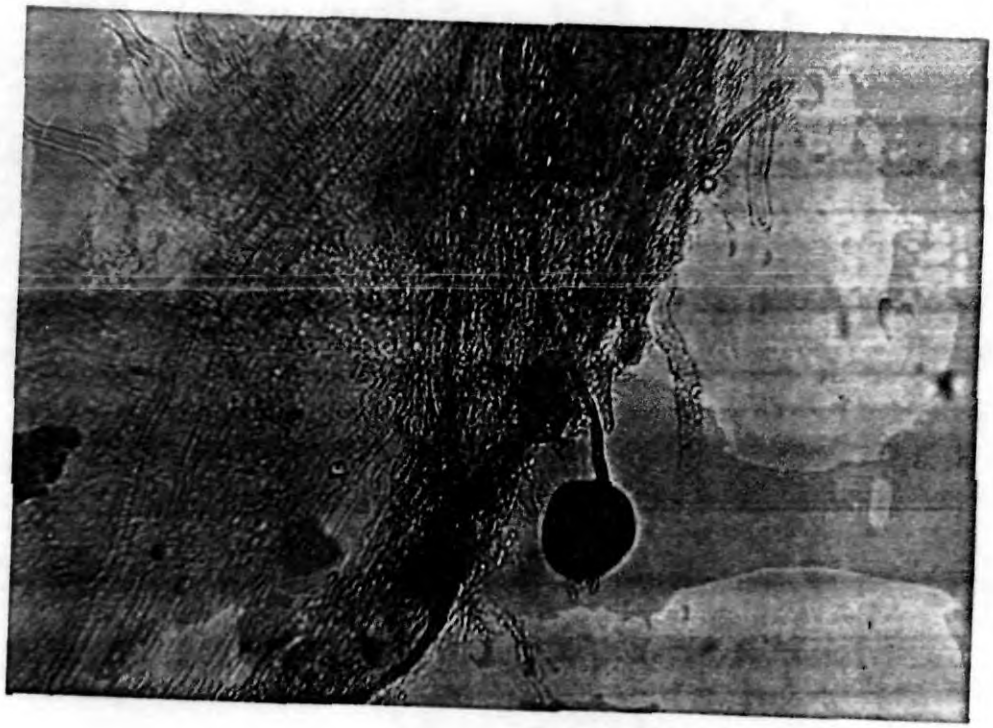

Plate 27 **Vesicles of *G. fasciculatum***

Table 13

Root colonisation and spore count after 40 days in soil upon *G. fasiculatum* inoculation on various host plants (Mean of 5 replications)

Treatments	Per cent infection	Spore count (Vermiculite soil mix ml^{-50})
P. phaseoloides	82	428
S. bicolar	92	882
Z. mays	88	826
P. polystygon	86	780

Chapter 5

Discussion

5 DISCUSSION

5.1 Distribution and Isolation of VAM

VAM association in *P. phaseoloides* and its role in biological nitrogen fixation is well established (Waidhyanatha *et al.*, 1979). However, there exists wide variation in the degree of VAM colonisation in roots and distribution of VAM spores in soil (Hayman, 1978). In the present study also such variations are recorded. A positive correlation was observed with respect to soil pH and VAM spore count. Palakkad soil having a pH of 5.8 contained maximum count of VAM spores while Kottayam soil the pH of which is 4.5 had the minimum spore count. A number of factors are considered to affect the population of VAM fungi in soil (Kruckelmann, 1975; Tarja Lehto, 1994) of which soil pH is believed to be most important factor (Bethlenfalvay, 1992). Kruckelmann (1975) also found that VAM spore population was more influenced by soil pH than by any other factors as observed in the present study.

All the 16 soil samples showed considerable variation in the species distribution of VAM fungi. *Glomus* spp. was comparatively more in all the soils. Rekharani and Mukerji (1987) while studying the species distribution of VAM fungi found that Indian soils are rich in *Glomus* spp. Soil moisture and temperature are also reported to determine the population densities of different species. *Glomus* spp., in general, prefer low temperature (18° to 20°C), soil moisture upto -31 bars. But *Gigaspora* spp. prefer a soil temperature of 25-35°C and affected even at -10 bars soil moisture (Koske, 1981). Low temperature and high moisture

prevailing in soils of rubber fields could be attributed to the difference in the population of *Glomus* spp. and *Gigaspora* spp.

Leguminous cover crops play a vital role in Hevea rubber cultivation due to their multifarious beneficial activities (Potty *et al.*, 1980). *P. phaseoloides* is the most popular crop in rubber plantation and its efficiency depends on the presence of optimum level of nutrients (Mathew *et al.*, 1978) and efficient prokaryotic microsymbiont, the *Bradyrhizobium* sp. VAM is another partner of the tripartite association in leguminous plants and it is considered to be essential for maximizing biological nitrogen fixation (Crush, 1974; Hayman and Day, 1978). VAM association in *P. phaseoloides* is well established and the latter fails to establish in the absence of compatible fungal partner (Waidyanatha *et al.*, 1979). It emphasises the need for investigating the distribution of VAM and using the efficient ones for maximum beneficial activity in *P. phaseoloides* which contributes substantially to the productivity.

5.2 Effect of Inoculation of Different VAM Isolates on *P. phaseoloides*

Comparative evaluation of the efficiency of different VAM fungi on *P. phaseoloides* revealed that all the VAM fungi promoted the growth of this cover crop and some species of VAM were comparatively more efficient in increasing growth and nutrient contents like NPK in tissues under sterile as well as unsterile conditions. The plants grown under sterile condition responded well to the VAM inoculation. In the absence of VAM the growth was stunted proving the role and importance of VAM in the establishment of *P. phaseoloides*. The failure of higher plants to grow normally in the absence of VAM was well established in many crops (Gerdemann, 1975; Hayman, 1978) especially under sterile condition. Compared to

unsterile condition, plants grown under sterile condition did not show much growth improvement irrespective of VAM species used. This might be due to better establishment of mycorrhizae in unsterilised soil. Soil bacteria are reported to influence the germination of spores of *G. epigaeus* and thereby increase VAM colonisation and growth of plants (Daniels and Trappe, 1980). Mosse (1981) also reported that in sterilised soil the native microflora has been destroyed and hence unfavourable for the germination of mycorrhizal spores and infection which is in confirmity with the findings of this study. Pot culture experiments conducted earlier in many crops using sterilised soils (Mosse and Hayman, 1971; Gerdemann, 1975) showed that VA mycorrhizal inoculation can drastically improve plant growth. Plant improvement under unsterile condition as observed in the present study was also reported by Mosse and Hayman (1971) and Bagyaraj and Manjunath (1980). Out of 11 selected VAM fungi, *G. fasciculatum* registered maximum beneficial effect on *P. phaseoloides* followed by *A. laevis*. The superiority of *G. fasciculatum* over other species in improving plant growth was well established (Kormanik *et al.*, 1982; Habte and Aziz, 1985). Under field conditions, VAM development is influenced by VAM species, plant under consideration and edaphic and environmental factors. VAM species vary considerably in their efficiency to infect and influence plant growth (Carling and Brown, 1980). In nature, a root system is typically colonized by more than one fungal species. Host response also differs with fungal species (Carling and Brown, 1980; Wilson, 1988) and with geographic isolate within a species (Bethlenfalvay *et al.*, 1989). The response range may also be due to changes in efficiency of different endophytes during the growing season (Daft *et al.*, 1981), to varying uptake of or exclusion capabilities of different fungi for different elements (Menge *et al.*, 1982; Victoria *et al.*, 1993) or even a change in soil environment itself during the season (Bazin *et al.*, 1990).

VAM inoculated plants, in general, and those inoculated with *G. fasciculatum* and *A. laevis* in particular show better growth parameters, nutrient uptake as well as higher biochemical constituents. The mechanism of improved plant growth caused by mycorrhizal inoculation has been investigated by many workers. Greater soil exploration by mycorrhizal roots as a means of increasing phosphate uptake is well established and the same phenomenon is also observed in the present study. They also improve the uptake of elements like Zn, Cu, S, etc. The other beneficial effects are their role in biological control of root pathogen, biological nitrogen fixation, hormone production and greater ability to withstand stress (Varma, 1979; Bagyaraj, 1984; Cooper, 1984). All these factors cumulatively influence growth of plants upon inoculation with selected mycorrhizal endophyte which vary from nothing to three-fold (Ruehle and Marx, 1979) and confirms the results obtained in this study.

The existence of a positive relationship between biomass production and per cent colonisation as observed in the present study, tends to uphold observations of many scientists that VA mycorrhizal colonisation enhances the plant growth through increased P uptake (Gerdemann, 1975; Hayman and Mosse, 1971; Tinker, 1975). The growth of *P. phaseoloides* is directly related to root colonisation, mineral uptake, nitrogen fixation and biochemical constituents. Plant macronutrients N, P and K have profound influence on the physiological activities and biomass production. In leguminous plants like *P. phaseoloides*, phosphates, potassium and trace elements contribute to the nodulation and nitrogen fixation by *Bradyrhizobium* sp. (Gibson, 1976; Munns and Mosse, 1980). The substantial increase in VA mycorrhizal colonisation and nodule production in *P. phaseoloides* ultimately contributed to enhanced N content leading to increased plant growth. Similar enhancement in nodule and biomass was observed by Bagyaraj *et al.* (1979) in

Physiological, chemical and physical mechanism of P uptake by VAM fungi were postulated, of which the most valid one is the hypothesis that hyphae ramifying into the rhizosphere from infected roots increase the effective P absorbing surface of the root by exploring larger volume of soil. It has been thought that this additional surface area and the distribution of P absorbing sites on the hyphae in the soil might wholly account for the superior absorbing capabilities of efficient VA mycorrhizal roots. Recent studies with tomato roots show that VAM root not only have more P absorbing sites, but also these sites on mycorrhizal roots have a greater affinity for P (Cress *et al.*, 1979). The difference in P uptake among different VAM fungi colonising *P. phaseoloides* observed in the present study might be due to the difference in root colonisation capability, mycelial extension and P absorbing sites.

All VAM fungi used in this study enhanced the concentration of sugars, amino nitrogen and phenols in *P. phaseoloides*. *G. fasciculatum* inoculated plants registered the maximum levels of these components in the tissues. Next to N, P is considered to be an essential nutrient and it plays a major role in the physiological activities of plants. Phosphate occupies a key position in the energy transfer (ATP-ADP) and promotes various activities in plants. The enhanced levels of sugars, amino nitrogen and phenols could be attributed to higher levels of P uptake as noticed in the present study. More the P uptake more is the quantity of various biochemical constituents. P uptake also indirectly increases the amino nitrogen level by augmenting the extent of nodulation and nitrogen fixation (Subba Rao and Krishna, 1988). The increased levels of sugars in tissue might be due to enhanced photosynthetic rate as well as larger area of leaf exposed to sunlight as evidenced by increased biomass and rapid growth in *G. fasciculatum* inoculated plants. This

finding is in confirmity with that of Nemac and Guy (1982) who observed enhanced level of sugars upon VAM infestation while Young *et al.* (1972) recorded increased amino acids.

Phenol is the another component, the quantity of which is influenced by VAM inoculation and the maximum was registered in *G. fasciculatum* inoculated plants. Enhanced phenol levels upon VAM colonisation as observed in the present study was also recorded earlier by Krishna (1981). Phenols that impart defence mechanism against the invasion of plant pathogens are synthesized by various biochemical pathways; sugars and amino acids serve as precursors for phenols synthesis via shikimic acid and acetate pathways (Stafford, 1974).

Photosynthesis is the primary physiological activity of plants and is influenced by many factors. Nitrogen either biologically fixed at nodule site or applied as manure/fertiliser ultimately contribute to increased photosynthetic rate (Lugg and Sinclair, 1981). In the present study, VAM inoculation caused substantial increase in chlorophyll content as well as the rate of carbon assimilation which could be attributed to the enhanced nitrogen in tissues under the influence of VAM. Similar observations were also made by Pang and Paul (1980) and Kucey and Paul (1982). *P. phaseoloides* inoculated with VAM, in general, and *G. fasciculatum* and *A. laevis* in particular promoted the absorption of potassium. In succession with nitrogen and phosphorus, potassium is given its full recognition as a fertiliser element. Evans and Sorger (1966) reported that potassium is the only univalent cation generally indispensable for all living organisms and it is involved in the activation of a number of enzymes. Potassium has a major role on photosynthesis which has been established with a wide range of higher plants (Moss

and Peaslee, 1965; Peaslee and Moss, 1966; Cooper *et al.*, 1967; Hartt and Burr, 1967). Obviously, the increased biomass upon VAM inoculation especially *G. fasciculatum* and *A. laevis* could be due to the enhanced level of chlorophyll and photosynthetic rate. *G. fasciculatum* inoculated plants having maximum photosynthetic activity are also rich in soluble sugars and amino acids indicating that such compounds originated from photosynthesis. These results of the present study clearly indicate the usefulness of VAM in *P. phaseoloides* establishment in rubber plantations.

The present study emphasises the need for the use of selected VAM fungi as there exists wide variations among different VAM isolates in influencing the growth of *P. phaseoloides*. Two isolates showing maximum beneficial effect in *P. phaseoloides* i.e., *G. fasciculatum* and *A. laevis* are considered for detailed studies of agronomical importance.

5.3 Effect of Inoculation of *G. fasciculatum* and *A. laevis* on Root Colonisation, Growth and Some Biochemical Constituents of *P. phaseoloides* at Different Intervals after Planting

It has been well established that VAM inoculation leads to enhanced root colonisation, growth, nodulation and nitrogen fixation in *P. phaseoloides* (Waidyanatha *et al.*, 1979). In the present study, VAM inoculation progressively increased root colonisation upto 50 days and throughout the experiment the infection per cent of VAM inoculated plants are higher than the uninoculated plants which get infected by native VAM fungi. Root colonisation irrespective of VAM inoculation steadily increased upto 50th day. Hayman (1970) and Sullia and Chandranath (1991) reported a steady increase of root colonisation by VAM fungi upto the end of the growing season in many cultivated legumes. However, a lag phase in root

colonisation and a three phase infection system was recognised by Sutton (1973) and Saif (1977). Sutton (1973) also noted an initial lag phase of 20-25 days in the case of field grown *Phaseolus* beans and soybean and such phenomenon was attributed to rapid growth of the seedlings and the time required for spore germination, germtube growth and penetration of the host plant root. In the second phase, lasting 30-35 days, extensive mycorrhizal development coincided with most shoot growth and copious spread of external mycelium leading to multiple infections which are in confirmity with the result of the present study. It seems likely that for the symbiosis to be established, molecular signaling events must precede to various physiological and anatomical changes in both the symbiosis during initial changes upto 10th day.

Growth and uptake of P and K in *P. phaseoloides* increased with age of the seedlings. Nodulation was recorded from 20th day of growth and increased progressively. Stimulation of the activity of *Rhizobium* which depended on adequate supply and uptake of P and VAM infestation was well established (Smith and Daft, 1977; Smith *et al.*, 1979). It is possible for the existence of such influence of P on *Bradyrhizobium* sp. in the nodulation of *P. phaseoloides*. Concomitant with the increase in nodulation there was an increase in the nitrogenase activity and nitrogen content in VAM inoculated *P. phaseoloides* plants which confirm the findings of Smith and Daft (1977) and Kucey and Paul (1982) who also made such observations.

VAM association not only augmented absorption of nutrients but also lead to considerable difference in biochemical constituents (Gianinazzi and Gianinazzi, 1984) of plants due to changes in the mineral nutrients as well as symbiosis. Important biochemical constituents influenced by VAM association are carbohydrates (Nemac and Guy, 1982), amino acids (Krishna and Bagyaraj, 1983)

and phenols (Krishna, 1981; Morandi *et al.*, 1984; Lakshmanan, 1987). In the present study, phenols, sugars and amino nitrogen content of VAM inoculated plants were more than the uninoculated control plants from the 20th day of inoculation and confirms the above findings. Invariably all the three treatments registered lower levels of phenols, sugars and amino nitrogen in the 20th day samples. The explanation for such phenomenon could be depletion of stored nutrients in the cotyledons during the initial establishment of seedlings (Bewley and Black, 1978; Mayer and Poljakoff Mayber, 1975) and switch over to the self reliance on photosynthesis. Noggle and Fritz (1986) also reported such decreased carbohydrate levels before the plants are supported by leaf photosynthesis and the uptake of water and inorganic solutes from soil.

Apart from this hypothesis for reduced sugars, amino acids and phenols, it can also be attributed to enhanced respiration (Hayman, 1982) due to VAM infection process. Increased respiratory activity of higher plants due to injury by pathogens or saprophytes is a common physiological phenomenon in higher plants (Goodman *et al.*, 1967; Brenneman and Black, 1979) and such activity takes place at the expense of energy reserves like sugars and amino acids (Akazawa and Uritani, 1962). Utilisation of simple carbohydrate and amino acids by VAM fungi seems to be yet another passive action for reduced level of sugars and amino acids on 20th day, the active stage of symbiosis (Bevege *et al.*, 1975). After 20 days of infection the level of sugars, starch, amino nitrogen and phenols showed an ascending trend irrespective of VAM inoculation. Similar trends were also made by Sutton (1973) and he hypothesised that such change was due to most shoot growth and copious spread of external mycelium leading to multiple infection. Young *et al.* (1972) observed an enhanced amino acid content in corn plants due to increased VAM infestation. Leguminous plants exhibit tripartite symbiotic association involving

VAM fungi and *Rhizobium*, a unique phenomenon which results in enhanced nitrogen fixation (Mane *et al.*, 1993). Nitrogen is the precursor for amino nitrogen and atmospheric nitrogen fixed by *Bradyrhizobium* sp. as evidenced by escalated nitrogenase activity and total nitrogen level in tissues contributed to amino nitrogen pool of *P. phaseoloides*.

The increased level of sugars, starch and amino nitrogen would be contributing to the formation of phenolics in *P. phaseoloides* as suggested by Neish (1964). Phenols are also synthesised in plants at the expense of amino acids, tyrosine and phenylalanine by the enzyme tyrosine ammonia lyase (TAL) and phenylalanine ammonia lyase (PAL) (Vance *et al.*, 1980) and forms yet another precursor for phenols in plants.

5.4 Effect of Different Levels of Rock Phosphate and Inoculation of *G. fasciculatum* and *A. laevis* on VAM Colonisation, Nutrient Content and Growth of *P. phaseoloides*

Phosphorus is one of the essential nutrients of plants and is doubly essential for leguminous plants as it is required for nitrogen fixation in large amounts (Munns and Mosse, 1980). One of the functions of VAM in plants is the absorption of soil P. Colonisation of plant roots by VAM fungi has been studied in relation to P nutrition (Bagyaraj and Sreeramulu, 1982). Such studies have clearly indicated that there is an inverse relation between root colonisation and P content in soil (Graham *et al.*, 1981; Schwab *et al.*, 1983). In the present study, increased application of rock phosphate beyond 50 per cent recommended level considerably reduced VAM colonisation. Baylis (1967), Mosse (1971), Azcon *et al.* (1978) and Miranda *et al.* (1989) have in fact shown that high P levels decreased and finally eliminated mycorrhizal infection from soil which are in confirmity with the present findings.

High soil P levels are known to result in root P concentration that may inhibit mycorrhizae and reduce external hyphae of VAM in soil (Sanders, 1975). Other workers (Abbott and Robson, 1977; Same *et al.*, 1983; Abbott *et al.*, 1984; Miranda *et al.*, 1989) observed a stimulating effect of low soil P application on root colonisation and they have suggested that the growth of VAM fungi may be influenced by the supply of P in soils naturally very deficient in this nutrient.

Spores, the infective propagules production is as important as root colonisation. VAM spore population in the present study, irrespective of VAM species, increased with the increase in root infection. Studies conducted in other crops in Australia and elsewhere (Mosse and Bowen, 1968) add additional support to the present observations.

Inoculation of *P. phaseoloides* with *A. laevis* and *G. fasciculatum*, significantly increased dry weight of shoot, nodule weight and nitrogenase activity in the absence of added phosphate and application of rock phosphate at 50 and 100 per cent of recommended dose. So also in the case of N, P and K. At all levels of rock phosphate addition, *G. fasciculatum* showed better growth, nodulation, nitrogenase activity and nutrient content than *A. laevis*. The species difference of VAM fungi in promoting the growth of plants at different levels of P is not uncommon. Plenchette *et al.* (1981) clearly proved the VAM species variation on growth of plants raised under different levels of P. The growth, nodulation and nutrient content of *P. phaseoloides* inoculated with VAM fungi at 50 per cent and 100 per cent of recommended dose of rock phosphate was almost same. Such observations with respect to growth and nutrient uptake especially P, K, Zn and Cu due to mycorrhizal inoculation at different levels of P application has been reported

by earlier workers (Hayman, 1980; Govinda Rao *et al.*, 1983; Chandrashekara *et al.*, 1995).

The degree of response of *P. phaseoloides* to VAM inoculation at 100 per cent recommended level of P was less when compared to 50 per cent P application. The decreased response to VAM inoculation at higher levels of P is an important point to be taken into consideration. Earlier studies also showed that plants infected with VAM are known to be more effective in the uptake of P from rock phosphate and soil low in available P than the uninoculated plants (Mosse, 1973). There is evidence that P from poorly soluble rock phosphate may be available only to mycorrhizal plants (Powell and Daniel, 1978). This is attributed to an increased effective surface area of P absorption in VAM which compensates for the low mobility of P in soil (Tinker, 1975). A factor undoubtedly important in fungal efficiency, but we do not measure, is the amount or intensity of hyphae distributed in the soil mix. Its external hyphae are correlated with internal hyphae (Tisdall and Oades, 1979), the difference among fungus species may be due to difference in volume of soil explored, since for the low P treatment, root colonisation was correlated with plant growth. Zones of P deficiency is estimated to be 1-2 mm around a root, would have taken place at low P, and mycorrhizal fungi may explore soil at least 8 mm distant from the roots (Rhodes and Gerdemann, 1975). This distance may vary if different fungal species produce different amounts of hyphae.

It is also observed in the present study that plants inoculated with *A. laevis* and *G. fasciculatum* showed enhanced nitrogenase activity and N content. *P. phaseoloides* being a legume, fixes atmospheric nitrogen in association with *Bradyrhizobium* sp. (Kothandaraman *et al.*, 1993). Biological nitrogen fixation is an energy consuming process and hence require more P. On an average 16 moles of

ATP are required for every mole of N fixed. The reduced rate of nitrogen fixation due to low level of P is well illustrated in *S. guyanensis* grown in very low phosphate (2 ppm Olsen P level) soil from Brazilian cerrado where the combination of *G. fasciculatum* and rock phosphate greatly increased nodulation and N_2 fixation in addition to P uptake and growth (Mosse *et al.*, 1976). This increased P uptake by VAM stimulated the activity of *Bradyrhizobium* which is well known to depend on an adequate supply of P. Studies with lucerne showed that the effects of mycorrhizae on nodulation and nitrogenase activity preceded those on growth (Smith and Daft, 1977; Smith *et al.*, 1979).

Taking into consideration the performance of different isolates in the pot culture studies and giving importance to the growth, biomass production, nodulation and nitrogenase activity, nutrient content and the extent of P fertiliser could be saved, the isolate of *G. fasciculatum* appears to be the most promising mycorrhizal fungus for *P. phaseoloides*.

5.5 Impact of *Azotobacter* sp., *Beijerinckia* sp. and *B. circulans* on Root Colonisation by *G. fasciculatum* and Growth, Nutrient Content and Rhizosphere Microbial Population of *P. phaseoloides*

It has been proved beyond doubt that VAM association of higher plants modify the saprophytic and pathogenic microflora (Tinker, 1982). VAM colonized roots have intimate contact with surrounding soil and subject to interaction leading to physicochemical reactions of soil, microbiological activities including plant pathogens (Sutton and Sheppard, 1976; Tisdall, 1991). Foster and Nicolson (1981) analysed microbial composition of soil aggregates in VAM inoculated soils and identified a range of fungi, bacteria, actinomycetes and algae including cyanobacteria.

In the present study inoculation of *Azotobacter* sp., *Beijerinckia* sp. and *B. circulans* with and without *G. fasciculatum* significantly increased root colonisation. Enhanced VAM colonisation of *P. phaseoloides* resulted in increased uptake of P and K, growth improvement, nodulation and nitrogen fixation and the importance of these activities are discussed earlier in Chapter 5.2.

In the absence of VAM inoculation *Azotobacter* sp., *Beijerinckia* sp. and *B. circulans* increased the natural VAM infestation. Under natural condition, several events take place in VAM colonisation including VAM spore germination, growth of mycelia through the soil, stimulation and attachment of infective hyphae to the root, penetration of the root, establishment of biotrophy and secondary hyphal streak outside and inside the root. Non-symbiotic nitrogen fixing bacteria and phosphate solubilising bacteria are reported to interact favourably with VAM fungi, either native or introduced at one or more of these stages (Bagyaraj and Menge, 1978; Mohandas, 1987; Brown and Carr, 1984). Such favourable activity of beneficial bacteria and VAM fungi were investigated in depth by Tilak *et al.* (1987a and 1987b). They isolated *Azospirillum* spp. from surface disinfected mycorrhizal roots of onion and from surface disinfected spores of *G. fasciculatum* and other species of *Glomus*. The increased VAM infestation in *P. phaseoloides* might also occur due to the production of plant hormones by free living N_2 fixers. The involvement of plant hormones, especially cytokinin in mycorrhizal development has been suggested by many workers (Allen *et al.*, 1980, 1982; Barea and Azcon-Aguilar, 1982) and the stimulation of VAM infection in *P. phaseoloides* and is growth by free living nitrogen fixers could involve hormonal interaction.

The enhanced VAM infestation due to bacterisation in control plants having native population of VAM fungi in tomato and alfalfa was reported by Azcon *et al.* (1978) which confirms the observation made in this study.

Dual inoculation of *P. phaseoloides* with *G. fasciculatum* and any one of the beneficial bacteria i.e., *Azotobacter* sp., *Beijerinckia* sp. and *B. circulans* registered higher rate of root colonisation and subsequent beneficial action than the corresponding bacterial inoculation treatment without VAM. It proves beyond doubt the synergistic action of these microorganisms with VAM in improving plant growth. Similar interactions have also been observed between *A. paspali* and VAM fungi in *Paspalum* (Barea *et al.*, 1973) and between *A. chroococcum* and *G. fasciculatum* in *Festuca arundinaceae* (Ho and Trappe, 1979) and lettuce (Brown and Carr, 1980). Manjunath *et al.* (1981) conducted a triple interaction study between free living nitrogen fixing bacterium *Beijerinckia mobilis* phosphate solubilising fungus *Aspergillus niger* and *G. fasciculatum* and found a synergistic beneficial effect on the growth of onion with all the three microorganisms.

The increased root colonisation by dual inoculation of VAM and phosphobacterium as observed in the present study has already been established by Barea *et al.* (1975), Pathiratna *et al.* (1990), Heggo and Barakah (1993) and Singh and Singh (1993). Barea *et al.* (1976) demonstrated that a very high proportion of the phosphate solubilising bacteria tested, produced IAA, GA_3 and cytokinin and they are considered to augment root colonisation by VAM fungi. It is needless to find reasons for the augmented growth, nodulation, nitrogen fixation and nutrient uptake in *P. phaseoloides* with more root colonisation by VAM under the influence of *Azotobacter* sp., *Beijerinckia* sp. and *B. circulans* as these activities were directly proportional to VAM colonisation. The increased growth, nodulation, nitrogen fixation and nutrient uptake by *P. phaseoloides* inoculated with VAM and beneficial

bacteria might be through increased VAM formation and direct action of these bacteria by nitrogen fixation, phosphate solubilisation and production of growth promoting substances.

Both *Azotobacter* sp. and *Beijerinckia* sp. inoculation with and without *G. fasciculatum* significantly increased growth, nodulation, nitrogen fixation and nutrient uptake. These two bacteria are well known non-symbiotic nitrogen fixers which improve plant growth (Bagyaraj, 1984). Bagyaraj and Menge (1978) studied the interaction between *A. chroococcum* and VAM fungus *G. fasciculatum* in tomato and found a synergistic effect on plant growth and nitrogen fixation. Such phenomenon might also have taken place in *P. phaseoloides* inoculated with *G. fasciculatum* and non symbiotic nitrogen fixing bacteria. The beneficial effect on plant growth by free living N_2 fixing organisms was attributed to hormone production, rather than, or in addition to nitrogen fixation as suggested by Bagyaraj and Menge (1978).

Among the various combinations, *G. fasciculatum* and phosphobacteria combination was found to be sound and improve the growth, nodulation, nitrogen fixation and nutrient uptake in plants as evidenced from the results of the present study. Raj *et al.* (1981) also reported similar effects while studying the dual inoculation of *G. fasciculatum* and phosphobacteria in finger millet. They also proved that the phosphate solubilising bacteria rendered more P soluble while VAM enhanced P uptake and concluded that with combined inoculation there was a synergistic effect on P supply and plant growth.

Dual inoculation with *G. fasciculatum* and phosphobacteria in *P. phaseoloides* resulted in more N content than the plants receiving *G. fasciculatum* and either of *Azotobacter* sp. or *Beijerinckia* sp. The augmented N content in the tissues of these plants did not coincide with the trend in nitrogenase activity. The

enhanced level of N might be due to increased absorption of N by the ramification of extramatrical mycelia as evidenced by increased root colonisation in this treatment. VAM usually increase the growth of plants solely by enhancing nutrient uptake (Abbott and Robson, 1984) by increasing site of nutrient absorption (Cooper, 1984). He also reported that nitrogen absorption by plants is directly related to VAM colonisation. The results of the present study is in line with these earlier findings. The phosphobacteria also reported to produce hormones (Barea *et al.*, 1976) and vitamins (Baya *et al.*, 1981) and such compounds might also contributed to the growth *P. phaseoloides* and subsequent physiological activity.

Corresponding to the increased VAM infestation and growth of *P. phaseoloides* under the influence of different beneficial soil bacteria there was an increase in the rhizosphere population of saprophytic bacteria, fungi and non-symbiotic N_2 fixers and the maximum being in dual inoculation. Such increase in microbial population is well established (Bagyaraj and Menge, 1978; Secilia and Bagyaraj, 1987) and the physiology of host plant is reported to be responsible for such changes. Rambelli (1973) and Linderman (1988) postulated that the photosynthetic rate increases and the partitioning of photosynthate to shoot and root changes. The nutritional status of the host tissue changes in response to altered uptake of minerals from the soil and this in turn can change structural and biochemical aspects of root cells that can alter membrane permeability and the quality and quantity of root exudation. Altered exudation changes the composition of microorganisms in the rhizosphere now appropriately called mycorrhizosphere.

Azotobacter spp. population is less in acidic rubber growing soils. Alexander (1967) also observed the absence or reduced population of *Azotobacter* spp. in soils of low pH. But upon its inoculation there was 86 x 10^2 bacterial cells

per gram of soil. In the presence of VAM fungi 23 per cent increase of *Azotobacter* spp. population was recorded and this shows selective compatibility of *G. fasciculatum* and *Azotobacter* sp. and has much agronomic importance in acid soil in which the population of latter was less. Population of *Beijerinckia* spp. in acid soil is also poor (Kothandaraman, 1979) as seen in the present study. But there is positive response upon inoculation with this bacteria and phosphobacteria with and without *G. fasciculatum*. However in the presence of *G. fasciculatum*, *Beijerinckia* sp. recorded maximum count. Endomycorrhizae in the roots are reported to have direct effect on the exudation of substances from the roots which in turn affect the distribution of microorganisms in the rhizosphere (Mukerji and Subba Rao, 1982). The root exudates contain numerous substances including sugars (Subba Rao, 1977). Kothandaraman (1979) observed enhanced growth of *Beijerinckia* spp. when simple sugars were made available. The observed increase in *Beijerinckia* spp. population could be due to the increase of sugars in the root exudates of *G. fasciculatum* inoculated *P. phaseoloides*.

Increase in phosphobacterial count upon VAM colonisation in association with phosphobacteria as observed in the present study was reported by Barea *et al.* (1975). The superiority of phosphobacteria over others in promoting the growth of *P. phaseoloides* might be due to their unique property of solubilisation of bound phosphate in acidic soil, the vital nutrient for nodulation, nitrogen fixation and growth.

Though there was considerable changes in the population of bacteria and fungi upon root colonisation of *P. phaseoloides* with *G. fasciculatum* with and without beneficial bacteria, the population of actinomycetes were unaltered. VAM fungi and soil actinomycetes are generally have an antagonistic effect on each other,

each suppressing the growth and multiplication of other in the rhizosphere (Krishna *et al.*, 1982). The unaltered and lesser population of actinomycetes might be due to the influence of VAM present in soil and introduced *G. fasciculatum*.

5.6 Screening of Host Plants for Mass Multiplication of VAM

Mass production of inoculum of VAM appears to be a bottleneck for detailed investigation of these fungi in promoting plant growth and field application. Since VAM fungi are obligate symbionts, they need living host for proliferation and the magnitude of multiplication differ with the plants under consideration. In the present study good root colonisation and VAM spore count in soil-vermiculite mixture was recorded when *S. bicolor* was used as host plant. Compared to other host plants tested *S. bicolor* produced more root biomass. High susceptibility of *S. bicolor* to VAM colonisation as well as the light weight of vermiculite soil mixture prove to be a better means of mass production of inoculum for various studies and distribution for farmers. Hayman (1982) also found such effect and included *S. bicolor* as one of the host plant for VAM bio-fertiliser production.

In the light of the results obtained from the present study, it is concluded that the growth and nutrient content of *P. phaseoloides* could be enhanced by dual inoculation with *G. fasciculatum* and either of *Azotobacter* sp., *Beijerinckia* sp. or phosphobacteria. This study also indicated the possibility of substantial reduction in the use of rock phosphate. *S. bicolor* is found to a better host plant for mass multiplication of *G. fasciculatum*.

Chapter 6

Summary

6 SUMMARY

This study essentially relates to the association of VAM fungi with *Pueraria phaseoloides*, the most commonly used cover crop for rubber. Natural rubber (*Hevea brasiliensis*) is generally grown in acid soils. Soils collected from different rubber growing regions in Kerala showed variation in pH ranging from 4.5 to 5.8. All the soils tested contained VAM spores. The spore count of VAM fungi varied from 214 to 428 in 50 ml of soil and it has a positive relation with changes in soil pH. The common genera of VAM fungi recorded in rubber growing soils include *Glomus*, *Gigaspora*, *Acaulospora* and *Sclerocystis*. The frequency of occurrence *Glomus* spp. was more followed by *Acaulospora* spp. and *Sclerocystis* spp. Only a few spores of *Gigaspora* spp. was recorded.

Thirty morphologically different VAM spores were selected and studied. Considerable differences were observed in their root colonising ability and in influencing the growth of *P. phaseoloides*. Eleven VAM fungi excelled in root colonisation and growth of *P. phaseoloides*. These eleven isolates, belonged to seven species of *Glomus*, two species of *Acaulospora* and one each of *Gigaspora* and *Sclerocystis*.

Two isolates of VAM fungi, i.e., *G. fasciculatum* and *A. laevis* were superior to others in their infectiveness and growth promoting capacity upon inoculation in *P. phaseoloides*, of which the former species was better than the later when studied under sterile and unsterile conditions. Inoculation of *P. phaseoloides* with these fungi led to enhanced growth, nutrient content, biochemical constituents and nitrogenase activity.

VAM infection manifested from the 10th day of inoculation with *G. fasciculatum* and *A. laevis*. The magnitude of root colonisation and spore count in the corresponding soil was maximum in *G. fasciculatum* treatment. Such plants also registered better growth and weight of both shoot and root, nodule number and weight, nitrogenase activity, NPK content and biochemical constituents like phenols, sugars and amino nitrogen. *G. fasciculatum* was closely followed by *A. laevis*.

The studies on the effect of rock phosphate on VAM development, growth and nutrient uptake upon inoculation with *G. fasciculatum* and *A. laevis* showed that application of 50 per cent of recommended dose of rock phosphate was optimum for maximising the beneficial effect for *P. phaseoloides*. The influence of VAM on various parameters was not much pronounced when the level of rock phosphate was beyond 50 per cent.

Studies on the interaction of *G. fasciculatum* with either *Azotobacter* sp., *Beijerinckia* sp. or phosphobacteria (*Bacillus circulans*) showed enhanced root colonisation by VAM fungi. Inoculation of any one of these microorganisms also showed a positive response in *P. phaseoloides* which was not as effective as dual inoculation with VAM. The VAM spore count also tended to increase by inoculation of *G. fasciculatum* in *P. phaseoloides* with and without the beneficial bacteria.

Concomitant to VAM root colonisation, there was an increase in all the growth parameters and nutrient content of *P. phaseoloides* in treatments involving either bacteria or VAM fungi. Dual inoculation was superior to individual inoculation with either *G. fasciculatum* or beneficial bacteria. All the treatments increased the microbial population comprising of saprophytic bacteria, fungi, nonsymbiotic nitrogen fixing bacteria and phosphobacteria. Actinomycetes population was not altered due to dual inoculation.

The levels of NPK in tissues of *P. phaseoloides* exhibited an increase upon dual inoculation. Inoculation with individual bacteria and VAM fungi also increased NPK contents, but not as marked as in the case of dual inoculation.

Among the four plant species tested for mass multiplication of VAM fungi, maximum root colonisation as well as VAM spore counts in soil-vermiculite mix was recorded in the case of *S. bicolor*. Hence mass multiplication of VAM is possible when soil and vermiculite are used for establishing *S. bicolor*.

The present study clearly indicates that rubber growing soils contain different groups of VAM fungi with varying potential. *G. fasciculatum* used in this study is efficient in root colonisation, growth stimulation and nutrient enrichment.

Application of rock phosphate at 50 per cent recommended dose is sufficient in plants inoculated with *G. fasciculatum* and the effect is comparable to that of 100 per cent rock phosphate application with VAM. *G. fasciculatum* is also compatible with other beneficial microorganisms and significantly improved the growth of *P. phaseoloides*. All these characters prove that *G. fasciculatum* is an ideal VAM fungus for use as biofertiliser in the establishment of *P. phaseoloides* in rubber plantations.

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Annexure

Annexure 1

Composition of Media Used

1. Congo red yeast extract mannitol agar medium for *Rhizobium*

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
$CaCO_3$	3.0 g
Yeast extract	0.5 g
Agar	15.0 g
Distilled water	1000 ml

2. Soil extract agar medium for bacteria

Soil extract	100 ml
K_2HPO_4	0.5 g
Glucose	1.0 g
Agar	15.0 g
Distilled water	900 ml
pH	7.0-7.2

1000 g of soil and 1000 ml distilled water were autoclaved for 1 h with a pinch of calcium carbonate. The extract was filtered and used for the preparation of medium.

3. Martin's rose bengal streptomycin agar medium for fungi

Dextrose	10.0 g
Peptone	5.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Rose bengal	30.0 mg
*Streptomycin sulphate (1% solution)	0.3 ml ml^{-100} of media
Agar	15.0 g
Tapwater	1000 ml
pH	5.5

*Streptomycin was added to the medium just before plating.

4. Kenknight's agar medium for actinomycetes

Glucose	1.0 g
KH_2PO_4	0.1 g
$NaNO_3$	0.1 g
KCl	0.1 g
$(NH_4)_2SO_4$	0.1 g
$MgSO_4.7H_2O$	0.1 g
Agar	15.0 g
Distilled water	1 litre
pH	7.2

5. Jensen's agar medium for *Azotobacter*

Sucrose	20.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.1 g
$CaCO_3$	2.0 g
NaCl	0.5 g
Na_2MoO_4	0.005 g
Agar	15 g
Distilled water	1000 ml
pH	6.5-7.0

6. Becking's agar medium for *Beijerinckia* sp.

Glucose	20.0 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4.7H_2O$	0.5 g
$FeCl_3.6H_2O$	0.05 g
$CaCl_2.2H_2O$	0.05 g
$MnSO_4.2H_2O$	0.005 g
$ZnSO_4.6H_2O$	0.005 g
$CuSO_4.5H_2O$	0.004 g
Na_2MoO_4	0.005 g
Distilled water	1000 ml
Agar	15.0 g
pH	6.5-6.9

7. Apatite agar medium for phosphobacteria

Yeast extract	0.2 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4.7H_2O$	0.1 g
KCl	0.2 g
Glucose	10.0 g
Soil extract	200.0 ml
Agar	15.0 g
Distilled water	800 ml
K_2HPO_4 (10%)	6 ml ml^{-100}
$CaCl_2$ (10%)	4 ml ml^{-100}

K_2HPO_4 and $CaCl_2$ were sterilised separately and poured to the medium just before plating.

Annexure 2

Preparation of Reagents

1. Folin-Ciocalteu reagent

Dissolved 100 g sodium tungstate ($Na_2Wo_4.2H_2O$) and 25 g sodium molybdate ($NaMoO_4.2H_2O$) in 700 ml water in 1 litre flask. Added 50 ml 85 per cent *ortho* phosphoric acid (H_3PO_4) and 100 ml conc. HCl and boiled under reflux gently for 10 h. Cooled and added 150 g lithium sulphate (Li_2SO_4) dissolved in 50 ml water and 4-5 drops of liquid bromine. Boiled the mixture without condenser for 15 minutes to remove the excess bromine. Cooled, diluted to volume with water and filter. The reagent, golden yellow in colour was stored in brown bottles. It is stable for many months. Just before use, dilute one volume of this stock solution with 2 volumes of water.

2. Reagent 'A'

In 800 ml of the glass distilled water 25 g of anhydrous sodium carbonate, 25 g of sodium potassium tartarate (Rochelle salt), 20 g of sodium bicarbonate and 200 g of anhydrous sodium sulphate were dissolved and diluted to 1 litre with glass distilled water.

3. Reagent 'B'

To 100 ml of glass distilled water, 15 g of copper sulphate and 1-2 drops of concentrated sulphuric acid were added.

4. Arsenomolybdate colour reagent

To 450 ml of glass distilled water, 25 g of ammonium molybdate, 21 ml of concentrated sulphuric acid and 3 g of sodium arsenate dissolved in 25 ml of glass distilled water were added and the mixture was kept in an incubator at 37°C for 48 h. The reagent was stored in a glass stoppered brown bottle.

5. Citrate buffer 0.2 M (pH 5.0)

Exactly 21 g of pure citric acid was dissolved in 200 ml of 1 N sodium hydroxide in a standard flask and the volume was raised to 500 ml with glass distilled water.

6. Ninhydrin reagent

To 500 ml of the citrate buffer at pH 5.0, 800 mg of hydrated stannous chloride was added. The solution was mixed with 20 g of recrystalised ninhydrin, dissolved in 500 ml methyl cellosolve as the solutions were mixed together. Fresh reagent was prepared on the day of use.

7. Diluent solution

Equal volume of glass distilled water and n-propanol were mixed and used.