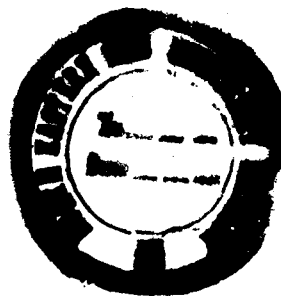


STUDIES ON INTERACTION BETWEEN VAM FUNGI AND FRANKIA IN NON-LEGUMINOUS TREE SPECIES (ALNUS Sp.) OF NORTH-EAST INDIA

MAHENDRA PRADHAN



THESIS SUBMITTED IN FULFILMENT OF
THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BOTANY

To



DEPARTMENT OF BOTANY
SCHOOL OF LIFE SCIENCES
NORTH-EASTERN HILL UNIVERSITY
SHILLONG (INDIA)
JUNE, 1993

RECEIVED, LIBRARY
Acc. No. 103147
Acc. by. M. P. Pradhan
Date 27/10/2000
Class by
Sub Reading by
Enter by
Presented by

per

DS
582.16095416
PRA

Grams ; NEHU

Phone : Off 23390

North - Eastern



Hill University

PROFESSOR R. R. MISHRA
M. Sc., Ph. D., F. N. A. Sc., F. N. I. E.

DEPARTMENT OF BOTANY
SCHOOL OF LIFE SCIENCES
SHILLONG - 793014

and

Dr. G.D. SHARMA
Reader.

C E R T I F I C A T E

We certify that the thesis entitled "Studies on interaction between VAM fungi and Frankia in non-leguminous tree species (Alnus sp.) of North-East India" submitted by **Mahendra Pradhan** for the degree of **Doctor of Philosophy** of the North-Eastern Hill University, Shillong, embodies the record of original investigation carried out by him under our supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of Ph.D. degree. This work has not been submitted for any degree of any other university.

G. D. Sharma

Signature of Supervisor

R. P. Mishra

Signature of Supervisor

Place: Shillong

Date : 25th July 1993.

ACKNOWLEDGEMENT

It is a matter of great privilege for me to express my deep sense of gratitude to Professor R.R. Mishra, F.N.A. Sc., F.N.I.E. and Dr. G.D. Sharma, Reader, Department of Botany, N.E. Hill University, Shillong for their expedient guidance, valuable suggestions and constant encouragements throughout the course of present investigation.

I would like to acknowledge my thanks to the Ex-Head Prof. Y.S. Chauhan, Prof. P. Tandon, Head, Department of Botany, NEHU, Shillong and other faculty members for their valuable suggestions.

My heartfelt thanks are due to all my friends and well-wishers, Dr. A.K. Shukla, Dr.S.C. Tiwari, Dr.S.R.Joshi, Mr. P.B. Gurung, Mr. Highland Kayang, Mr.Debashish Dutta, Mr.C.S. Rao, Mr. S. Jyrwa, Dr. Pratima Rao, Dr. A.L.S. Rajee, Dr. U.K. Sahoo, Mr. Bhawani Chamling, Mr. Neil Chhetri, Mr. A. Kharmawphlang, Mr. Patea, Kong Rit and other friends too numerous to name, for their wholehearted cooperation and encouragements.

I would like to acknowledge my gratitude to Mr. P.C. Rumba and Mrs. Leena Rumba, whose best wishes and constant inspirations bestow me this distinction.

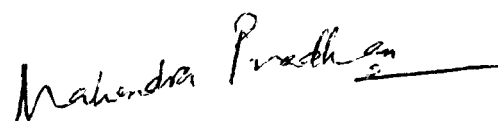
My thanks are also due to Mr. P.B. Das, Mr. B.K. Das, Mr. K. Sohlang, Mr. N.P. Choudhary and Mr. Saili for their respective contributions.

I am greatly indebted to my parents, brothers and sisters, without whose contributions, present distinction would not have been possible.

The financial assistance from The University Grant Commission (New Delhi) in the form of Research Fellowship is gratefully acknowledged.

Place : Shillong

Date : 25th July '93

A handwritten signature in cursive script, reading "Mahendra Pradhan", followed by a horizontal line.

(MAHENDRA PRADHAN)

CONTENTS

	Page
General Introduction	1
Review of Literature	11
Climate, Soil and Flora	76
Chapter I	79
Seasonal changes in the morphology and population of microsymbionts (<i>Frankia</i> and vesicular-arbuscular mycorrhizal (VAM) fungi associated with nitrogen fixation in <i>Alnus nepalensis</i> D. Don woodland.	
Chapter II	124
Isolation, cultivation and characterization of <i>Frankia</i> from root nodules of <i>A. nepalensis</i> D. Don growing in field conditions.	
Chapter III	153
Isolation and characterization of vesicular-arbuscular mycorrhizal (VAM) endophytes from <i>A. nepalensis</i> D. Don forest stand.	
Chapter IV	160
Interaction between <i>Frankia</i> and <i>Glomus mosseae</i> on P uptake, nitrogen fixation and seedling growth of <i>A. nepalensis</i> under various soil pH regimes.	

Chapter V	184
Interaction between <i>Frankia</i> and <i>G. mosseae</i> on P uptake, nitrogen fixation and growth performance of <i>A. nepalensis</i> seedlings under various soil organic matter levels.	
Chapter VI	206
Interaction between <i>Frankia</i> and <i>G. mosseae</i> on P uptake, nodulation, nitrogen fixation and their influence on seedling growth of <i>A. nepalensis</i> at different levels of rock phosphate amended soil.	
General Discussion	226
Summary	251
References	263

GENERAL INTRODUCTION

Soil represents one of the most dynamic living entities which harbour a variety of microbes like Protozoa, algae, fungi, bacteria, actinomycetes etc. Some of the microbes in course of time have evolved from saprophytic to specialized symbiotic forms eg: mycorrhiza, *Rhizobium* and *Frankia* which are directly involved in plant nutrition. The genus *Frankia* (Lechevalier and Lechevalier, 1980) belongs to actinomycetes and is able to fix atmospheric nitrogen in root nodules of non-leguminous (actinorhizal) plants (Callahan et al., 1978). So far 24 genera of woody non-leguminous angiosperms have been reported to form symbiotic association with *Frankia* (Dixon and Wheeler, 1986). Unlike legumes, actinorhizal plants however, possess a wide ecological tolerance which extends from temperate to subtropical and tropical zones of the globe. The potential biological nitrogen fixation by actinorhizal plants in forests has gained much attention in present days especially to minimize the use of costly inorganic nitrogenous fertilizers. Further, biological fixation of nitrogen by *Frankia* nodulated plants

represent a substantial contribution to the global nitrogen cycle (Torrey, 1978) especially in the forests, wetlands and abandoned fields of the temperate and tropical regions of the globe (Bond, 1976) which is comparable to leguminous system in agricultural ecosystems. Besides, the actinorhizal plants have recently been increasingly exploited for reclamation of hostile wastelands, (Perinet *et al.*, 1985; Wheeler *et al.*, 1986), softwood production (Leikola, 1976; Gordon and Dawson, 1979; Pregnet and Camire, 1985), short term forestry (Gordon *et al.*, 1979), improving soil fertility (Tarrant and Trappe, 1971; Mikola *et al.*, 1983; Dawson, 1986), nurture plants (Thiagalingam, 1983; Turner *et al.*, 1978), wind breaks (Geary, 1983; Midgley *et al.*, 1983) and stabilization of lands (Silvester, 1977; Midgley *et al.*, 1983).

Under the natural conditons however, it is difficult to visualize a habitat in which both nitrogen fixing and vesicular - arbuscular mycorrhizal (VAM) microendophytes exist in isolation. In majority of the instances their ecological niche overlap depending upon the availability of nutrients to the host and consequently resulting into continuous interactions among themselves and host plants. Similar to legumes, the root systems of some actinorhizal species have been reported to form symbiotic association with VAM fungi (Williams and Aldon,

1976; Williams, 1979; Rose, 1980; Clelland *et al.*, 1983; Gardner, 1986). *Frankia* and VAM fungi associated with non-leguminous plants ameliorate plant growth especially by supplying N and P to the host hence afford an ecological advantage over plants forming single symbiosis or without symbiosis. In fact, the plants forming tripartite associations are true pioneer species in colonizing nutrient-deficient and hostile habitats (Daft and Hacskeylo, 1976).

The role of mycorrhizal fungi in plant nutrition has received a great deal of attention which pertains to the mineral nutrition especially the uptake of non labile P. An adequate supply of P is important for satisfactory nodulation and nitrogen fixation by *Rhizobium* nodulated legumes (Demeterio *et al.*, 1972) which may be accomplished by mycorrhizal symbiont (Carling *et al.*, 1978). Besides, mycorrhizal fungi may also aid in the uptake of other elements like Cu, S, Zn and Mn (Starks, 1971; Mosse, 1973; Gray and Gerdemann, 1973; Mosse *et al.*, 1976; Kothari *et al.*, 1991) which may consequently lead to enhanced nodulation and nitrogen fixation by legumes (Crush, 1974; Daft and El-Giahmi, 1976; Smith and Daft, 1977). In non-leguminous system too, similar enhancement of nodulation, nodule activity and plant growth characteristics in response to VAM

symbiosis under nutrient deficient conditions has been observed (Rose and Youngberg, 1981; Gardner *et al.*, 1984; Russo, 1989; Jha *et al.*, 1993). The tripartite association involving non-legume-*Frankia*-VAM fungi represents one of the most complex systems in which each microsymbiont interacts between themselves and with the host plant. No information is presently available on the seasonal^{al} nitrogen fixation by root nodules in actinorhizal system involving VA mycorrhizal symbiont in terms of *in vivo* morphology of microendophytes (*Frankia* & VAM fungi) which may add to present meagre knowledge on *in vivo* interaction between endophytes under field conditions where an array of limiting factors is operational.

Direct effects of *Rhizobium* and VAM fungi on each other are evident (Bayne *et al.*, 1984; Bethlenfalvay *et al.*, 1985; Brown and Bethlenfalvay, 1987) and exhibit both nutritional as well as non-nutritional, host mediated effects (Harris *et al.*, 1985; Brown and Bethlenfalvay, 1987; Brown *et al.*, 1988). Since the demonstration of importance of VAM fungi in nitrogen fixation (Asai, 1944) most of the interest in the VAM fungi-*Rhizobium* has gained attention in the role of former in the acquisition of P under P stress (Barea and Azcon-Aguilar, 1983). This role is clearly important and may influence nodule activity either through the enhanced

nutritional status of the host plant (Cluett and Boucher, 1983; Carling *et al.*, 1978) or may directly or preferentially stimulate nodule function (Barea and Azcon - Aguilar, 1983; Van Nuffelson and Schenck, 1984). Further, the effect of *Rhizobium* on VAM fungi has been indicated (Pacovsky *et al.*, 1986) which may be mediated by production of polysaccharides by *Rhizobium* (Azcon-Aguilar *et al.*, 1980) or directly affecting metabolism of mycorrhizal fungus (Paccini *et al.*, 1988). However, in contrast to leguminous system, very little is known about the effect of VAM fungi on *Frankia* (Fraga-Beddiar and Tacon, 1990) and absolutely nothing on effect of latter on former.

Fixation of atmospheric nitrogen by actinorhizal tree species contributes a substantial amount of N to the forest ecosystems (Bond, 1967; Ferguson, 1971; Torrey, 1978) and a large proportion of latter is accreted to soil system by annual litter-fall (Tarrant and Miller, 1963; Zavitkovski and Newton, 1971; Debell and Radwan, 1978) thus contributing to existing soil organic matter (Tarrant and Miller, 1963; Bormann and Debell, 1981). This process may lead to elevated level of organic matter which may eventually influence the growth, survival, infectivity and effectivity of VAM fungi (Danielson and Visser, 1989) and *Frankia* microendophytes.

Besides, plant phenolics released from organic matter are known to affect *in vitro* growth and survival of both *Frankia* (Perradin *et al.*, 1983; Vogel and Dawson, 1985) and VAM endophytes (Pederson *et al.*, 1991). Presently no investigation is available on the effect of organic matter on growth, survival, infectivity, effectivity of endophytes and their interactions in tripartite association.

Soil pH has been known to affect survival and effectivity of symbiotic *Frankia* (Seilor and McCormick, 1982; Burggraaf and Shipton, 1982; Favre-Raynaud *et al.*, 1986; Smolander and Sundman, 1987; Smolander *et al.*, 1988) and VAM fungi (Kruckelmann, 1973; Graw, 1979). Further, the optimal pH requirement may also differ from microendophyte to microendophyte and between host plant and microendophytes. Effectiveness of tripartite association involving legumes may be severely affected by soil acidity (Newbould and Rangeley, 1984). But in non-legumes, studies in this field *are* lacking and warrant more endeavour. This is practically important as the supporting soil system is constantly becoming acidic due to rapid replenishment of exchangeable cations with increasing soil N content (Crocker and Major, 1955; Debell and Radwan, 1983) and may exert harmful effect on the growth, survival and effectivity of mycorrhizal symbionts (Danielson and Visser, 1989) which is important in terms of nutrients

uptake, nitrogen fixation and survival of host plants colonizing the degraded ecosystems. The increasing soil acidity may also influence growth, survival and nodulation by *Frankia* (Rodriguez -Barrueco *et al.*, 1968; Akkermans and Houwers, 1979; Smolander and Sundman, 1987) which is closely associated with N nutrition of pioneer actinorhizal species.

The importance of VAM fungi in P uptake by plants growing in infertile and high P-fixing soils (Mosse, 1973; Powell and Daniel, 1978) leading to improve plant growth is mainly due to capacity of mycorrhizal fungi to absorb and transfer P to host root beyond the depletion zone (Rhodes and Gerdemann, 1978; Li *et al.*, 1991). The ability of fungus in the uptake and efficient utilization of P from sparingly soluble (unavailable) rock phosphate sources under acidic conditions with consequential improved nodulation and nitrogen fixation by legumes is evident (Mosse *et al.*, 1976; Pairunan *et al.*, 1980). This is particularly important in tropics and subtropics in which many P-deficient soils occur in developing and underdeveloping countries where cost and rapid fixation of soluble fertilizers preclude their use. Besides, the increased application of soluble P may inhibit mycorrhizal colonization and spread thus affecting P nutrition which may be detrimental to nodulation, nitrogen fixation and growth of legumes (Asimi *et al.*, 1980).

Therefore, the application of rock P may serve as excellent alternative to soluble P fertilizers in order to alleviate plant productivity. In contrast to leguminous system,

- Studies involving non-legume-*Frankia*-VAM fungus symbioses, their interaction and overall effect on host plant growth under unavailable P source are unknown and warrant investigation which may be helpful in the future programmes on reclamation of nutrient-deficient habitats where P forms one of the major factor limiting plant growth.

North-Eastern region of the country which is rich in plant diversity is recently being threatened by continuous encroachment of forested areas, shifting cultivation (Jhum) and other human activities. However, the hills of the region are moist and favour development of variety of plant species among which actinorhizal species of *Alnus nepalensis* D.Don, *Myrica esculanta* Buch.-Ham.ex D.Don, *Coriaria nepalensis* Wall and *Elaeagnus* spp. grow luxuriantly on nutrient deficient soils and represent a dominant vegetation along with pine forest. *A.nepalensis* is more versatile, hardy and economically important species particularly colonizes hostile soil conditions of Eastern Himalaya. It fixes substantial amount of atmospheric nitrogen consequently improving N status of fragile forest ecosystem (Sharma and Ambasht,1984;

Sharma, 1988). However, investigation on characterization of two endophytes (*Frankia* and VAM fungi) of this region has not been accomplished although some information on VAM fungi from forested areas is available (Sharma *et al.*, 1986). These studies are important to alleviate productivity of actinorhizal tree species.

Keeping in view the above objectives, the present investigation has been carried out as outlined below :

- Seasonal study of microsymbionts (symbiotic *Frankia* and VAM fungi) morphology associated with seasonal changes in nitrogen fixation (nitrogenase activity) by *Alnus nepalensis* D. Don under field conditions.
- *In vitro* isolation, cultivation and characterization of *Frankia* and VAM fungal microendophytes from root nodules and forest soils supporting *A. nepalensis*.
- Investigations leading to interactions between *Frankia* and *Glomus mosseae* under various levels of :
 1. Soil pH,
 2. Soil organic matter and
 3. Unavailable phosphate source (Rock phosphate)

and their effect on nodulation, nitrogenase activity, P uptake and growth of *A. nepalensis* seedlings under controlled conditions, were carried out.

REVIEW OF LITERATURE

FRANKIA SYMBIOSIS :

First report of root nodules in non-leguminous plants was made by Mayen (1829) who observed that alder growing in deep shadow or proximity of water had a remarkable tendency to produce nodules.

Woronin (1866) observed inter-cellular hyphae which formed round swellings. He proposed the name *Schinzia alni* for the nodule parasite on alder.

Brunchorst (1886) concluded that a microbe was indeed involved in the nodule formation in actinorrhizae and proposed a new name to the organism, *Frankia subtilis*.

Moller (1890) proposed a new species *Frankia brunchostii* on the basis of cytological differences between the structures of microendophytes in *Ainus* and *Myrica*.

Shibata and Tahara (1917) provided evidences for an actinomycete-like organism in the actinorrhizal nodules, which was confirmed later on by Krebber (1932) and Schaede (1933, 1938 and 1962).

Mowry (1933) demonstrated the susceptibility of various species of *Casuarina* to the same nodule endophyte and carried studies in cross inoculation among four actinorrhizal plant species.

Ferguson and Bond (1953) provided evidences for the fixation of nitrogen in the root nodules of host inoculated with nodule suspension. Besides, nodulation occurred freely over pH range of 5.4 - 7.0 and subsequent better plant growth in the pH range 4.2 - 5.4. Further, higher oxygen requirement of root nodules was shown.

Ziegler and Huser (1963) showed that root nodules of *Comptonia peregrina* possess nitrogen-fixing ability and focused the role of *Comptonia* in improving the N status of forest soils in North America.

Employing isotopic ^{15}N as a tracer, Delwiche *et al.* (1965) revealed that excised nodules of *Ceanothus spp.* exhibited a strong ability to fix atmospheric nitrogen.

Investigations of Daly (1965) confirmed that *Alnus rugosa* was capable of contributing greatly to nitrogen level of the soil at a rate excess of 150 lb/acre/year.

Becking (1965) opined the possibility of geographical adaptations of the nodule endophyte.

Rodriguez-Barrueco (1966) found that *A. jorellensis* inoculated with nodule homogenate grew vigorously and fixed atmospheric nitrogen while plants inoculated with nodule suspension of *A. glutinosa* formed abundant nodules but fixed little or no nitrogen.

Gardner and Bond (1966) observed marked specialization of the nodule symbionts of particular host species occurred in *Myrica* much more than in *Alnus*.

Wheeler (1969) reported the occurrence of diurnal variation in nitrogen fixation in actinorhizal plants even under the constant temperature conditions. Wheeler (1971) demonstrated that extracts of nodules contain sucrose, glucose and fructose.

Angulo Carmona (1974) amply described the process of formation of an infection thread-like structure in *Alnus rubra*.

Becking (1970) proposed a sub-division of *Alnus* type endophytes into nine species with the generic name

Frankia based on cross-inoculation barriers between plant families.

Lalonde and Devoe (1975) reported polymorphic endophytes in the cells of root nodule enveloped by a membrane called host plasma-lemma. They added that the perforations through which endophyte penetrated the host cells may be a result of enzymatic degradation regulated by penetrating hyphae.

Lalonde and Knowles (1975) revealed that the endophyte capsule was composed mainly of granular and fibrillar non-sulphated, de-esterified polygalacturonic acid or its salts. They indicated that multivesicular bodies were responsible for the synthesis of capsule surrounding the endophyte.

Wheeler and Lawrie (1976) demonstrated that a very small fraction of ^{14}C accumulated in the infected cells of nodule indicating that carbon compounds arriving in these cells are rapidly utilized.

Van Dijk and Merkus (1976) showed that in spore⁺ nodules sporangia begin to develop at a short distance behind the nodule lobe apex in the region where vesicles, hyphae and nodule cells are no longer viable. Besides, they recognized

two types of nitrogen-fixing nodules in actinorhizal plants based on the extent of spore formation by endophyte. However, no difference in the tissue organization in the spore⁺ or spore⁻ nodules was noted.

Akkermans *et al.* (1977) and Van Straten *et al.* (1977) detected nitrogenase activity in the root nodule homogenate prepared from *Alnus*, *Hippophae*, *Shepherdia* and *Myrica* under anaerobic conditions when supplied with exogenous Mg - ATP and Na₂SO₄ as reductant.

Field studies of Johnsrud (1978) showed that annual rate of nitrogen fixation by *A. incana* reached as high as 434 Kg N/ha and was affected by temperature and light intensity.

Gordon and Wheeler (1978) indicated that nitrogenase activity of root nodules was determined both by host as well as *Frankia* genotype.

Torrey (1978) observed that *Frankia* strains isolated from *Alnus*, *Myrica* and *Comptonia* nodulated freely and established effective symbioses among themselves.

Mian and Bond (1978) demonstrated that onset of

103147



nitrogenase activity in young nodules of alder coincided with the production of symbiotic vesicles by the endophyte and concluded that vesicles were the site of nitrogen fixation.

Van Dijk (1978) concluded that the difference between root-nodules in actinorhizae with or without sporangia was caused by genetic differences of the nodule symbiont.

The first successful isolation of root-nodule endophyte from *Comptonia* was made by Callahan *et al.* (1978) employing enzymatic maceration and selective incubation. The filamentous bacteria grew slowly in yeast extract medium, induced actively nitrogen-fixing nodules and promoted plant growth vigorously without the exogenous supply of N.

Sprent *et al.* (1978) observed seasonal variation in the nitrogenase activity in *Myrica gale* which reached its peak during late July-August and declined in October.

Wheeler *et al.* (1979) demonstrated that acetylene reduction activity of alder was maximum at 15 - 30 % oxygen. They found high activities of peroxidase and O-diphenyl-oxidase in cell cytoplasm which showed no relationship with nitrogenase activity but with cellular senescence.

Van Dijk (1979) concluded that degree of nodulation depends on soil conditions and endophyte strain.

Roelofsen and Akkermans (1979) detected hydrogenase activity only in summer and concluded that it was confined to microsymbiont.

Akkermans *et al.* (1979) reported the property of vesicle clusters to reduce acetylene when incubated anaerobically in the presence of ATP and dithionite. They concluded that the final steps in the formation of citrulline takes place in host cytoplasm.

Hall *et al.* (1979) on the basis of growth response of alder species showed significant host genotype/actinomycete strain interactions.

Dawson and Gordon (1979) found significant correlation between leaf area, nitrogen fixation and between photosynthetic rate and nitrogen content of inoculated *A. glutinosa* plants. They indicated that genetic variations in the host as well as the source of the endophyte affected the rate of nitrogenase activity and photosynthesis in black alder.

Baker *et al.* (1980) demonstrated non-infectivity of strain EUII isolated from *Elaeagnus umbellata* on *Ceanothus* species.

Torrey and Tjepkema (1979) observed that in *Myrica* and *Comptonia* root hair infection was followed by pre-nodule formation thereafter secondary nodules were formed sparsely and in a precisely ordered sequence.

Benson *et al.* (1980) showed that hydrogenase activity in the vesicle clusters may be driven by a range of artificial electron acceptors.

Bhuwaneshwari *et al.* (1980) developed an inoculation technique to establish loci of the infection process by *Frankia*.

Ingestad (1980) found that a continuous supply of combined N in small doses stimulated both nitrogen fixation and growth of alder seedlings.

Akkermans and Roelofsen (1980) revealed that vesicle clusters suspension had higher nitrogenase and hydrogenase activity when incubated anaerobically and concluded that the fixed nitrogen is excreted as ammonia into host cytoplasm.

Houwers and Akkermans (1980) found that many soils devoid of alders had low numbers of endogenous *Frankia* or none at all. Further, they showed that nodule homogenate inoculum prepared from spore⁺ nodules was thousand times infective than those prepared from spore⁻ nodules.

Baker and Miller (1980) discovered the macrofossil of actinorhizal root nodule dating to about 11500 radiocarbon years old in North Vermont, U.S.A. From the qualitative differences observed among the root nodule endophytes, they concluded that the fossil nodules contained actinomycetous endophytes morphologically similar to that of extant *Elaeagnaceae*.

Baker et al. (1981) demonstrated that endophyte of *Alnus* and *Comptonia* exhibited similar whole cell sugar patterns and sero-group and were totally dissimilar to those exhibited by *Frankia* isolates from *Elaeagnus* group. They distinguished *Frankia* isolates into two serotypes; serotype 1 (isolates from *Alnus*, *Comptonia*, and *Myrica*) and serotype 2 (isolate from *Elaeagnus*).

Burggraaf et al. (1981) isolated *Frankia* from actinorhizal nodules by employing selective incubation coupled with double agar layer technique.

Akkermans *et al.* (1981) detected that a variety of enzymes associated with tricarboxylic acid cycle were active in vesicle clusters.

Normand and Lalonde (1982) reported the occurrence of diversity of *Frankia* isolated from actinorhizal plants of different provenances and suggested that spore⁺ or spore⁻ characteristics could contribute in establishing the taxonomy of the genus.

Schwintzer *et al.* (1982) observed the seasonality of nitrogenase activity of root nodules of *Myrica gale* and strong relationship between occurrence of vesicles and nitrogenase activity.

Shipton and Burggraaf (1982) showed that growth of *Frankia* isolates from various actinorhizal plants was severely affected by water potential.

Dillon and Baker (1982) evaluated symbiotic effectiveness between *Frankia* isolates from various actinorhizae and actinorhizal plants in terms of nitrogen fixing abilities. They concluded that efficiency of nitrogen fixation was mainly determined by compatibility of host and

Frankia strains.

Huss-Danell *et al.* (1982 a) suggested that glyoxylate cycle enzymes active in *Frankia* Avc II when grown in media with Tween 80 were repressed in the nodule symbiosis. Huss-Danell *et al.* (1982 b) concluded that a more complex mechanism of ammonium influence than a simply short term reduction in supply of carbon compounds to the nodules may occur.

Burggraaf and Shipton (1982) showed that optimal temperature requirement for growth of most *Frankia* isolates was between 30-35° C.

Ching *et al.* (1983) revealed that symbiotic *Frankia* from *Alnus spp* possess aerobic metabolism by virtue of presence of cytochrome system.

Dixon and Wheeler (1983) reviewed the effect of abiotic environmental factors on nitrogen fixation by actinorhizal plants.

Employing crushed nodule inoculum, Coyne (1983) noted inter-genetic specificity in the family *Casuarinaceae*

for host-endophyte relationships.

Shipton and Burggraaf (1983) reaffirmed that optimal growth of *Frankia* isolates from *Casuarina*, *Hippophae* and *Myrica* occurred at 30°C. Growth was generally favoured at pH 6 and 8 and declined below -6 bars water potential.

Quispel *et al.* (1983) made the comparison of lipid requirement of some *Frankia* isolates and concluded that root lipid extracts from alder were essential for the isolation of *Frankia* from *Alnus glutinosa*, *Hippophae rhamnoides* and *Myrica gale*.

Perradin *et al.* (1983) observed that ferulic acid, O-coumaric acid and β -coumaric acid were effective in inhibiting growth and decreasing the number of *Frankia* sporangia. They suggested that these active phenolic compounds present in actinorhizae might act as "chemical mediators" between host cells and symbiotic *Frankia*.

Based on DNA homology *Frankia* strains were distinguished into two genogroups i.e. those with relatively high homology to strain Ar 14 and those with low homology to strain Ar 14 (An *et al.*, 1983).

Benson and Hanna (1983) distinguished 43 alder

isolates on the basis of protein patterns of whole cell lysates.

Granhall *et al.* (1983) demonstrated that repeated application of inorganic N did not suppress nitrogenase whereas an initial large N application effectively inhibited nodulation and nitrogenase activity and finally concluded that mode of N application was more important than the total amount applied.

Tisa *et al.* (1983) found that *Frankia* isolates EAN 1 pec, Cp11 and ACN 1⁹ utilized propionate for growth except *Eu11c*. The size and number of vesicles were greater in media without ammonia and the sporulation of all isolates was C-source dependent.

Akkermans *et al.* (1983) observed that *Frankia* cells grown with tween 80 or acetate as C sources contained glyoxylate cycle enzymes but repressed in media with propionate as well as in symbiotic stages in nodules.

Knowlton and Dawson (1983) revealed that infection of *A. rubra* root by *Frankia* isolates doubled the number of nodules in the presence of *Pseudomonas cepacia*. Further, they observed the interaction between seedling,

substrate pH and bacteria in the promotion of nodulation.

Burggraaf and Valstar (1984) concluded that host plant selects *Frankia* strains during infection and nodule formation.

Baker and O'Keefe (1984) isolated *Frankia* from the root nodules of actinorhizal plants and soil samples using sucrose density fractionation and simple N- free medium.

Moiroud *et al.* (1984) showed the inability of *Frankia* isolates to grow at temperature below 15°C irrespective of their origin from cold areas. However, according to them, *Frankia* remained viable even at low temperature.

Seiler and Johnson (1984) observed that moisture stress greatly reduced shoot, root, nodule and total dry weight and nitrogenase activity of actinorhizal plants.

Lechevalier and Ruan (1984) demonstrated the diversity of *Frankia* strains isolated from *Comptonia* and *Ceanothus*.

Murry *et al.* (1984) showed that propionate was

metabolized via the propionyl-coenzyme A pathway. Further, propionate was considered most efficient carbon source in the induction of nitrogenase activity by *Frankia*.

Sharma and Ambasht (1984) reaffirmed the seasonality of nitrogen fixation in root nodules of *Alnus nepalensis*.

Studies of Van Dijk (1984) showed the presence of *Frankia* from areas where host plants are growing and its absence in areas without a living host plant.

Gauthier *et al.* (1984) suggested that there may be a continuum between typical strains of Rhamnales and some strains of Casuarinaceae.

Horriere (1984) demonstrated significant variation in the abilities of *Frankia* strains to produce protease, urease and β -glucosidase.

Lechevalier (1984) categorized *Frankia* isolates from various actinorhizae into two physiological categories.

Hafeez *et al.* (1984) isolated two *Frankia* strains from root nodules of *Alnus nitida* from North Pakistan.

The result of Carpenter *et al.* (1984) indicated

that both host and endophyte genotype may affect the efficiency of host-endophyte symbiosis.

Lopez and Torrey (1985) showed the presence of several tricarboxylic acid cycle enzymes in the vesicle clusters.

Vander Bosch and Torrey (1985) opined that the ability to form sporangia in symbiotic stage is controlled by endophytic strain rather than host genotype or host-endophyte combination.

Reddell and Bowen (1985) revealed that more than one *Frankia* strain may coexist in same nodules.

Jiaben et al. (1985) concluded that *Frankia* isolates from *Alnus* and *Elaeagnus* show considerable variation and divided them into two groups.

An et al. (1985) revealed that restriction fragments banding patterns were unique for each *Frankia* isolate and suggested that this method was efficient in distinguishing morphologically indistinguishable isolates.

Meesters et al. (1985) showed that most of the nitrogenase activity was located in the vesicles of the

nodule endophyte.

Vogel and Dawson (1985) observed that none of *Frankia* isolates had the ability to utilize phenolic acids as carbon sources.

Vernaud *et al.* (1985) developed a new spot inoculation technique for the nodulation of host close to the point of inoculation.

Weber (1986) after surveying several alder stands in Finland concluded that both spore⁺ and spore⁻ nodule types co-existed in the alder forests.

St. Laurent and Lalonde (1986) successfully isolated 30 *Frankia* isolates from root nodules of *Myrica gale* and characterized them on the basis of morphological and biochemical criteria. They observed that 12 strains were infective on original host and *Alnus glutinosa* while 5 were ineffective but effective in *Elaeagnus* and *Hippophae*.

Righetti *et al.* (1986) studied the effect of soil moisture, P, K and micro-nutrients in the nodulation of actinorhizal plants.

Faure-Raynaud *et al.* (1986) showed that growth of most of the *Frankia* isolates was pH dependent.

Huss-Danell and Frej (1986) recorded *Frankia* in soils devoid of host plants for decades.

Wheeler *et al.* (1986) reported that both *Alnus rubra* and *A. glutinosa* were equally effective in return of N to the ecosystem. Further, the isolated *Frankia* from *A. rubra* or *A. glutinosa* varied morphologically and in growth characteristics.

Sellstedt (1986) found no difference in plant biomass between nitrogen fixing and nitrate fed alders.

Sundstrom and Huss-Danell (1987) revealed that nitrogenase activity of actinorhizal plants was severely affected by drought stress.

Immuno-gold labeling studies conducted by Meesters (1987) showed that nitrogenase activity was located in *Frankia* vesicles but not in hyphae.

Vikman and Huss-Danell (1987) found that cyanide inhibited oxygen consumption in vesicles, ^{but} was not affected by salicylhydroxamic acid, an inhibitor of alternate respiratory pathway indicating that *Frankia* has an aerobic metabolism.

Torrey (1987) proposed that the environment within the nodule determines the formation of spores.

Mackay *et al.* (1987) and Sheppard *et al.* (1987) observed that mineral N and soil factors may influence the host-*Frankia* combination.

Parsons *et al.* (1987) concluded that outer wall of *Frankia* vesicles was thickened by multiple liquid-like monolayers in proportion to partial ambient oxygen level.

Guofan and Tingxiu (1987) observed that soil pH and calcium carbonate were the main variables influencing nodulation and nitrogen fixation in purple coloured soil.

Tisa and Ensign (1987) revealed the indispensable role of calcium in both vesicle development and nitrogenase activity by *Frankia* strains isolated from *Elaeagnus* and *Ceanothus*. Further, they suggested that cyanide insensitive respiration may be involved in protecting nitrogenase inside the vesicles from oxygen.

Smolander *et al.* (1988) demonstrated that survival of *Frankia* in soil at pH > 6.0 was better than at pH

< 4.5. [^]Smolander *et al.* (1986) concluded that the stimulatory effects of clay on *Frankia* may be due to adsorption of inhibitory compounds secreted by alder.

Huss-Danell and Hahlin (1988) showed that addition of ammonium to alder strongly reduced ¹⁴C content in vesicle clusters.

Lalonde *et al.* (1988) demonstrated that *in vitro* sporulation by *Frankia* was determined by the endophyte strain infecting the host.

Harrison and Seilor (1988) showed that direct moisture stress exerted no effect on acetylene reduction and nodule sugar content.

Huss-Danell *et al.* (1989) observed a strong correlation between nitrogenase activity in alder and number of hours of sunshine under field conditions.

Murry and Lopez (1989) found that in pure cultured *Frankia* hydrogen stimulated hydrogenase activity at supra optimal oxygen concentrations.

Rytter (1989) noticed that coarse roots of alder

were found close to the trunk of trees while fine roots were evenly distributed in the stand. Further, he attributed 90 % of the below ground biomass in the uppermost soil profile to availability of soil oxygen in peaty soil.

Hahn *et al.* (1989) proposed a new definition of the family Frankiaceae based on RNA catalogs and reverse transcriptase sequencing.

Simonet *et al.* (1989) delineated 5 closely related *Frankia* from alder hosts on the basis of presence of plasmids and southern hybridization to a nif AB probe or to a cryptic *Frankia* probe.

Berry *et al.* (1989) identified five Indole compounds secreted by *Frankia* under defined cultural conditions by using gas chromatograph and mass spectrometry technique. Their result indicated that utilization of indole-3 - ethanol by plants led to modification of pattern of root primordium initiation.

Seguin and Lalonde (1989) detected pectolytic activity in the nodule and culture filtrates of some *Frankia* strains.

Hennessey *et al.* (1989) concluded that nitrogenase

activity was limited by xerotolerance of host rather than that of endophyte.

Observations of Tjepkema and Murry (1989) revealed that nodules of *Casuarina* possess a barrier to oxygen diffusion as supplied by host tissue and not by symbiotic *Frankia*.

Dobritsa and Stupar (1989) observed multiple infection of actinorhizal plants with various *Frankia* strains and also the presence of more than one strain in a single nodule.

Kohls and Baker (1989) concluded that the inhibition of nodulation by nitrate in actinorhizal plants resulted from suppression of root hairs.

Abeysekera *et al.* (1990) suggested that symbiotic *Frankia* obtain sugar via degradation of pectic substances or polysaccharides which are constituents of capsule surrounding *Frankia* in alder hosts.

Observation of Huss-Danell and Bergman (1990) proved that onset of nitrogenase synthesis in vesicles occur at certain stages of the vesicle development.

Kurdali *et al.* (1990) suggested that both spore and spore⁻ *Frankia* strains coexist in the same root system however, spore⁻ exhibited greater specific nitrogenase activity than that of spore⁺ *Frankia* strains. They concluded that competitive ability varies with strains and that the effectiveness is affected even if nodulated plants are transplanted into soil.

Faure-Raynaud *et al.* (1990) reported the diversity of *Frankia* isolates from nodules of *Alnus rubra* by comparing the protein and other enzyme patterns.

Lundquist and Huss-Danell (1990) assigned the loss of nitrogenase activity of intact plants to the deactivation and reduced level of enzyme in the *Frankia* vesicles.

Lundquist and Huss-Danell (1991) proved that the loss of nitrogenase activity in actinorhizal plants subjected to darkness was largely due to loss of Mo-Fe protein in the symbiotic vesicles of *Frankia*.

Kurdali and Domenach (1991) reported that the rate of nodulation of host was affected by nutrient supply and *Frankia* population, which decreased with soil depth.

Maneaur and Torrey (1971) concluded that root exudates of hosts facilitate the infection process by stimulating germination of *Frankia* spores.

Perry *et al.* (1991) investigated the ultrastructural details of nodules of *Cowania subtintegra* and noted that endophytic actinomycete was present in host cortical cells in the form of septate hyphae and elliptical vesicles but not in sporangial form.

Investigations of Bosco *et al.* (1992) revealed that *Frankia* strains isolated from an *Elaeagnus species* were able to cross the inoculation barrier and infect *Alnus species* as well.

VESICULAR- ARBUSCULAR MYCORRHIZAL (VAM) FUNGAL SYMBIOSIS :

Reissek in the year 1849, for the first time described the presence of a septate fungus in the roots of orchid, *Neottia nidus*. However, the identity and the significance of fungus was not recognised.

Frank (1885) introduced the term "mycorrhiza" to designate symbiotic association of plant root and the fungus. He divided mycorrhiza into two broad categories, *ie.* ectotrophic and endotrophic.

Garrett (1950) remarked that the mycorrhizal fungi have evolved from aggressive parasites by progressive selection of non-lethal varieties so that symbiotic stage became indefinitely prolonged.

Mosse (1953) observed hyphal interconnection between fungal sporocarp and strawberry roots. Mosse (1956, 1957) successfully established mycorrhizal infection by using surface-sterilized spores / sporocarps and observed better growth of the mycorrhizal plants.

Gerdemann and Nicolson (1963) were able to isolate endogonaceous spores from soil employing wet sieving and

decanting technique.

Baylis (1967) reported phycomycetous mycorrhiza in several tree species including gymnosperms from a rain forest in New Zealand. Baylis (1967) also justified the ecological significance of mycorrhiza in forest where P availability was low.

Mosse and Bowen (1968) published a key for the identification of different spore-types based on the characteristics of cytoplasm, wall structure of spore, presence or absence of attached hyphae, nature of attachment and colour of spores.

Hayman (1970) found that mycorrhizal infection and endogone spores population in wheat were influenced by seasons and soil treatments. Hayman (1970) observed maximum number of spores and highest mycorrhizal infection during summer.

Phillips and Hayman (1970) developed a technique to clear roots in KOH solution and staining in trypan blue for the rapid assessment of mycorrhizal infection in roots.

Hayman and Mosse (1971) observed significantly large increase in the shoot dry weight in onion and *Caprosoma*

plants inoculated with *Endogone* under P-deficient soil.

Daft and Nicolson (1974) reported the mycorrhizal association in plants growing on coal strips. They found infections in those plants to be heavy and stressed the ecological significance of mycorrhizae in such nutrient deficient harsh habitats.

Gerdemann and Trappe (1974) published a monograph on the family Endogonaceae with segregation of genus *Endogone* into seven distinct genera and provided the detailed description of the species found in north-west Pacific.

An experiment of Rhodes and Gerdemann (1975) revealed that the absorption of P and its translocation to host by hyphae of *Glomus fasciculatum* which extended beyond the P depletion zone of mycorrhizal onion to at least 7 cm from the root surface.

Redhead (1975) studied the effect of ecological factors on mycorrhizal association of *Khaya sp.* and concluded that environmental variables like moisture level, light and nutrient supply exerted influence on infection and sporulation by VA mycorrhizal fungus.

Powell (1975) using labelled P, found no significant difference in specific activities of absorbed P in mycorrhizal and in non-mycorrhizal plants.

Kinden and Brown (1975) investigated on the ultra-structure of VA mycorrhizal root of ^{yellow}poplar and showed the occurrence of inter and intra-cellular vesicles. They (1975) further demonstrated the formation of intra-cellular haustoria-like arbuscules in the host root cells.

Khruscheva (1977) found that P and N decreased the occurrence of vesicles whereas addition of K to N and P fertilizer increased its presence in wheat. The addition of lime to NPK fertilizer proved beneficial for mycelial growth but decreased the process of vesicles formation. He finally concluded that vesicle formation was dependent on mineral metabolism and physio-biochemical processes within the host plant.

Becker and Gerdemann (1977) described a colorimetric method for quantification of VAM based on yellow pigments in infected roots in onion.

Sanders *et al.* (1977) found that out of four VA endophyte inoculated on onion, three enhanced the growth, whereas one showed no growth response.

Abbott and Robson (1977) investigated the distribution and abundance of different VA endophytes in cultivated soils of Australia. They (1978) evaluated the effect of three introduced VAM fungi in field soil infested with four indigenous VA endophytes and found that introduced endophytes differed in their ability to increase growth and P content of subterranean clover which were related to rate of mycorrhizal development when limited by P supply.

Cooper and Tinker (1978) by means of radioactive and split plate culture techniques observed that the amount of P, S and Zn translocated was in the ratio of 35:5:1 whereas the mean influx in the hyphae was in the ratio of 50:8:1.

Rhodes and Gerdemann (1978) studied translocation of Ca and P using tracer technique. They found that mycorrhizal root can explore P and Ca at a greater distance from root surface than non-mycorrhizal one. They added that Ca is translocated to roots through mycorrhizal hyphae less rapidly than P.

Ratnayake *et al.* (1978) proposed a hypothesis that P inhibition of mycorrhizal symbiosis is associated with a membrane mediated decrease in root exudation.

Menge *et al.* (1978) proved that concentration of P was the regulatory factor in the determining colonization, infection and spore production by VAM fungi.

MacDonald and Lewis (1978) reported acid phosphatase activity in and growing fungal structure of *Glomus mosseae*.

Powell and Daniel (1978) succeeded in introducing *G. tenuis* into the soil already infested with the indigenous mycorrhizal fungi and was the most efficient fungus in stimulating P uptake. Mycorrhizal plants were more efficient in recovering added P fertilizers than their non-mycorrhizal counterpart.

Gianinazzi-Pearson and Gianinazzi (1978) reported a close correlation between the mycorrhiza specific phosphatase activity and development of the infection and host growth.

Carling *et al.* (1979) found that the number of infection units in young soyabean seedlings increased with the increase in *Glomus fasciculatum* inoculum till a maximum was attained.

Skipper and Smith (1979) observed that overall cultivar response in unlimed soil at pH 5.1 was greater for *Gigaspora gigantea* and at pH 6.2 for *Glomus mosseae*.

Graw (1979) observed that *Tagetes minuata* and *Guizotia abyssinica* reacted differently to changes in soil pH and to fertilization with various P in spite of colonization with same strain of *Glomus macrocarpum*.

Dichotomous synoptic keys for the identification of mycorrhizal fungi were published by Hall and Fish (1979).

Gianinazzi *et al.* (1979) observed a significant acid phosphatase activity in little vacuolated, immature terminal arbuscule whilst strong alkaline and α -naphthyl phosphatase and β -glycerophosphatase activity was localized within vacuoles of mature arbuscules and intercellular hyphae. Further, in the root cells neither acid nor alkaline phosphatase distribution was found to be modified by mycorrhizal formation.

Owusu-Bennoah and Wild (1979) found that inoculation with *Glomus mosseae* increased seedling growth and P content in both irradiated or non - radiated soils. Successive autoradiographs showed that for the mycorrhizal

and nonmycorrhizal roots, most P uptake was within 0.2 and 0.1 cm respectively of the root surface.

Hasel-Wandter (1979) found a close relationship between VAM infection and host vigour. She observed that infection level increased with decrease in altitude of sampling site.

Mishra and Sharma (1979) studied the mycorrhizal status of ~~the~~ forest trees and distribution of *Endogone* species in the humid forests of Meghalaya.

Williams (1979) identified spores of three VA fungal species i.e. *Glomus*, *Gigaspora* and *Acaulospora* sp. isolated from the rhizospheric² soils and roots of two actinorhizal plants i.e. *Cercocarpus montanus* and *Purshia tridentata* growing at several locations in North and South America.

Bagyaraj and Manjunath (1980) found that inoculation of cotton, cowpea and *Eleusine coracana* with *Glomus fasciculatum* in soil with low P availability significantly increased root and shoot weight, P and Zn content of plants but Mn was similar to that of non-mycorrhizal plants.

Jenson and Jockobsen (1980) found abundant VAM infection in barley roots grown in least soil P and least infection in roots with high soil P. Infection also decreased with increasing nitrogen fertilizer. They found a close relationship between spore count and infection intensity.

Chambers et al. (1980) observed decreased VAM development in young clover seedlings which was attributed to addition of combined N. However, NH_4^+ was found to be more effective than NO_3^- .

Johnson et al. (1980) also found reduced infection by *Glomus fasciculatum* and *G. mosseae* in response to high N fertilization.

St. John (1980) found a strong association between root depth and mycorrhizal infection. Surface roots were much more likely to be infected by VAM fungi than deeper ones.

Rose (1980) after surveying 25 flowering non-legume plant species from seven angiosperm~~ii~~ families concluded that these non-legumes form mycorrhizal association with *Gigaspora*, *Glomus*, *Acaulospora* and *Entrophospora* and

other undescribed VAM taxa. Spores of first three genera of fungi were found most frequently from soil sievings.

Rose and Trappe (1980) described 3 new *Glomus* species associated with an actinorhizal shrub.

Giovannetti and Mosse (1980) reviewed various methods of assessment of VAM infection in plants and calculated the standard errors for four methods used extensively by most workers.

Nemec (1981) histologically demonstrated the distribution of dehydrogenase in young arbuscule, hyphae and vesicles. He also detected peroxidase and catalase activities in senescing arbuscules.

Mishra *et al.* (1981) observed highest mycorrhizal infection and growth when 40 or more endogonaceous spores were inoculated per plant.

Cooper and Tinker (1981) concluded that the translocation of P in the hyphae occurred by protoplasmic streaming. They however, did not rule out the possibility of bulk flow of hyphal content under a water potential gradient.

Wang and Hayman (1981) found that level of

ammonium nitrate in *Trifolium* did not affect mycorrhizal colonization but was adversely reduced in onion.

According to Gianinazzi-Pearson *et al.* (1981) the principal role of VAM fungi is the utilization of soil P more efficiently where the source of P for both mycorrhizal and non-mycorrhizal plants remains the same.

Biermann and Linderman (1981) proposed a method for the assessment of VAM using less number of root segments. According to them, the result can be obtained within confidential limit of 10 % of the mean.

Chilvers and Daft (1981) observed morphological diversities of VAM infections in different genera of host plants. They did not observe any relationship between spore population and VAM infection but indicated that mycorrhizae were significantly less likely to bear root hairs.

Kariya and Toth (1981) investigated the ultrastructure of mycorrhizal association between *Zea mays* and *Glomus fasciculatum*. They observed that in response to penetration of cortical cell walls thin out and form a "collar" around the endophyte. Host cell cytoplasm was never observed to be in direct contact with endophyte but

was separated by an interfacial zone consisting of host plasmalemma, interfacial matrix and fungal cell wall. Further, the arbuscules were formed by repeated branching of endophyte and its deterioration started from finer branches and proceeded backward to the point of penetration.

Azcon and O'Campo (1981) observed that concentration of nutrients in plant tissues did not affect level of VA infection in wheat cultivars inoculated with *Glomus mosseae*. They further noticed that lack of sugar exudation from the roots of non-host wheat plants was solely responsible for the absence of mycorrhizal infection.

Positive response in growth of *Nicotianum tabaccum* inoculated with *Gigaspora marginata* and significantly greater number of spores were detected in soils with lowest fertilization level (Cnios, 1981).

Manjunath and Bagyaraj (1981) further studied the effect of different components of VAM inoculum on the growth of onion and suggested that both extra-matrical chlamydospores and infected root segments stimulated the plant growth but the latter was more efficient compared to former component.

The VAM fungal inoculum potential was

significantly reduced below 30 cm depth and approached nil at less than 1 meter soil depth (Schwab and Reeves, 1981)

Smith and Walker (1981) suggested a model which allows determination of rates of infection per unit length and time and the rate of fungal growth in the cortex, which may be useful in distinguishing environmental effects in phases of colonization (in soil, at root surface and within the root). They further found that root tip is nearly 10 times more infectible than average of whole root.

Trappe (1982) proposed a synoptic key for the identification of genera and species of zygomycetous mycorrhizal fungi.

Bonfante-Fasolo and Grippiola (1982) based on their electron microscopic studies on symbiotic association between *Glomus fasciculatum* and *Vitis sp.* concluded that there occurred a gradual simplification of the wall components from the extra-radical hyphae to the intra-radical one.

Hogberg (1982) studied the mycorrhizal infections in various tropical forest ecosystems of Tanzania and reported a dominance of endomycorrhizae.

Stahl and Christensen (1982) found that relative prominence of different VAM fungi was related to the geographic locations, abiotic factors and identity of plant symbiosis.

Bethlenfalvay *et al.* (1982) determined fungal biomass both extra and intra-radical by chitin assay technique and found that the intra-radical mycelium increased throughout the life span of host whereas extra-radical one was maximal till onset of soybean pods and decreased thereafter. The ratio of extra-and intra-radical hyphae also decreased through association. They considered that this ratio to be an index of usefulness to host growth and its high value coincided with the significant growth enhancement of host.

Whittingham and Read (1982) showed that direct transfer of nutrients between living source and sink plants occurred by connecting mycorrhizal hyphae.

Marx *et al.* (1982) on the basis of ultrastructural studies and localization of ATPase activities in arbuscules, proved that the nutrient exchange in VA mycorrhizae occurs across the living host fungus interface and P transfer from fungus to host by active transport mechanism in the finer

branches of arbuscules.

Kucey and Paul (1982) determined the biomass of mycorrhizal fungi associated with bean roots by converting the fungal biovolume to biomass using conversion factor of 0.35 cm^{-3} .

Azcon *et al.* (1982) proved that soil nutrients influenced mycorrhizal development by acting in the establishment of infection rather than spread within the root system.

Buwalda *et al.* (1982 a) showed that in early phase of infection, the rate of spread is not significantly altered by the presence of greater length of uninfected roots and concluded that the initial relative growth rate of VA fungus was mainly limited by its internal metabolism rather than by availability of substrate. ^{Buwalda *et al.*} (1982 b) also found that inherent rate of spread of fungus was not markedly altered by application of P.

Smith (1982) reported that the inflow of P (moles P absorbed/unit length/unit time) into mycorrhizal roots from soil exceeded that of non-mycorrhizal roots over a range of P levels. High rates of P uptake were associated with reduction in R/S ratio.

Allen (1982) observed increased water transport and reduced resistance to water inflow with mycorrhizal infection. He suggested that one major factor involved may be increased absorptive root surface area provided by VAM fungal hyphae.

Observation of Same *et al.* (1983) showed that the increased P supply decreased the per^{cent} root length infection and concentration of soluble carbohydrate within the roots. Further per^{cent} root length infection was closely related with concentration of soluble carbohydrate within the root.

Hepper (1983) stated that application of nitrate resulted in higher level of VA infection by *Glomus mosseae* at high P level. At high P concentrations, infection was inhibited but at low concentration, infection level was related to N content of the host. Treatments with low N resulted in few arbuscules and intercellular hyphae.

Bolan *et al.* (1983) demonstrated that in clover there was a marked threshold level of P application below which the non-mycorrhizal plants took up little P and grew poorly. VAM inoculation eliminated this threshold even when large amounts of iron-hydroxide were present.

Nielson and Jenson (1983) showed that VAM increased growth of shoot, P concentration and P uptake at all P levels but was most pronounced at low soil P level. Further, they noted that VAM increased N and P uptake decreased Ca, Mg, Zn concentration in shoots.

Biermann and Linderman (1983) showed that intra-radical vesicles increased inoculum potential of mycorrhiza.

Daft and Hogarth (1983) working with maize and onion indicated that *Glomus caledonium* was less efficient than *G. claram* and suggested a mixed inocula for effective field inoculations.

Schwab *et al.* (1983) were of the opinion that apparently P nutrition of host does not affect pre-penetration stages of mycorrhiza formation. External hyphal growth following initial penetration of host was reduced by P nutrition. Further, subsequent ability of fungus to form secondary infections was decreased resulting in overall decrease in mycorrhizal formation normally in high P.

Olivers *et al.* (1983) observed that variation in mobility pattern of soluble alkaline phosphatases in the extracts of mycorrhizal roots depended upon VA endophyte

involved. They suggested that these enzymes were of fungal origin.

Wilson *et al.* (1983) developed a immuno-flourescent antibody technique employing which they distinguished VAM fungi from non symbiotic fungi.

Hall *et al.* (1984) indicated that VAM stimulated the shoot growth of *Lolium perenne* L. greatest and with highest level of applied P and/or N in nutrient (P and N) deficient soil. They suggested that applied N and possibly P resulted in mycorrhizae making a more important contribution to plant P or Cu status.

Buwalda *et al.* (1984) concluded that the host regulates the rate of growth of the mycorrhizal fungus.

Smith *et al.* (1985) assayed the enzymes in the mycorrhizal and non-mycorrhizal root and shoot extracts and concluded that VAM fungi were able to assimilate ammonium via *glutamine synthetase* activity.

Graham and Timmer (1985) demonstrated that addition of superphosphate reduced root colonization and growth response of root stock of *Carizo citrange* and sour

orange. They showed that rock phosphate has a long term available P compared to superphosphates which leached easily. No further P fertilization was necessary if rock phosphate was used and VAM inoculated in soil-less media.

Franklānd and Harrison (1985) reported that VAM infection in *Acer* was highly correlated with soil factors i.e. pH, organic matter, P and Fe and the growth of the host had greater influence on VA fungal infection than soil factors.

Kurakov (1985) reviewed the effects of environmental variables in endomycorrhizal formation in plants and distribution of VAM fungi in nature.

Davis and Young (1985) observed that different VA fungal species behaved differently in their effectiveness in tolerating liberal fertilization as well as their potential for stimulating or depressing plant growth.

O'Campo and Azcon (1985) found no direct relationship between sugar concentration in root and VAM infection in wheat and concluded that sugar in root extracts of these plants may not be a decisive factor in plant susceptibility to mycorrhizal infection.

Abbott and Robson (1985) were of the view that differences among isolates of VAM fungi in distribution of hyphae in the soil may be as important as differences in length of external hyphae when selecting fungi that are effective at increasing nutrients level.

Sen and Hepper (1985) characterized 6 species of *Glomus* by subjecting spore extracts to polyacrylamide gel electrophoresis and selective enzyme staining technique.

Smith and Roncadori (1986) reported that *Glomus intraradices*, *G. ambisporum*, and *Gigaspora marginata* generally stimulated plant growth equally at 24 and 30^o C but *G. ambisporum* was slightly more effective at 36^o C.

Schubert and Hayman (1986) found reduced mycorrhizal infection at increasing P levels but the rate of colonization and infection plateau varied with different VA endophytes. *Glomus mosseae* was effective at low and medium P whereas *G. caledonium* at high, medium and low P. They finally concluded that analysis of VAM symbiosis with single endophyte cannot always be used as a basis for generalization about VA fungi and that selection of field inoculants should take special account of endophyte behaviour in soils given excess P.

Azcon *et al.* (1986) demonstrated that significance of VAM in growth and P uptake by soyabean and provided evidence of a key role of this symbiosis in P cycling in natural calcareous soils.

Noordwijk and Hairiah (1986) surveyed the VA mycorrhizal association in rain forest of North Sumatra and depicted that VAM infection of most root samples of mixed vegetation was negatively correlated with both pH and P availability.

Smith *et al.* (1986) indicated that VAM infection can occur both immediately behind the root apex and upto a considerable distance (55 mm long) behind the apex in 16 days old plants.

Aldwell and Hall (1986) observed that *Glomus masseae* successfully competed against *Acaulospora laevis* and spread through the soil at approximately 600 mm per year. Application of P fertilizers to the soil did not impose any effect on the rate of migration.

Sharma *et al.* (1986) recorded eight species of Endogonaceae belonging to *Glomus*, *Gigaspora* and *Sclerocystis*. *Gigaspora gregaria* and *S. microcarpus* were

reported by them for the first time from the forest of North-East India.

Schubert *et al.* (1987) noted that the total extra-radical mycelium of VAM fungi increased steadily and reached maximum length 8 weeks after inoculation unlike inability rate which decreased from an initial value of 100 % to less than 15 % after a period of 5 to 8 weeks. Thus, they suggested that the viability of extraradical mycelium may be an important factor influencing nutrient uptake and translocation by VA mycorrhiza.

Dodd *et al.* (1987) found increased acid phosphatase activity in mycorrhizal wheat and onion though this increase was different in plants inoculated with different VA endophyte. In wheat, this increase in phosphatase activity induced by *Glomus geosporum* and *G. mosseae* became apparent between 25 and 51 days after sowing.

Wright *et al.* (1987) characterized *G. occultum* by application of monoclonal antibodies, which react strongly with spores and hyphae. Further, they ~~also~~ indicated the occurrence of various strains of VAM fungi from various habitats.

Sylvia (1988) indicated that production of active hyphae in abundance lagged several weeks behind that of root colonization.

Gemma and Koske (1988) observed a pre-infection stimulation of root growth which they attributed as non-nutritional benefit of the VAM symbiosis.

Dixon *et al.* (1988) reported decrease in exudation of reducing sugars and amino acids from citrus seedlings roots as soil and root P increased. They found less exudation of reducing sugars and amino acids from mycorrhizal than non - mycorrhizal roots.

Sainz and Ames (1988) reported significant improvement in dry matter production of red clover and P uptake by VAM fungi in acid soils. The two VAM fungi investigated possessed varying degree of effectiveness.

Rosendahl *et al.* (1988) quantified VAM fungi by polyacrylamide gel electrophoresis technique and referred to it as most effective compared to root staining and glucosamine assay.

Avio and Giovannetti (1988) demonstrated that cellulose sources reduced rate of infection by all VAM

endophytes tested. However, the effectiveness of cellulose in reducing VAM colonization was influenced by the addition of nitrogenous fertilizers.

Baas *et al.* (1989) concluded that biotic factor in rhizospheric soil affected uptake of P and under stress conditions, latter can be compensated by VAM fungi.

Davidson and Visser (1989) investigated the effect of forest soil acidification on mycorrhizal development and revealed that only a restricted number of VAM species could cause infection in acid soil. However, the mycorrhizas formed by some endophytes under such conditions did not impart beneficial role to the host.

Amijee *et al.* (1989) observed that addition of P to soil did not affect the rate of extension of roots but increased the initiation of lateral roots. Low content of P in soil also affected the fungal behaviour and increased the length of root available for colonization.

Manjunath *et al.* (1989) suggested that higher level of rock P was required to attain growth ~~comparable to~~ ^{than} that obtained with application of soluble P fertilizers.

Auto-radiographical studies of Tang *et al.* (1989)

higher than

showed that mycorrhizal infection enhanced ^{45}Ca absorption and transport was remarkably greater in mycorrhizal than non-mycorrhizal counterpart.

Bell *et al.* (1989) noticed that both P and Zn nutritions were affected by VAM activity but most prominent role of VAM fungi in oxisols soil was the uptake of P.

Gianinazzi-Pearson *et al.* (1989) reported that root exudates of clover increased *in vitro* spore germination and hyphal growth of *Gigaspora margarita*. In addition, flavonones and flavone induced rapid growth of spores at low concentration. They suggested that the presence of such chemicals in root exudates may promote cell to cell contact between VA symbionts during early preinfection phases.

Rajapakse *et al.* (1989) found that root mycorrhizal colonization was negatively correlated with medium P and shoot P concentration. They also showed the interspecific variation in plant growth in response to VAM inoculation at different P levels.

Paula and Sequiera (1990) concluded that suspension cultured *Pueraria* cells stimulate hyphal growth of VA endophyte by accumulating and leaking active metabolites

into the medium.

Antonio (1990) concluded that spore-like structure in the mycelium which ultimately forms appresorium in the modified environment at a root epidermal cell surface.

Sidhu *et al.* (1990) identified *Glomus fasciculatum* and *Scutellospora calospora* which colonized the roots of fast growing *Casuarina equisetifolia* in sand dunes and suggested the importance of these VAM fungi in the successful colonization of hostile sand dunes by *Casuarina* species.

Shnyreva and Kulayev (1990) demonstrated the production of xylanase in VA fungal cultures and stressed the possible participation of xylanase secretion in the process of penetration of host roots and its effect in host-endophyte relationship in VAM symbiosis.

Sequiera *et al.* (1990) attributed the ameliorative effects of VAM on growth of host plants in highly acid or overlimed soils was related to improved nutrient uptake.

Sylvia and Neal (1990) concluded that plant N stress may affect the resistance of the host plant by VAM fungi.

Smith and Smith (1990) in their review considered the implications of the involvement of mycorrhizal fungi in the nitrogen nutrition of the plants and also that in nature, plants may be nutritionally linked by mycorrhizal hyphae.

Spanu *et al.* (1990) found that during early infection stages, the specific activities of chitinase were higher in mycorrhizal roots than ⁱⁿ non-mycorrhizal ones. However, after the establishment of symbiosis reverse was true.

Eissenstadt and Newman (1990) found that 5 weeks old *Plantago* seedlings had more mycorrhizal infection when grown with larger mycorrhizal plants than when grown alone.

Morton (1990) studied the phylogeny of endogonaceae and subdivided it into two broad categories on the basis of structures formed in mycorrhizal roots. He hypothesized that they have evolved from a common arbuscular ancestor.

Bayetchko and Tewari (1990) concluded that the pattern of root colonization by *Glomus dimorphicum* was influenced by the host genome and that fungal morphology in the roots varied and not diagnostic for mycorrhizal species.

Braunberger *et al.* (1991) showed that the length of arbuscular colonized root length per appressorium was strongly reduced in response to increased shoot P concentration and concluded that this was mediated primarily through an inhibition of intra-radical development.

Li *et al.* (1991 a) demonstrated that hyphae of VAM fungi have the ability to form a P depletion zone and a zone of altered pH in the adjacent soil leading to considerable difference between soil condition at root-soil and hyphae-soil interface and that in the bulk soil. Li *et al.* (1991 b) have also shown that mycorrhizal plants have uniform P depletion zone extending upto 11.3 cm compared to non-mycorrhizal counterpart which extended to maximal of 1 cm into the outer compartment.

Data by Kothari *et al.* (1991) showed that VAM hyphal uptake and translocation to the host is important in increased acquisition of P and Zn by mycorrhizal plants.

Thompson *et al.* (1991) employed split root technique to separate plant and soil mediated effects of P in the formation of mycorrhizae by *Scutellospora* on its clover host and concluded that the effects of luxury amount of P on infection by *S. calospora* could be mediated through the soil.

The findings of Nair *et al.* (1991) suggest that formononetin and biochanin may act as a signal molecule in vesicular-arbuscular mycorrhizal symbiosis.

Gundersen (1991) in his review article has discussed the interactions of effects of excess N on nutrient deficiencies, loss of mycorrhiza and soil acidification and suggested that excess N may destabilize the forest ecosystem and contribute to forest decline.

Pedersen *et al.* (1991) observed that soil extracts of asparagus stand caused a reduction in root colonization and plant growth and ultimately affecting the survival of asparagus.

Sanders and Fitter (1992 a) investigated the seasonal pattern of mycorrhizal occurrence and morphology in various host plants under field conditions. They noted a inter-specific variation in infection amongst most of the plant species. Besides, temporal variation in hyphal density, arbuscule and vesicle density varied little with time. Sanders and Fitter (1992 b) did not observe relationship between VAM infection and shoot concentration of P, Cu, Zn and Mn or plant biomass and only in a few cases was nutrient inflow related to infection.

Gazey *et al.* (1992) noted that a critical length of mycorrhizal root was found to be associated with onset of sporulation which varied between different VAM fungi. Further, the latter behaved differently in terms of intensity of sporulation and length of external mycelium production.

Dillon (1992) demonstrated that the VAM fungi isolates showed considerable amount of host preference.

Jackobsen *et al.* (1992) showed that *Glomus* species transported most ^{32}P to shoot over soil-root distance shorter than 1 cm, while *A. laevis* transported longer than 1 cm.

Simon *et al.* (1992) obtained for the first time the DNA sequences from VAM fungi by directly sequencing amplified fragments of nuclear genes coding for small subunit and r-RNA. They suggested that this technique as coupled with molecular technique will facilitate rapid detection, identification and possibly quantify VAM fungi.

Garcia-Garrido *et al.* (1992 a) showed an increase in endo- and exoglucanase activities in VAM plants at the beginning of entry point formation and arbuscule development. They attributed some of cellulase activity to be of VAM origin. Garcia-Garrido *et al.* (1992 b) have suggested that endoglucanases may

be involved in the process of colonization of lettuce roots by *Glomus fasciculatum*.

Johnsen *et al.* (1992) concluded that the isolate of *Glomus intraradices* in symbiosis with cucumber was able to absorb N from growth medium and transport of it to the host over a distance of 5 cm or more.

SOIL ENZYMES

DEHYDROGENASE :

Levels of dehydrogenase activity of soils are considered to give some guide to the microbial activity of the soils and have been increasingly investigated since the introduction of the method for estimation of this activity (Lenhard, 1956).

Stevenson (1956) modified Lenhard's technique and presented evidences for the reliability of the dehydrogenase as a means of estimating total microbial activity in soils. He however, did not find relationships between dehydrogenase activity and bacterial numbers unless organic amendments were made to the soil.

Casida *et al.* (1964) detected relatively constant

dehydrogenase activity regardless of small fluctuations in microbial numbers. On addition of nutrients, it paralleled with the rapid multiplication of gram-positive bacteria and decreased subsequently when the metabolic activities of bacteria slowed down.

Skujins and McLaren (1968) compared the dehydrogenase activity of geologically preserved soils with freshly collected desert and cultivated soils and finally concluded that the activity did not reflect microbial number but to represent rate of overall metabolism.

Ross (1971) reported that formazon production was significantly greater in systems incubated anaerobically than in systems without complete anaerobiosis. Further, the addition of glucose and NAD together did not prevent decline in rate of formazon formation on prolonged incubation.

Casida (1977) while trying to explain microbial metabolic activities observed a close relationship between dehydrogenase activity and respiration.

Schinner and Gurschler (1978) reported that higher values for enzymes, soil organic matter and moisture were found in the top layers of soil and decreased with increase

in soil depth.

Smith and Pugh (1979) indicated that the dehydrogenase assay can provide a valid indication of soil microbial activities.

Burns (1982) investigated the soil enzyme activity and suggested that total activity of an enzyme in soil comprised of activities associated with soil constituents i.e. viable microbes, cell debris, clay and humic colloids.

Trevors (1984) reported that there was a high positive correlation between dehydrogenase activity and substrate concentration, incubation, temperature and soil pH. Further, a highly negative correlation between dehydrogenase and oxygen content existed.

Das and Mishra (1986) while investigating the dehydrogenase activity in pine forest could not establish correlation between dehydrogenase and total microbial population and suggested that these two were governed by different environmental factors.

Shuji and Takao (1986) established positive correlation among dehydrogenase activity, soil pH and

bacterial population.

Vekemans *et al.* (1989) investigated twenty Belgian agricultural soils for their biochemical and microbiological properties. In spite of very different nature of the characteristics studied, close relationship between soil respiration and glucose mineralization rates, biomass, carbon and enzymes like alkaline phosphatase and dehydrogenase was found.

Jha *et al.* (1992) showed that dehydrogenase activity showed a marked variation in soils sampled from different locations.

PHOSPHATASE :

Phosphatase activity provides an index of potentially available P in soil. Rogers *et al.* (1942) explained that acid phosphatase present in plant roots and acid soils was responsible for the hydrolysis of organic P in soil.

Golebiewska (1956) found that highest phosphatase activity was in surface soil and the activity decreased in low profile soil.

Keilling *et al.* (1960) and Hoffman and Eliar-Azar (1965) reported the existence of close correlation of phosphatase activity with organic C, moisture content and total N.

Khaziev and Burangulova (1965) found positive correlation between phosphatase activity and soil organic phosphorus content.

Greaves and Webley (1965) investigated the mechanism of organic phosphorus breakdown by microbes. They suggested that roots and microbes were major sources of phosphatase in soil.

Dixon and Webb (1966) found positive relation of phosphatase activity with Mg and suggested that it is due to Mg ions being natural activator of the majority of enzyme activities on phosphorylated substrates.

Casgrove (1967) hypothesized that soil phosphatases hydrolyse the phosphate-esters to inorganic P available to higher plants and soil microbes.

Parkinson *et al.* (1986) indicated that phosphatase activity decreased along depth due to decreasing microbial

population with increasing depth.

Tabatabai and Bremner (1969) emphasized the influence of pH on phosphatase activity.

Neal (1973) observed the marked influence of vegetation on soil phosphatase activity .

Ladd and Butler (1975) suggested that positive relationships with organic C content is probably associated with the fact that phosphatase like other enzymes, become bound in humo-protein complex which protects the enzymes from degradation

Spiers and McGill (1979) reported that increasing P supply increased microbial synthesis of phosphatase .

Harrison and Pearce (1979) investigated the seasonal variation of phosphatase activity in woodland soils. They noted 2-3 peaks i.e. in summer, autumn and spring seasons respectively.

Nielsen and Eiland (1980) established a relationship between P fertility, phosphatase activity and ATP content in soil. They also suggested that there is a natural regulation of phosphatase activity in soil and it is

enhanced when ^{the} latter is depleted of available P.

Appiah and Thomas (1982) also assigned the low phosphatase activity to low organic content, lower microbial population and biomass.

Kulinska *et al.* (1982) could not establish correlation between microbial population and phosphatase activity. They also did not find correlation between soil water holding capacity and enzyme activities.

Stroo and Jencks (1982) reported significant positive correlation between phosphatase activity and soil respiration.

Frankenberger and Dick (1983) found significant positive correlation of alkaline phosphatase with respiration but in case of acid phosphatase such correlation was absent.

Harrison (1983) studied the relationships between the intensity of phosphatase activity and physio-chemical characteristics of soil and their possible interactions with soil depth, soil type, season, vegetation type or parent material in woodland soils. His result indicated that intensity of activity and soil properties differed with soil

depth, soil type, season or vegetation type.

Nohrstedt (1985) reported that the concentration of organic C was the strongest predictor of phosphatase activity.

Pang and Kolenko (1986) demonstrated that the activity of phosphatase depends on soil, geographical location and depth. Fertilization of soil with phosphates and urea depressed phosphatase activity.

Tarsar and Stores (1987) found that maximum phosphatase activity was at pH range of 5-6 and also suggested that the activity appeared to be dependent on the concentration of organic matter content of soil.

Baligar *et al.* (1988) studied the acid phosphatase activity in soils of the Appalachian region. They suggested that activity was more in moist soil compared to the air dried soil. They also observed a positive correlation with soil moisture, C and N contents of soils.

Tarafdar *et al.* (1989) correlated the acid phosphatase to the number of fungi. Both acid and alkaline phosphatase were significantly correlated with soil organic

matter, available organic and inorganic P and microbial populations.

Rajo *et al.* (1990) revealed that phosphatase activity was concentrated in the larger soil fractions and was associated with plant debris and less humified organic matters.

Bonmati *et al.* (1991) observed a strong correlations between soil phosphatase activity with total N and organic C.

Fox and Comerford (1992) noted a significant acid phosphatase activity in the upper horizons of the soil of various locations. They also found that P fertilization decreased former's activity to a large extent.

FRANKIA-VAM FUNGI-NON LEGUME INTERACTION :

Rose and Youngberg (1981) showed for the first time that dually inoculated (*Frankia* and VAM fungi) *Ceanothus* seedlings exhibited higher dry weights of shoots and roots, number of nodule, nodule weight, nitrogenase activity as well as higher level of N, P and Ca in plant tissues composed to singly inoculated counterpart.

Gardner **et al.** (1984) by using various experimental inoculation regimes demonstrated significant improvement in nodulated mycorrhizal *Hippophae rhamnoides* compared to singly inoculated plants with respect to plant growth, uptake of P and nitrogenase activity when grown under nutrient deficient conditions.

Russo (1989) investigated the interaction between *Frankia* and VAM on growth and nitrogen fixation of *Alnus acuminata* seedlings under three different P levels. He indicated that at low P level alder seedlings inoculated with both *Frankia* and VAM fungus, *Glomus intraradices* showed significantly higher acetylene reduction activity and overall plant growth over singly inoculated plants.

Berlinger and Torrey (1989) demonstrated that *Comptonia* and *Myrica* plants inoculated with actinomycete *Frankia*, with VAM fungus *Glomus intraradices* or with *Frankia* and *Gl. intraradices* together were infected by *Frankia* only. Uninfected *Comptonia* developed well when supplied with mineral nutrients but degenerated when watered with solution lacking combined N or with deionized water.

Fraga-Beddiar and Tacon (1990) demonstrated that VAM fungus markedly improved nodule number, their weight and

nitrogen fixation at various P levels. They noted that VAM improved nitrogen fixation by undetermined mechanisms rather than by providing P to the host. These unknown mechanism contribute to an increased number of *Frankia* infection and increased nodule tissue.

Visser *et al.* (1991) in their field investigation revealed that shoot height, and weight, root weight, nodule weight and mycorrhizal root length of dually inoculated *Elaeagnus* and *Shepherdia* were manyfold greater than for their uninoculated counterparts. The shoot productivity was highly correlated with nodule status and the soil amendments (peat and mineral soils) variously affected symbiont development and plant growth in *Elaeagnus* and *Shepherdia* seedlings.

Raman and Elumalai (1991) surveyed the mycorrhizal and actinorhizal status of *Casuarina equisetifolia* in the coastal region of Madras and found that plants were more mycorrhizal and produced more nodules at soil pH more than 7.5.

Jha *et al.* (1993) also noted synergistic effect of VAM fungi on nodulation and nitrogen fixation by *Frankia* under P deficient conditions which decreased with increase in soil P concentration.

Climate, Soil and Flora of the Study Site.

Present field investigation was carried out at upper Shillong located at 1726 mMSL, latitude $25^{\circ} 34'$ N and longitude $91^{\circ} 56'E$ in East Khasi Hills, Meghalaya (Plates 1 & 2). The climate of the site is sub-tropical monsoonic type.

Based on meteorological conditions, a year may be broadly sub-divided into spring, rainy, autumn and winter seasons. The spring season extends from March to April which is characterised by high velocity of wind, less humidity and moderate temperature. In fact, this season represents a period of transition between winter and rainy season. The rainy season extends from May to September. Generally maximum rainfall (approximately 70 - 80%) occurs during June to August. Further, optimal conditions of moisture, temperature, sunlight and other climatic factors mark this season, leading to maximal growth of plants in this season. Autumn, a transitory period between rainy and winter, extends from October to November. This period is generally characterized by less or scanty rainfall, dropping down of



Plate 1. Distant view of the forest stand of Alnus nepalensis D. Don
at Upper Shillong



Plate 2. A closer view of the forest stand of A. nepalensis.

temperature and humidity conditions. Finally, winter onsets with the approach of December and persists till the end of February. The climatic variables like temperature, rainfall and humidity drop down to minimal value in this season.

The average seasonal rainfall ranged from 32 to 2009 mm during study period. The average minimum and maximum temperatures varied from 6.3 to 23.5 °C, 6.9 to 23.5 °C and 7 to 22.6 °C during 1989, 1990 and 1991 respectively. The relative humidity varied from 61 to 90 %, 59 to 92 % and 56 to 93 % during 1989, 1990 and 1991 respectively (Fig. 1).

Soil:

Greater Shillong plateau falls under the Gondwana (Mesozoic) period. The plateau is constituted by rocks of Pre-Cambrian age consisting of rocks of hard crystalline granites, gneiss and granulites (Singh, 1971). The soil of the study site is red laterite under red or brown loam type, sandy loam laterite in texture (Sand 54.1%, silt 25.9% and clay 20%). The soil exhibits acidic reaction throughout the year.

Flora:

Present study site was a pure stand of Himalayan alder (*Alnus nepalensis* D. Don) spread over an area of about

7 hectares. The age of this forest grove is estimated to be about 40 years and extends from a hillock to a neighbouring valley. The estimated density of trees is 432 trees per hectare. In fact, the stand comprises mostly of established trees except in some pockets where stem cutting or/and self-regeneration has given rise to saplings. Other predominant understory plant species of the forest include *Rubus ellipticus*, *Litsea cubeba*, *Osbeckia crinata*, *O. nepalensis*, *Cassia mimosoides*, *Arundinella khasiana*, *Houttynia cordata*, *Haedychium aurantiacum*, *Trichosanthes spp.* and *Eupatorium adenophorum*.

Chapter	I	: Seasonal changes in the morphology and : population of microsymbionts (<i>Frankia</i> and : vesicular-arbuscular mycorrhizal (VAM) : fungi} associated with nitrogen fixation : in <i>Alnus nepalensis</i> D. Don woodland.

Introduction:

Non-leguminous alder, possessing actinorhizal nodules typically colonize nutrient-deficient habitats and efficiently fix atmospheric nitrogen (Becking, 1970; Johnsrud, 1978; Akkermans and Van Dijk, 1981; Sharma and Ambasht, 1984). Apart from diurnal changes, seasonal variation in nitrogenase activity (nitrogen fixation) of nodules is evident (Tripps *et al.*, 1979; Sharma and Ambasht, 1984). An array of soil, environmental and host factors such as temperature (Benecke, 1970; Akkermans, 1971; Wheeler, 1971; Becking, 1977), light influx (Wheeler, 1969) and photosynthate (Wheeler, 1971; Huss-Danell and Sellstedt, 1985; Pizzelle *et al.*, 1992) are known to influence the process of nitrogen fixation. In nature, vesicular-arbuscular mycorrhizal fungi often form association with many of these non-legumes (Daft and Hacskeylo, 1976; Williams and Aldon, 1976; Williams, 1979; Rose, 1980; Gardner, 1986). Apart from

increased uptake of P (Mosse, 1972) which is inevitably related to nitrogen fixation, mycorrhizal fungi also aid in the uptake of Cu, S and other trace elements (Starks, 1971; Mosse, 1973; Gray and Gerdemann, 1973; Mosse *et al.*, 1976; Rhodes and Gerdemann, 1978; Zaag *et al.*, 1979; Kothari *et al.*, 1991). It is indicated that enhancement of nodulation and nitrogen fixation is a response to improved nutritional status of the host plant rather than a direct effect on N uptake (Carling *et al.*, 1978) although some investigators have indicated the potential of mycorrhizal endophytes in N uptake directly from the soil (Ames *et al.*, 1983; Azcon and Barea, 1992; Johnsen *et al.*, 1992) .

The tripartite association involving non-legume-*Frankia*-VAM fungi represents one of the most complex systems in which each microsymbiont interacts between themselves and also with host plant. No information is presently available on the seasonal fixation of atmospheric nitrogen by *Frankia* in actinorhizal system involving VA endophyte in terms of *in vivo* morphological changes in symbiotic organisms which merits investigation and which may add a significant information to the tripartite symbiosis.

Besides, the role of P (Russo, 1989; Jha *et al.*, 1993), Ca (Tisa *et al.*, 1981) and Co (Dixon and Wheeler,

1983) in nodulation and nitrogen fixation by non-legume, the implication of mineral nutrients critical for nitrogen fixation under natural field conditions is lacking.

Alnus nepalensis D. Don, an actinorrhizal plant growing naturally or in plantations in hostile soil conditions of Eastern Himalaya possesses nitrogen - fixing ability (Sharma and Ambasht, 1984; Sharma, 1986), which in addition also grows luxuriantly in higher altitudes of North-East India. It is economically important especially in improving soil fertility, land reclamation, short term energy plantations, softwood production and soil stabilization.

Taking above facts into consideration, present investigation has been carried out to evaluate seasonal morphological changes of microsymbionts in relation to nitrogen fixation by *Alnus nepalensis* under field conditions.

Materials and methods :

The present investigation was carried out at upper Shillong (Study site) from July 1989 to April 1991. The sampling was conducted on every 16th day of July, October, January and April thus covering all four seasons of the year.

Collection of plant and soil samples :

The plant and soil samples were collected from five randomly chosen spots in *A. nepalensis* (Himalayan alder) grove. After removing the ground vegetation manually, the root nodules and mycorrhizal roots along with the soil were collected aseptically with the help of sterile trowel from 0-15 cm (D_1), 15-30 cm (D_2) and 30-45 cm (D_3) on volume basis i.e. 15 cm^{-3} . The composite samples obtained by mixing five samples were transferred to sterile plastic pouches, brought to the laboratory and stored at 4°C till further investigation.

Soil temperature :

On each sampling, soil temperature was recorded using a soil thermometer.

Soil moisture content :

The moisture content of the soil was determined by drying 10 g of freshly collected soil samples in a hot air oven at 105°C till the constant weight was obtained (24 h). The percentage soil moisture content was calculated using following formula:

$$\text{Moisture content (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

where,

W_1 = Initial weight of soil before drying

W_2 = Final weight of soil after drying

Soil pH :

Ten g . fresh soil was suspended in 50 ml distilled water in a beaker. The soil-water mixture was stirred for 20 min on a magnetic stirrer and pH of the soil suspension was read with electronic digital pH meter.

Organic matter :

Walkley and Black's (1934) rapid titration method was followed for the determination of soil organic matter. To 1.0 g sieved, dry soil (<0.2 mm) kept in a 500 ml conical flask, 10 ml of 1N $K_2Cr_2O_7$ was pippetted and was thoroughly mixed by swirling the flask. Then 20 ml of conc. H_2SO_4 was poured, thoroughly mixed and allowed to stand for 30 min at room temperature. Thereafter, the solution was diluted to 200 ml with distilled water and treated with 10 ml of 85 % H_2PO_4 alongwith 3 drops of

diphenylamine indicator. The solution was finally titrated with 1 N FeSO_4 solution. A control (blank) was maintained by adding above reagents only. Three replicates were used for each analysis. The organic matter content of the soil sample was determined according to the following formula :

$$\text{Organic matter (\%)} = \frac{B - S \times 0.003 \times 1.724 \times 100}{W}$$

where,

B = volume of FeSO_4 used for blank titration

S = volume of $\text{K}_2\text{Cr}_2\text{O}_7$ used for sample titration

W = weight of the soil sample (g)

1.724 = conversion factor from organic C to organic matter

Total nitrogen:

Total soil nitrogen was estimated by semi-micro ^K Kjeldahl method as suggested by Allen (1974). Half a g. of dry, sieved soil (<0.2 mm) was transferred to Kjeldahl flask and 2 g of K_2SO_4 -HgO catalyst (20:1) mixture and 3 ml conc. H_2SO_4 were added and heated mildly on the digestion rack till the frothing subsided. The sample was digested on strong heat till the solution became pale green to colourless. Thereafter 20 ml distilled water was added while the digest was still warm, mixed thoroughly and was distilled in the presence of 40 % NaOH. The released ammonia

was absorbed in 4 % boric acid indicator solution and finally titrated with N/140 HCl. The nitrogen content was estimated as follows:

$$\text{Nitrogen (\%)} = \frac{(T - B) \times N \times 140}{S}$$

where,

T	=	Sample titration
B	=	Blank titration
N	=	Normality
S	=	Sample weight (g)

Available phosphorus :

Olsen's extractant was employed for the extraction of available (NaHCO_3 extractable) phosphorus (Olsen *et al.*, 1954), which was prepared by dissolving 210 g NaHCO_3 in water and adding 100 ml 1M NaOH. Finally the solution was diluted to 5 L and pH adjusted to 8.5 ± 0.05 with 0.1 N HCl or NaOH.

Estimation of available P was done following molybdenum blue method (Allen, 1974). Five g sieved soil (< 0.2 mm) was transferred to 250 ml capacity polyethylene container to which 100 ml Olsen's extratant was added with a

pinch of activated charcoal and stirred for 30 min on a rotary shaker. A control was maintained which contained only Olsen's extractant and was treated in similar manner as to soil extract. The soil extract was filtered through Whatman No. 44 filter paper and was neutralized with 10 % H_2SO_4 containing 0.1 % nitrophenol indicator. Thereafter 2 ml of the filtrate was pipetted into 50 ml capacity volumetric flask to which 5 ml of distilled water and 1 ml of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ were poured and thoroughly mixed. Then, 1 ml of $SnCl_2$ solution was added, mixed properly, was diluted to 50 ml and finally left for 30 min at room temperature. The optical density of blue coloured solution was read at 700 nm and converted to known units through standard curve of P. The available phosphorus was calculated using the following formula :

$$\text{Available P (\%)} = \frac{C \text{ (mg)} \times \text{solution volume (ml)}}{10 \times \text{aliquot (ml)} \times \text{sample weight (g)}}$$

where,

C = concentration of P obtained from standard curve.

Exchangeable potassium :

Exchangeable soil potassium (K) was determined

following atomic absorption spectrophotometric method as suggested by Allen (1974). Ammonium acetate solution (1 M, pH 7.0) was used for the extraction of exchangeable bases i.e. K, Ca, Mg and Mn and was prepared by mixing 575 ml glacial acetic acid with 600 ml ammonia solution which was diluted to 10 L with distilled water. The pH of the solution was adjusted to 7.0 ± 0.05 with acetic acid or ammonia solution.

Ten g sieved soil was transferred to 1L capacity polythelene container containing 250 ml ammonium acetate extractant and was stirred for one h on a rotary shaker. The solution was filtered through whatman no. 44 filter paper and absorbance of the filtrate was recorded at 766.5 nm using K lamp in an atomic absorption spectrophotometer (Perkin Elmer 2380, USA). The potassium in the sample was determined according to the following formula:

$$\text{Exchangeable K (\%)} = \frac{C \text{ (ppm)} \times \text{solution volume (ml)}}{10^4 \times \text{sample weight (g)}}$$

Where,

C = ppm of K obtained from the standard curve.

Exchangeable magnesium :

Exchangeable soil magnesium (Mg) was estimated

following the atomic absorption spectrophotometric method (Allen, 1974). The soil extract used for the determination of K was used. The absorbance of the extract was read in an atomic absorption spectrophotometer using Mg lamp at 285.7 nm and was expressed in percentage. Exchangeable Mg was calculated using following formula :

$$\text{Exchangeable Mg (\%)} = \frac{C(\text{ppm}) \times \text{solution volume (ml)}}{10^4 \times \text{sample weight (g)}}$$

Where,

C = concentration of Mg obtained from the standard curve.

Exchangeable calcium :

Exchangeable soil calcium (Ca) was determined using the same extract employed for the estimation of K and Mg and following atomic spectrophotometric method (Allen, 1974). However, absorbance of the extract was read at 422.7 nm using Ca.lamp.

Extractable manganese :

Extractable soil manganese (Mn) was estimated employing the same extract used for determination of exchangeable cations and following atomic absorption spectrophotometric method (Allen, 1974). The absorbance of

the extract was read at 279.5 nm using Mn lamp.

Determination of enzyme activities of the soil :

Total phosphatase activity :

Soil phosphatase activity was assayed following the method of Tabatabai and Bremner (1969). To 1.0 g sieved (<0.2 mm) soil, 4 ml of modified universal buffer (pH 6.5), 0.25 ml toluene and 1 ml 0.115 M disodium p-nitrophenyl phosphate hexahydrate were added and shaken vigorously. The flask was plugged with cotton plug and finally incubated at 37^o C for 1 h. Thereafter, 1 ml of 0.5 M CaCl₂ and 4 ml of 0.5 M NaOH were added to the flask and mixed thoroughly. The control was maintained using foresaid reagents only. The soil suspension was filtered through Whatman no.1 filter paper and absorbance of the filtrate was read at 420 nm in a spectrophotometer (Hitachi 220, Japan). The amount of p-nitrophenyl released was estimated from a standard curve of p-nitrophenol and was expressed as umoles of p-nitrophenol released g⁻¹ dry soil h⁻¹.

Dehydrogenase activity :

Dehydrogenase activity of soil sample was assessed by using 2,3,5 triphenyl tetrazolium chloride (T.T.C.) reduction technique as suggested by Casida (1977). One g

of freshly collected soil was transferred to clean dry test tube to which 0.1 g CaCO_3 and 1 ml of 1% w/v T.T.C. were added and mixed well. The test tube was plugged with a rubber stopper and incubated at 30°C for 24 h. The T.T.C. reduced to triphenyl formazon as a result of dehydrogenase activity was extracted with methanol. The soil of the tube was quantitatively transferred to a Whatman No. 1 filter paper with small quantity of methanol and washed into 50 ml capacity volumetric flask until 50 ml filtrate was obtained. Three replicates were maintained in each determination and a control was maintained which contained only above reagents. The absorbance of the pink-coloured filtrate was read at 485 nm on a spectrophotometer. The dehydrogenase activity was calculated using the standard curve of triphenyl formazon and was expressed in terms of μg formazon formed g^{-1} dry soil 24 h^{-1} .

Assessment of nitrogenase activity :

Nitrogenase activity of root nodules of *A. nepalensis* was assessed following acetylene reduction technique (Stewart, *et al.*, 1967; Hardy, *et al.*, 1968). The assessment of nitrogenase activity was carried out on each sample. Immediately after collection, the root-nodules were separated manually from soil, washed in a jet of tapwater, excised, transferred to 10 ml capacity serum

bottles and sealed with air-tight suba seal. One millilitre of air was withdrawn with the help of syringe, replaced with 1 ml of acetylene (to impart 10% v/v acetylene) . The samples were incubated for 1 h at room temperature and the aliquots of the gas phase (1 ml) were withdrawn and C₂H₄ was analysed in a gas chromatograph (Tracor 540).

The apparatus was fitted with flame ionization detector and 2.13 m stainless steel 0.32 mm diameter column packed with Porapack R. The temperature programme was isothermal; injector 64°C, column 54°C and detector 100°C. All gases used were of purity grade in which following flow rates were maintained : H₂ - 25 ml min⁻¹; O₂ - 100 ml min⁻¹ and N₂ - 25 ml min⁻¹.

The nitrogenase activity was determined using following formula :

$$\text{Nitrogenase activity} = \frac{\text{C}_2\text{H}_4 \text{ area of the sample} \times \text{vol. of gas phase} \times \text{vol. of sample injected}}{\text{Area of C}_2\text{H}_4 \times \text{g}^{-1} \text{ dry nodule} \times \text{incubation period}}$$

Determination of root nodule biomass :

The root nodules were manually separated from soil, thoroughly washed in a jet of water, excised followed by subsequent washing with Tween 80 and water. The

soil free nodules was then placed on filter paper fold to allow excess moisture to be absorbed. Dry weight of nodule was estimated after oven drying the root-nodules at 60°C till constant weight using electronic balance (Sartorius 6110) and was expressed in g per 15 cm⁻³ soil volume.

Population of root nodules :

The soil-free root nodules were further separated into various age class groups i.e. young, medium and old / senescing ones on the basis of their size, shape and colour. Nodule number of each age group was recorded and was expressed as population of nodule of each age group per unit soil volume (15 cm⁻³). A count of total nodule population was also recorded.

Morphology of symbiotic *Frankia*

1. Light microscopy :

The root nodules collected on each occasion were washed thoroughly, excised and soil free nodules were fixed in FAA (formalin, 5 parts : 90% ethyl alcohol, 90 parts : acetic acid, 5 parts) for 48 h. Further processing of root nodules was done following the method of Johansen (1940). Ten FAA fixed nodules were then randomly selected and passed through ethyl alcohol gradients i.e. 50, 70, 80, 90, 95

and 100 % for 30 min each and were embedded in paraffin.

The solidified nodule blocks were trimmed, and cut into 5-10 μ m thick sections at regular intervals along longitudinal plane using a microtome (Weswox, MT 1090 A). The sections obtained were transferred into glass slides, flattened, depaerified with xylene and passed through a gradient of tertiary butyl alcohol (TBA) viz; 30, 50, 70, 80, 90, 95 and 100 % in a descending manner. It was stained with 0.1% toluidine blue in boric acid solution and again dehydrated in above TBA gradient followed by treatment with xylene. Then they were mounted on little amount of DPX and covered with clean coverslips. The slides were oven dried at 45-50°C for 24 h and observed on the phase contrast microscope (Leitz DX 20) for the evaluation of symbiotic *Frankia* in the nodule.

2. Scanning electron microscopy (SEM):

Ten randomly selected young root nodules sampled in each season were washed, excised, free hand sectioned (> 1 mm thick) and were fixed in FAA for 48 h at 4°C. The methods of Lalonde and Devoe (1975) was followed for the preparation of nodules for SEM investigation. The fixed nodules were then washed 3 times in sodium cacodylate buffer (0.2 M, pH 7.0), post fixed in 1% OsO₄ in 0.2 M

cacodylate buffer at 4°C for 2 h and passed through a dehydration series of acetone in water i.e. 30, 50, 70, 80, 90 and 95 % for 30 mins each. The samples were twice dehydrated in 100 % acetone for 30 mins, critical point dried using CO₂ and were coated with a thin layer of gold in an ion sputter (Jeol JFC 1100, Japan). The prepared samples were visualized under the scanning electron microscope (Jeol 35 CF, Japan) at an accelerating voltage of 15 KV.

Isolation of soil *Frankia* :

Soils collected from D₁, D₂, and D₃ depths on each sampling date from Himalayan alder forest were used for the isolation and enumeration of *Frankia*. They were isolated and estimated by following sucrose density fractionation technique (Baker and O'Keefe, 1984) with some modifications. Five g of fresh soil was transferred to sterilized capped 250 ml capacity conical flask and to it 50 ml steam sterilized distilled water was aseptically added, stirred for 10-15 min and allowed to stand for 30 min at room temperature. The soil solution was filtered through a sterile cotton pad in another set of sterile conical flask. Two ml of filtered soil solution was layered over each 60, 45 and 30 % steam sterilized sucrose solution in sterile graduated centrifuge tubes and was capped with flame sterilized aluminium foil. Thereafter, centrifugation

was done at 2,500 g for 15-20 min and the solution in the interface of 60-45 % sucrose was collected using Freeman's automated pipette and inoculated on to Petridishes using pour plate method (dilution 1:100). Different media i.e. standard agar with lecithin and Tween 80, Actinomycete isolation. agar, actidione agar base without actidione Himedia, India) and defined proportionate minimal (DPM) media were tested to isolate *Frankia* from the soil. The composition of DPM medium (Baker and O'Keefe, 1984) is as follows :

Macronutrients	Concentration (g/l)

KH ₂ PO ₄	1.0
MgSO ₄	0.1
CaCO ₃	0.01
Sodium propionate	1.2

Micronutrients	Concentration (mg/l) (Lalonde and Calvert, 1979).

H ₃ BO ₃	1500
MnSO ₄ · 7H ₂ O	800
ZnSO ₄ · 7H ₂ O	600
CuSO ₄ · 7H ₂ O	100
(NH ₄) ₆ Mo ₂₂ O ₇₂ · 4H ₂ O	200
CoSO ₄	10
Agar	10 g

All above media were steam sterilized at 15 psi for 30 min supplemented with micronutrients and treated with 400 mg/l cycloheximide and 15 mg/l of nystatin. The pH of the media was adjusted to 6.8 ± 0.05 before autoclaving. Preliminary test revealed that DPM medium was highly superior to other media. Therefore, further isolation of *Frankia* from soil was performed using only DPM medium. Micronutrient solution suggested by Lalonde and Calvert (1979) was more suitable than that of Hoagland (Hoagland and Arnon, 1950) for *Frankia* growth in synthetic media hence former was incorporated for further investigation. The slow growing, microscopic (<1mm diameter) and microaerophilic (growing submerged in solid agar) colonies were counted. The population of *Frankia* was determined using following formula :

$$\text{Frankia population} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Inoculum(ml)} \times \text{dry wt. of 1g fresh soil}}$$

Morphology of soil *Frankia* :

After 60 days of incubation, slow growing *Frankia* colonies were harvested aseptically with the help of sterile pasteur pipette and were transferred to clean glass slide. They were stained with 0.1 % of toluidine blue in boric acid

for 15 min mounted with the help of 2 - 3 drops of 5 % acidic glycerol and observed under the phase contrast microscope. Different structures of *Frankia* i.e. vesicles, sporangia and hyphae were noted.

Nodule nitrogen content :

Nitrogen (N) content of root-nodule was estimated by semi-microKjeldahl method (Allen, 1974). Oven dried, sieved (<0.2 mm) nodule tissue^{0.2g} was transferred to 500 ml capacity digestion flask and was treated with 2 g K_2SO_4 -HgO catalyst mixture and 3 ml of conc. H_2SO_4 and was digested till the colour of the extract becomes colourless or pale yellow. After digestion, the aliquot was diluted to 50 ml with distilled water and was distilled in the presence of 40 % NaOH.

The released ammonia was absorbed in 4 % boric acid indicator and finally titrated with N/140 HCl. Nitrogen content of nodule was determined as follows -

$$N (\%) = (T - B) \times N \times 140 / S$$

T - sample titration

B - blank titration

N - normality

S - sample weight (g)

Assessment of mycorrhizal infection :

On each sampling, the washed roots were cut into approximately 1 cm long segments. Hundred segments were randomly chosen and transferred to 100 ml conical flask containing 30 ml of 2 % KOH. The samples were cleared and stained following the method of Koske and Gemma (1988). After autoclaving at 121 C for 5 min with KOH, the roots were washed with distilled water, then decolourised with alkaline H_2O_2 and washed with 1 N HCl and water. They were stained with 0.05 % trypan blue and mounted on lactophenol in groups of 20 segments on microscopic slides for observations under phase contrast microscope.

The infection was assessed following root-slide technique of Read *et al.* (1976). The mycorrhizal infection was determined as follows :

$$\text{VAM infection (\%)} = \frac{\text{No. of infected root segments}}{\text{Total no. of root segments examined}} \times 100$$

Determination of mycorrhizal root biomass :

Fine mycorrhizal roots (<2 mm) separated from the soil by floatation method (Mckell *et al.*, 1961) were placed on a 60 μ m sieve followed by thorough washing under

2 jet of tap water. After several washings, the roots were transferred to filter paper to absorb extra moisture from the surfaces of roots. They were placed in a hot air oven at 60° C for 24 h and dry weight was determined on an electronic balance.

Determination of chitin assay :

The methods of Ride and Drysdale (1972) was followed for the assessment of chitin content in infected roots.

Hundred mg of dried and ground roots was boiled in conc. KOH (120 g dissolved in 100 ml distilled water) at 120° C for 30 min. The alkali was removed, employing 75 and 45 % ethanol. Subsequent removal of alkali was done through centrifugation with distilled water. The residue in the form of "chitosan" was deaminated with NaNO_2 , KHSO_4 and $\text{NH}_4\text{SO}_3\text{NH}_2$ which was finally reacted with 3 methyl-2 benzothiazolone - hydrazone hydrochloride (MBTH) and FeCl_3 . The absorbance of the solution was read at 650 nm and glucosamine HCl was also reacted with MBTH and FeCl_3 for comparison. The chitin content was determined from the standard curve of glucosamine HCl and was expressed in ug chitin mg dry root.

Morphology of symbiotic VAM fungi

1. Light microscopy :

The VA mycorrhizal roots of *A. nepalensis* sampled at each season was washed several times in water, which was then cut into 1 cm segments, cleared and stained as described earlier (Koske and Gemma, 1988). The cleared and stained mycorrhizal roots were mounted on lactophenol in a bunch of 20 root segments on clean glass slides. The root segments were viewed under a phase contrast microscope. Different structures of VAM fungi, i.e. vesicles and arbuscules were recorded.

2. Scanning electron microscopy (SEM) :

The soil-free mycorrhizal roots of various seasons were hand sectioned in transverse plane (> 2 mm), fixed in FAA for 48 h at 4 °C, washed 3 times in sodium cacodylate buffer and prepared similarly as described for root nodule. The gold coated root sections were mounted vertically on copper stubs and viewed in a scanning electron microscope at an accelerating voltage of 15 KV.

Assessment of endogonaceous spores :

The endogonaceous spores were isolated and

enumerated from soil samples by wet sieving and decanting technique of Gerdemann and Nicolson (1963). Ten g. of air dried soil was suspended in 2 L of tap water, stirred with glass rod for 5 min and was allowed to stand for 25-30 mins. Clear soil suspension was passed through a series of sieves of different pore sizes ranging from 500 to 60 μ m. The sievings were transferred to glass beakers and filtered through Whatman no. 1 filter paper. Each sieving collected on filter paper was scanned under a stereo-microscope for the enumeration of endogonaceous spores. A count of total fungal spores and their individual abundance was noted. They were picked up manually and identical spores were separated and stored in Ringer's solution. They were mounted on polyvinyl lactophenol (PVL) on glass slides. Ringer's solution and PVL medium were prepared as described below.

Ringer's solution :

Sodium chloride	= 7.5 g
Potassium chloride	= 0.075 g
Calcium chloride	= 0.1 g
(anhydrous)	
Sodium bicarbonate	= 0.1 g
(anhydrous)	

All above constituents were dissolved in 1L of water and pH of the solution was adjusted to 7.4 with 1N

163147.



NaOH/ 1N HCl.

PVL Medium :

Polyvinly lactophenol medium was prepared by dissolving 15 ml of PVA granules in 100 ml of distilled water on a water bath at 80 C for 48 h . The solution was mixed with 22 parts each of lactic acid and phenol. The resulting solution form PVL medium was used for permanent mount of endogonaceous spores. The endogonaceous spores were identified upto species level following the keys *proposed* by Hall and Fish (1982).

Statistical analysis :

Data for plant and soil materials were subjected to standard error (S.E) and lattic square design (LSD). The coefficient of correlation was calculated by using Karl Pearson's correlation coefficient (Zar, 1974).

RESULTS

Soil temperature :

A marked seasonal variation in the soil temperature was observed. The soil temperature was significantly ($P < 0.05$) high during rainy and low during

winter season. In a given season, the soil temperature of three soil depths i.e. D_1 , D_2 and D_3 also varied (Fig. 2).

Soil moisture :

Seasonality in the soil moisture content was evident, which was recorded significantly ($P < 0.05$) high during rainy and low during winter season. In rainy season, soil depths D_1 and D_2 contained more moisture content however, it was recorded more in soil depth D_3 during winter season (Fig. 3).

Soil pH :

The soil at all depths exhibited acidic reaction throughout the year but more acidic values were recorded from upper soil depths during winter. Generally, significant ($P < 0.05$) difference in the soil pH was recorded among various soil depths in any given season (Fig. 4).

Organic matter :

A distinct seasonal variation in the soil organic matter content was evident. Most of the organic matter was restricted to soil depth D_1 , which decreased with increase in soil depth. Further significantly ($P < 0.05$) high

content of organic matter was noted during rainy and low during winter season (Fig. 5).

Total nitrogen :

In general, total soil nitrogen was significantly ($P < 0.05$) high during rainy and low during winter. Like organic matter, soil nitrogen content was highest in soil depth D_1 and its content declined with increase in soil depth (Fig. 6).

Available phosphorous :

Available soil phosphorous also exhibited a distinct flux and was affected by seasonal changes. It was generally recorded significantly ($P < 0.05$) high during rainy and low during winter season. The soil phosphorous level was significantly ($P < 0.05$) high in soil depths D_1 and low in D_3 (Fig. 7).

Exchangeable potassium, calcium and magnesium :

Exchangeable potassium (K), calcium (Ca) and magnesium (Mg) exhibited the similar trend throughout the year. Their concentration was significantly ($P < 0.05$) high during rainy and low during winter season. However, unlike N and P, exchangeable cations were more in D_2 and D_3 soil depth

than D_1 during rainy and following seasons. Generally in most of the seasons significant ($P < 0.05$) variation in the exchangeable cations content was observed amongst the different soil depths (Fig. 8,9,10).

Extractable manganese :

Plant available Mn content of the soil also followed the same pattern as that of cations. However, more Mn was recorded in D_1 and decreased with increase in soil depth (Fig. 11).

Total soil phosphatase activity :

A distinct variation in the phosphatase activity of soil as modulated by seasonal changes of environmental conditions was observed. The soil exhibited significantly ($P < 0.05$) high phosphatase activity during winter and low during rainy season. In majority of instances, soil depth D_1 showed higher phosphatase activity than corresponding lower soil depths (Fig. 12).

Dehydrogenase activity :

The soil exhibited a marked change in the dehydrogenase activity as influenced by seasonal changes. Generally dehydrogenase activity of soil was significantly

($P < 0.05$) high during winter and low during rainy season. The activity was recorded higher in the soil depth D_1 which decreased exponentially in the subsequent soil depths (Fig. 13).

Nitrogen fixation rate of root nodule :

The nitrogen fixing (nitrogenase) activity of *A. nepalensis* root nodules varied markedly in various seasons. In the present investigation rate of nitrogen fixation by root nodules was recorded high during rainy season which ranged from 18.2 – 20.3 $\mu\text{moles C}_2\text{H}_4 \text{ g}^{-1} \text{ dry nodule h}^{-1}$ and dropped to a minimum value of 3.0 during mid-winter. A rise in the nitrogenase activity was evident on the arrival of spring and persisted till the end of autumn (Fig. 14).

Nodule biomass :

Rate of production of nodule by actinorhizal Himalayan alder varied markedly amongst various seasons of the year. Nodule production per unit area reached a peak stage during rainy season and was least during winter season (Fig. 15). However, nodules were encountered only from the soil depth D_1 and especially abundant in upper 0 – 10 cm soil. The root-nodules were completely absent in soil depths

D₂ and D₃.

Population dynamics of root nodules :

Total number of root-nodules was significantly ($P < 0.05$) high during rainy and low during winter season thus marking a clear seasonality in terms of total nodule population (Fig. 15). Qualitatively too, population of young and medium nodules was markedly dominant over old nodules during mid-rainy than that in other corresponding seasons of the year (Fig. 16). During winter, ratio of young + medium (active nodules) to old nodules decreased considerably and the reversed was true during rainy season. Pale-white and spongy nodules were formed abundantly on the advent of spring.

Morphology of *Frankia* in vivo :

The morphology of symbiotic *Frankia* in the young root-nodule was generally characteristic for each seasons of the year. Symbiotic *Frankia* usually colonized cortical cells of the nodule and extended till the endodermis or pericycle. Colonization by *Frankia* was maximum during winter and minimum during spring season. The percentage of cells containing *Frankia* vesicles in the apical zone of the nodule was markedly low and high during winter and rainy season

respectively (Fig. 17; Plate 3 & 4). In middle and basal zone of nodule, the percentage of colonized cells with vesicles was high during rainy season compared to following seasons (Fig. 17; Plate 5, 6, 7 & 8). However, the vesicle size increased in time where older part of nodule i.e. basal zone contained lesser and bigger sized vesicles. Production of sporangia in the middle and basal zone of nodule exhibited a clear cut seasonal variation. Maximum percentage of host cells with sporangia was observed during winter, followed by autumn season and total absence during spring season (Fig. 18; Plates 5, 6, 7 & 8). Further, basal part of nodule harboured more *Frankia* sporangia than middle whereas apical zone lacked sporangia at all seasons (Fig. 18; Plates 3, 4, 5, 6, 7 & 8).

Similarly, number of host cells with *Frankia* hyphae/microcolonies in various zones of nodule showed marked difference among various seasons (Fig. 19; Plates 3, 4, 5, 6, 7 & 8).

Morphology of *Frankia* in Vitro :

Frankia colony appeared after 7 weeks of incubation and exhibited slow growth on DPM medium. Their colony size was <1 mm in diameter and grew submerged in agar. The identifying morphological features which characterized

Frankia were the presence of thin $< / > 1$ μ m diameter, cushion-like hyphae, electron dense vesicles borne on a short stalk on parental hyphae and presence of typical sporangia (Plate 9).

***Frankia* population :**

The population of soil *Frankia* exhibited a distinct seasonal variation. Total platable *Frankia* propagules ranged from 562 to 10,982 g dry soil. Significantly ($P < 0.05$) high number of *Frankia* was recorded during rainy and low during winter season. Besides, majority of soil *Frankia* were confined to soil depth D_1 and decreased exponentially with increasing soil depth (Fig. 20).

Nitrogen content of root nodule :

A marked variation in the seasonal nitrogen content of nodule was noted. Nodule nitrogen content was statistically ($P < 0.05$) high during rainy and low during winter season (Fig. 21).

Mycorrhizal infection :

Mycorrhizal infection by VA endophyte was recorded high (>90 %) in all the seasons of the year. Besides, the seasonal variation in the intensity of the VAM

infection was not clearcut which, however, showed higher infection during winter and low during rainy season (Fig. 22). Further, like root nodules, mycorrhizal roots were confined only to soil depth D_j and absolutely absent beyond 15 cm depth.

Mycorrhizal root biomass :

The dry weight of fine mycorrhizal roots was markedly affected by seasons. Maximum biomass of mycorrhizal roots was recorded during winter (0.402 - 0.42 g per 15 cm soil volume) and was lowest (0.27 - 0.29 g per 15 cm) during rainy season (Fig. 23).

Chitin content :

The chitin content of VAM fungi in mycorrhizal roots of Himalayan alder showed a clearcut seasonal pattern. It was high, 63 ug mg^{-1} dry root during winter and declined to a minimal value of 32 ug during rainy seasons (Fig. 22). Chitin assay unlike mycorrhizal infection provided more clearcut picture. However, both the techniques led to a similar pattern of fungal colonization of host root.

Morphology of VAM fungi *in vivo* :

The morphology of VA endophyte colonizing mycorrhizal root was found to be distinctive in different seasons. The seasonal variation in colonization of outer and inner cortex was however, not distinct. Endophytic fungi produced more arbuscules in the rainy season than following seasons. Besides, vesicle population increased with the increase in time. Ratio between arbuscule and vesicles decreased and reached a minimal value during winter season (Fig. 24; Plates 10 & 11). In fact, arbuscules degenerated after growing seasons (April - October) and was almost absent in mid-winter. VAM fungal endophyte in some cases varied in the morphology especially the diameter of hyphae (Plate 11).

Endogonaceous spore dynamics :

Majority of the endogonaceous spores were confined to D_1 and D_2 soil depths which decreased exponentially with further increase in soil depth. Its number increased exponentially during winter and decreased during rainy season (Table 1). Various types of spores were encountered which showed varied abundance in various seasons and soil depth. *Glomus mosseae* was found to be dominant in almost all seasons (Table 2).

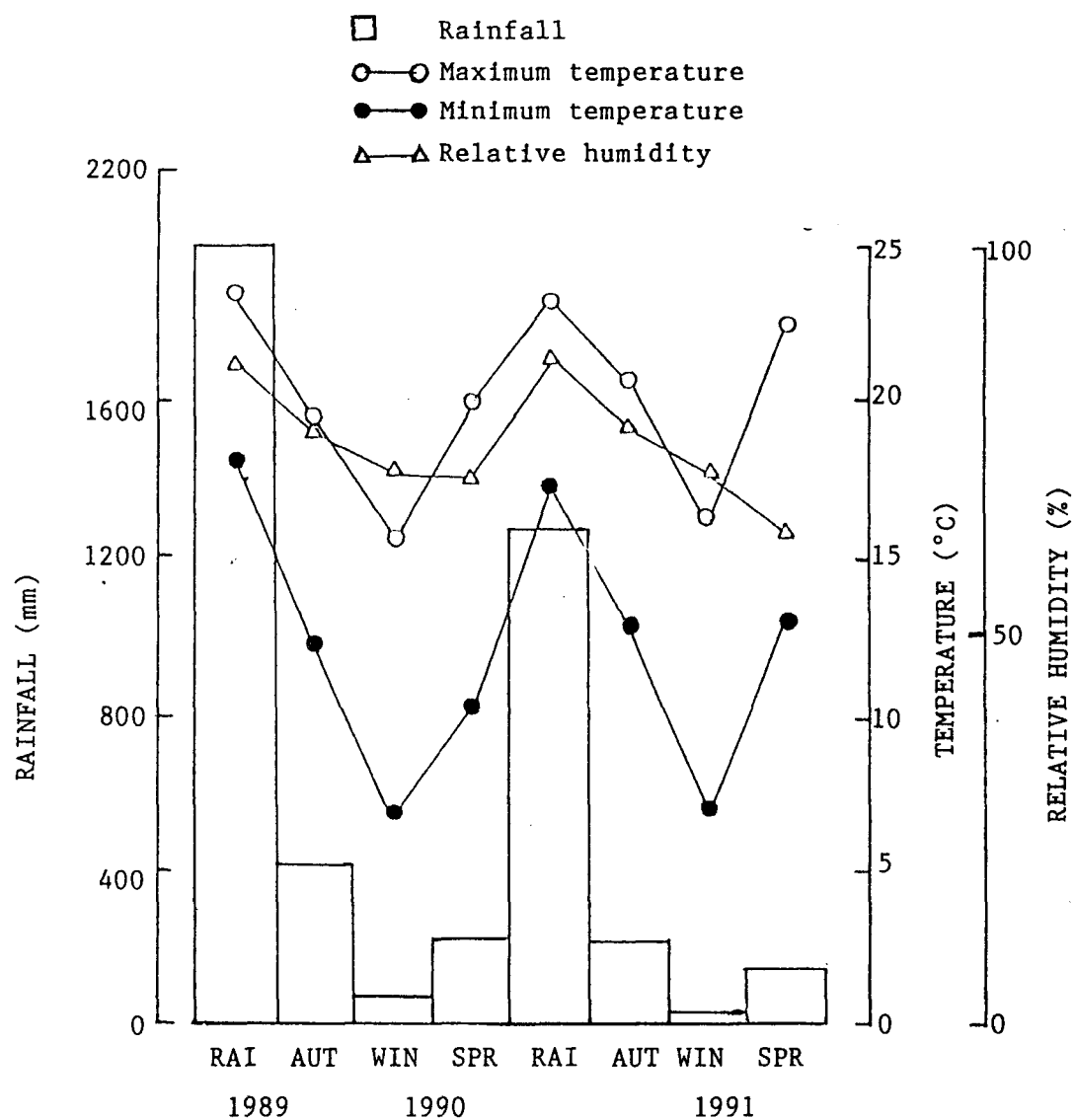


Fig.1. Meteorological data including rainfall, maximum and minimum temperature and relative humidity of the study site.

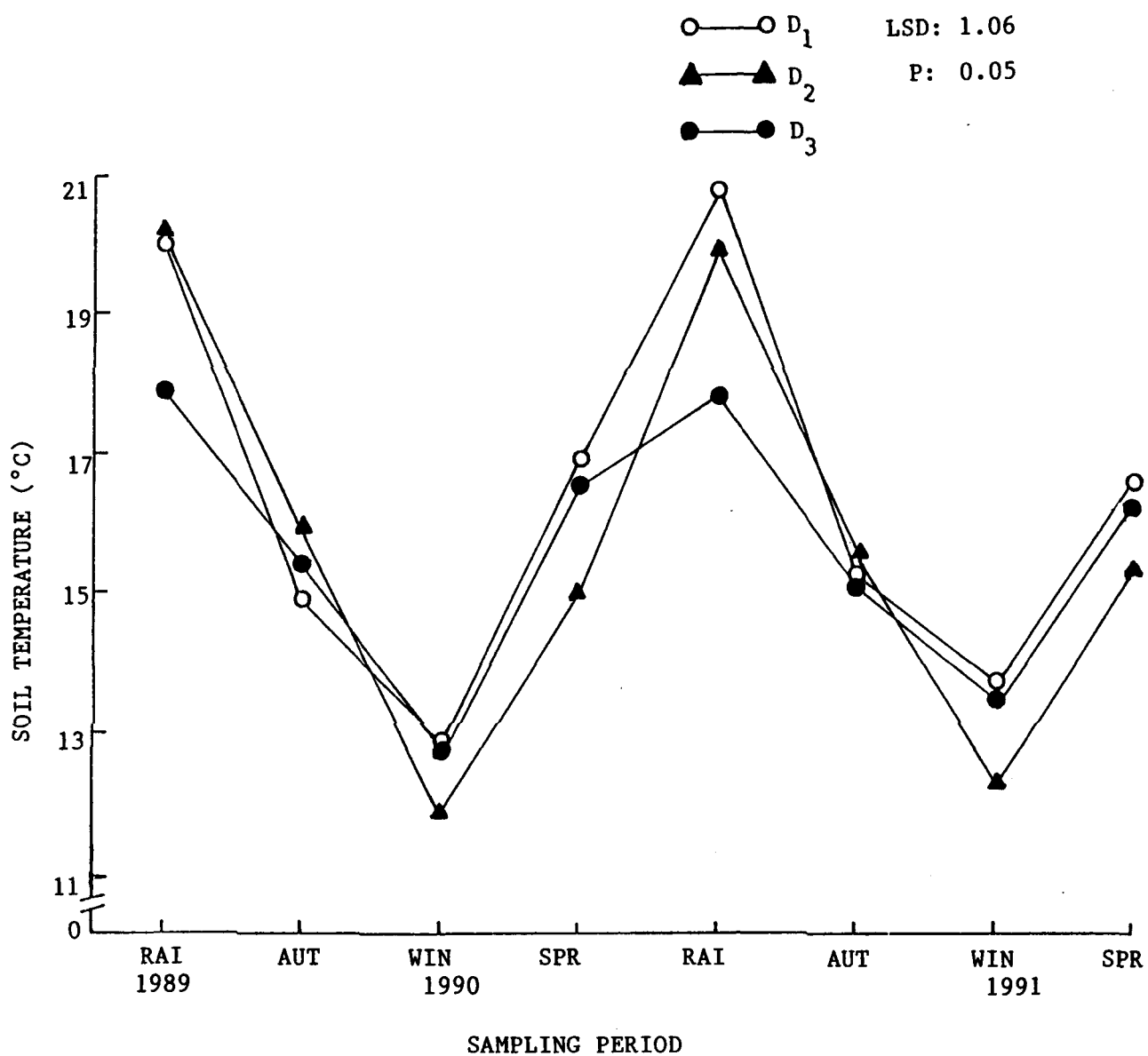


Fig.2. Variation in the soil temperature in various soil depths and seasons (D_1 = 0-15 cm; D_2 = 15-30 cm; D_3 = 30-45 cm).

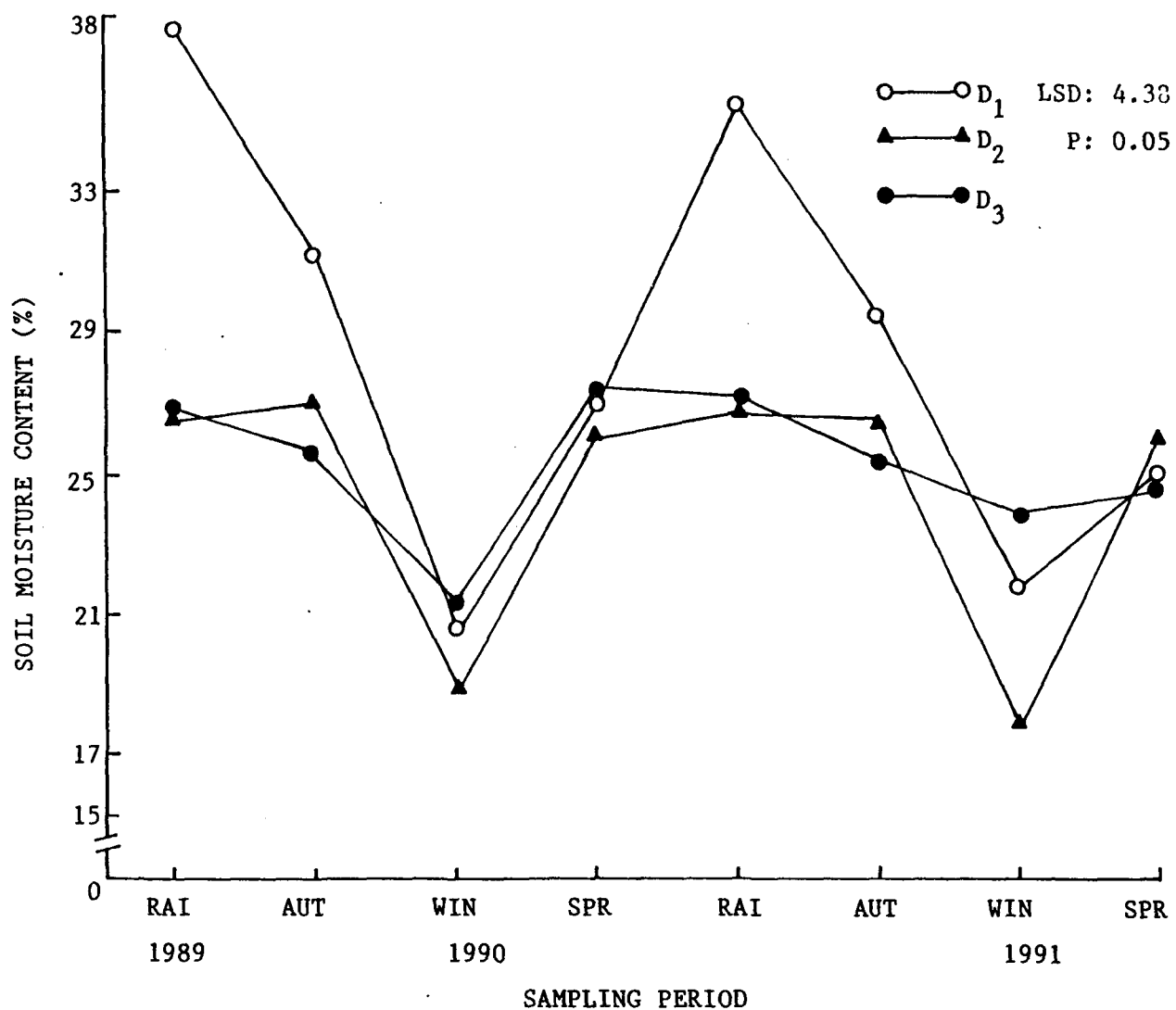


Fig. 3. Seasonal and depthwise changes in the soil moisture content (%) of Himalayan alder forest stand (D₁ - 0-15 cm; D₂ - 15-30 cm; D₃ - 30-45 cm).

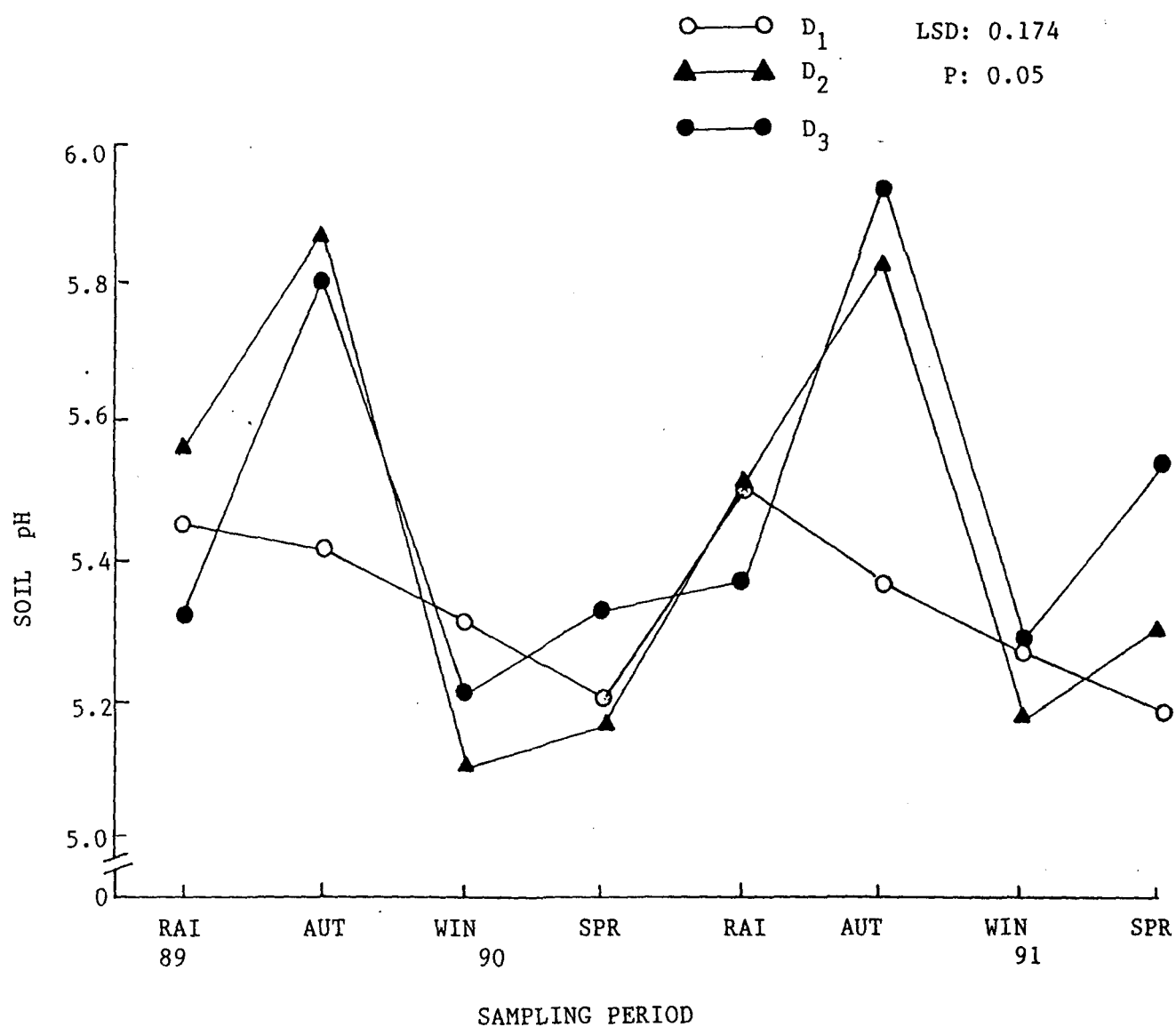


Fig.4. Seasonal and depthwise changes in the soil pH in A. nepalensis forest stand .

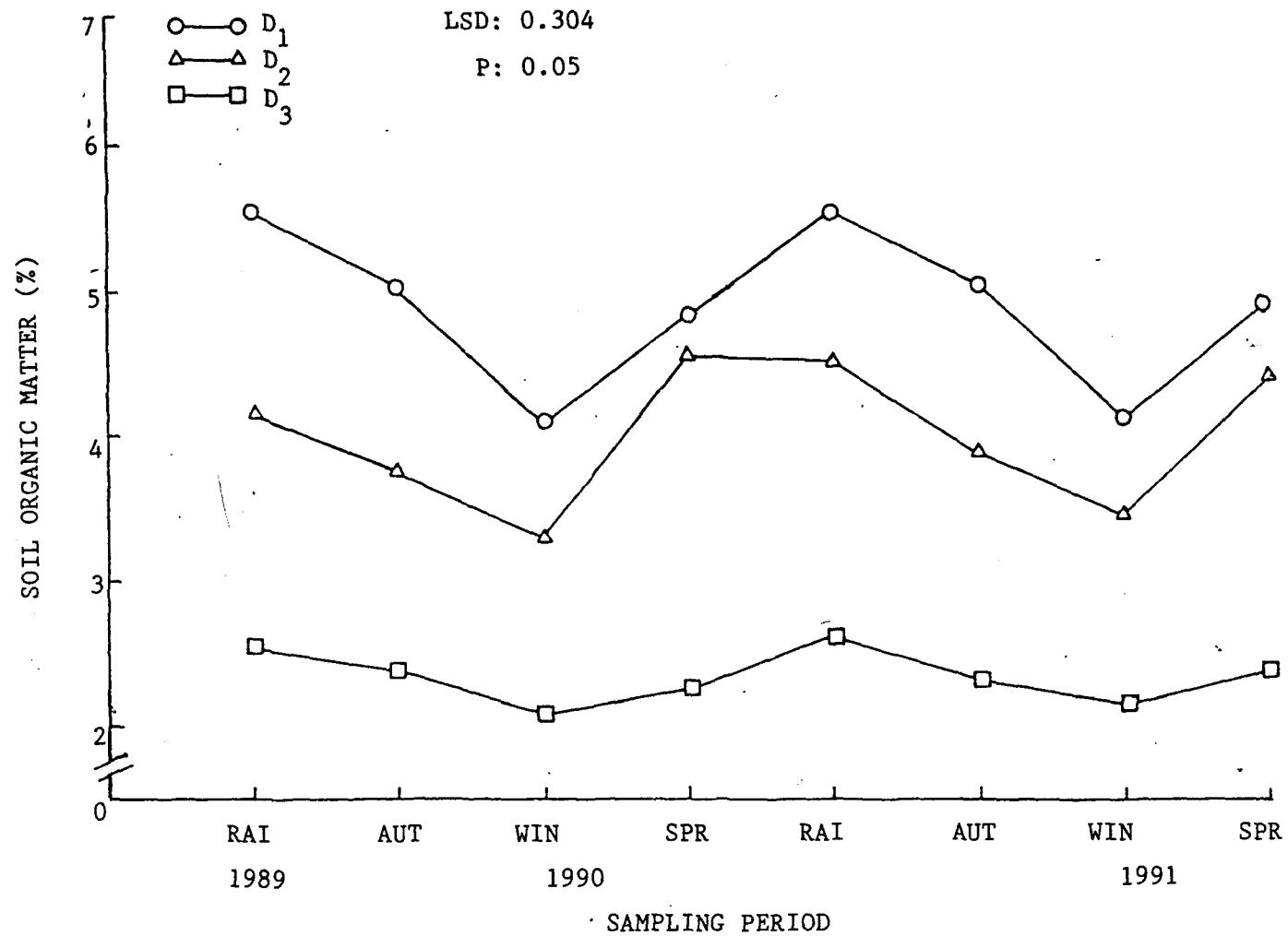


Fig.5. Seasonal changes in organic matter content (%) of soil at various soil profile (D₁, 0-15 cm; D₂, 15-30 cm; D₃, 30-45 cm).

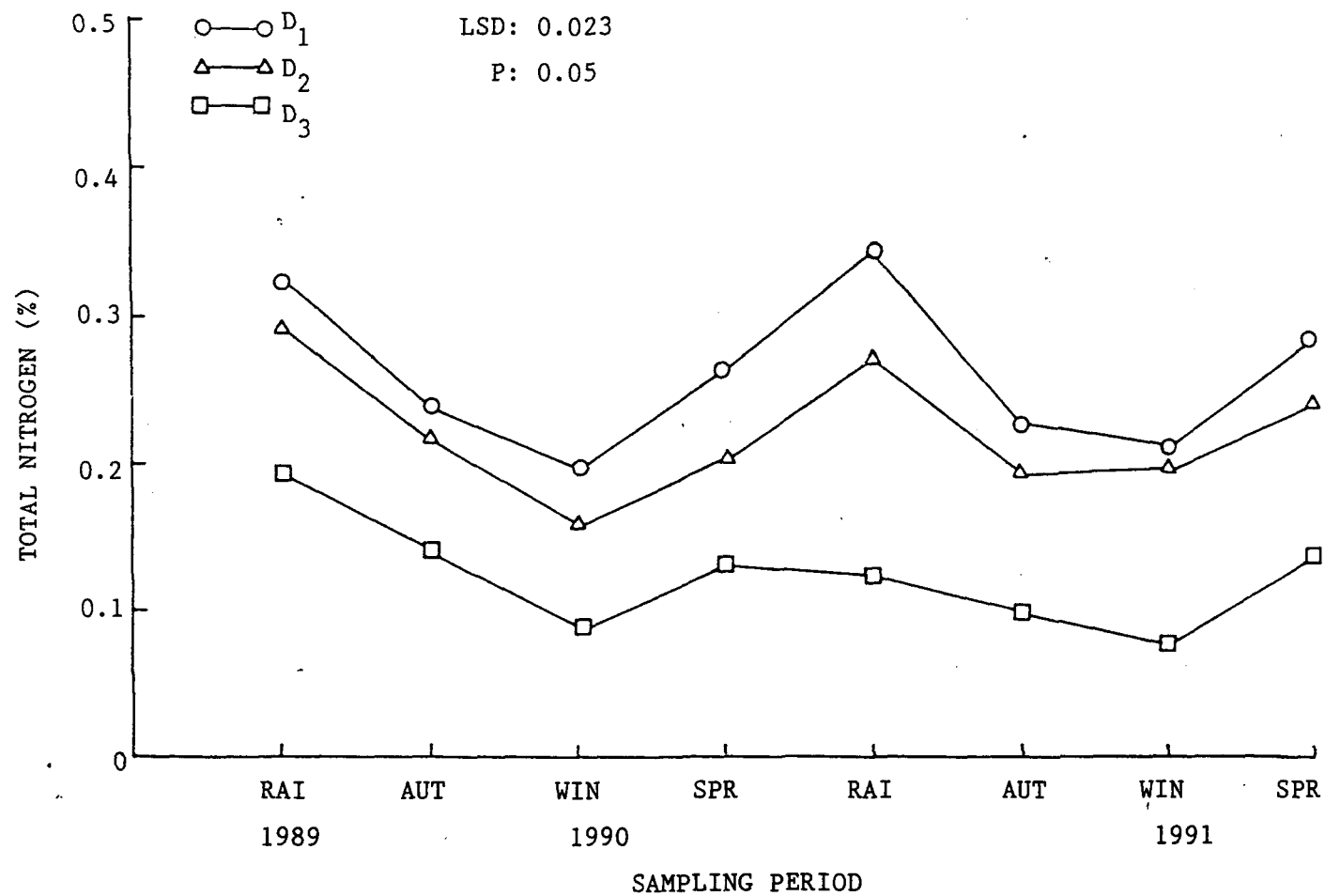


Fig.6. Seasonal variation in total nitrogen content (%) at various soil depths under A. nepalensis stand (D_1 , 0-15 cm; D_2 , 15-30 cm; D_3 , 30-45 cm).

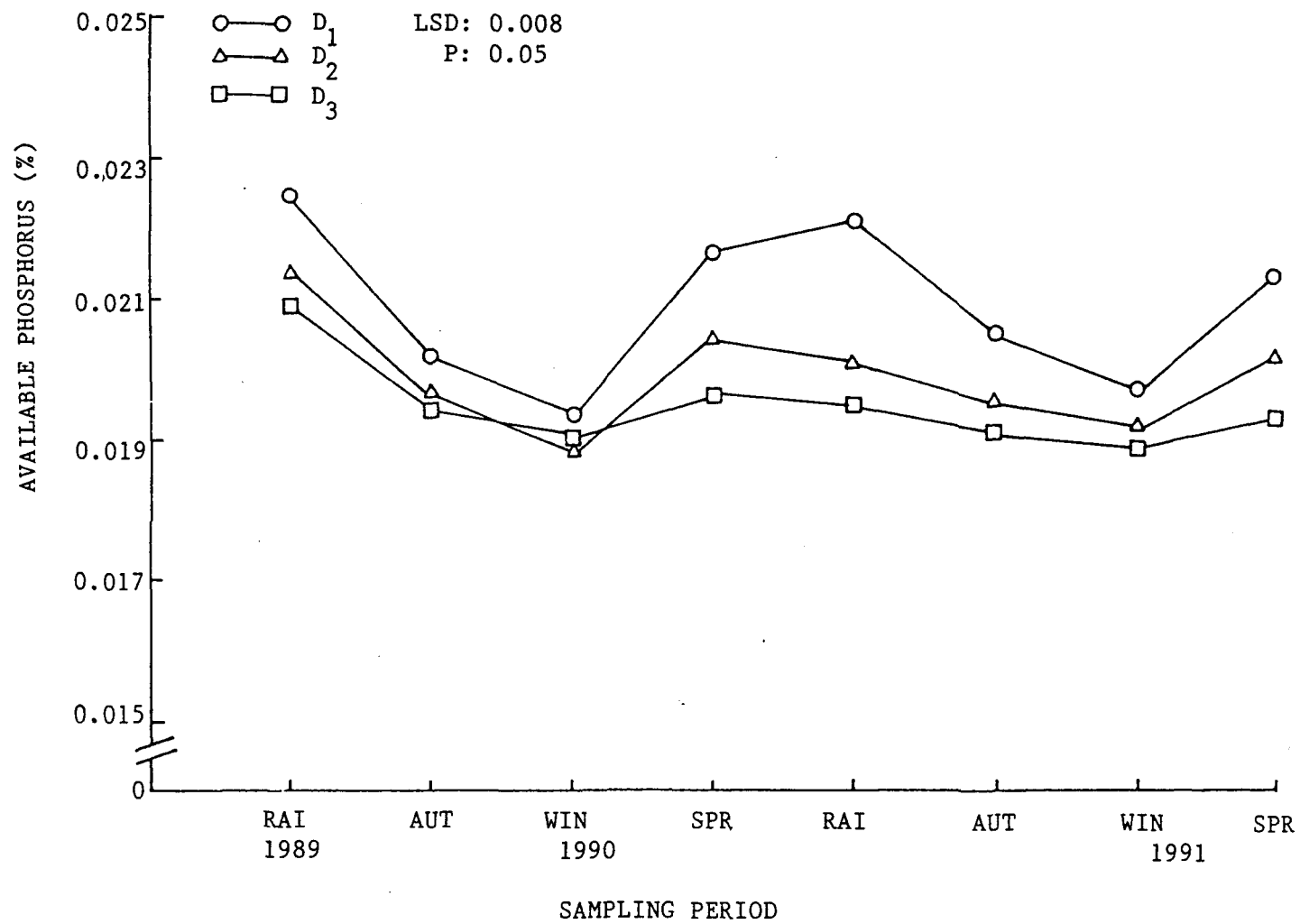


Fig.7. Seasonal variation in available phosphorus (%) content of soils of various depths (D₁, 0-15 cm; D₂, 15-30 cm; D₃, 30-45 cm).

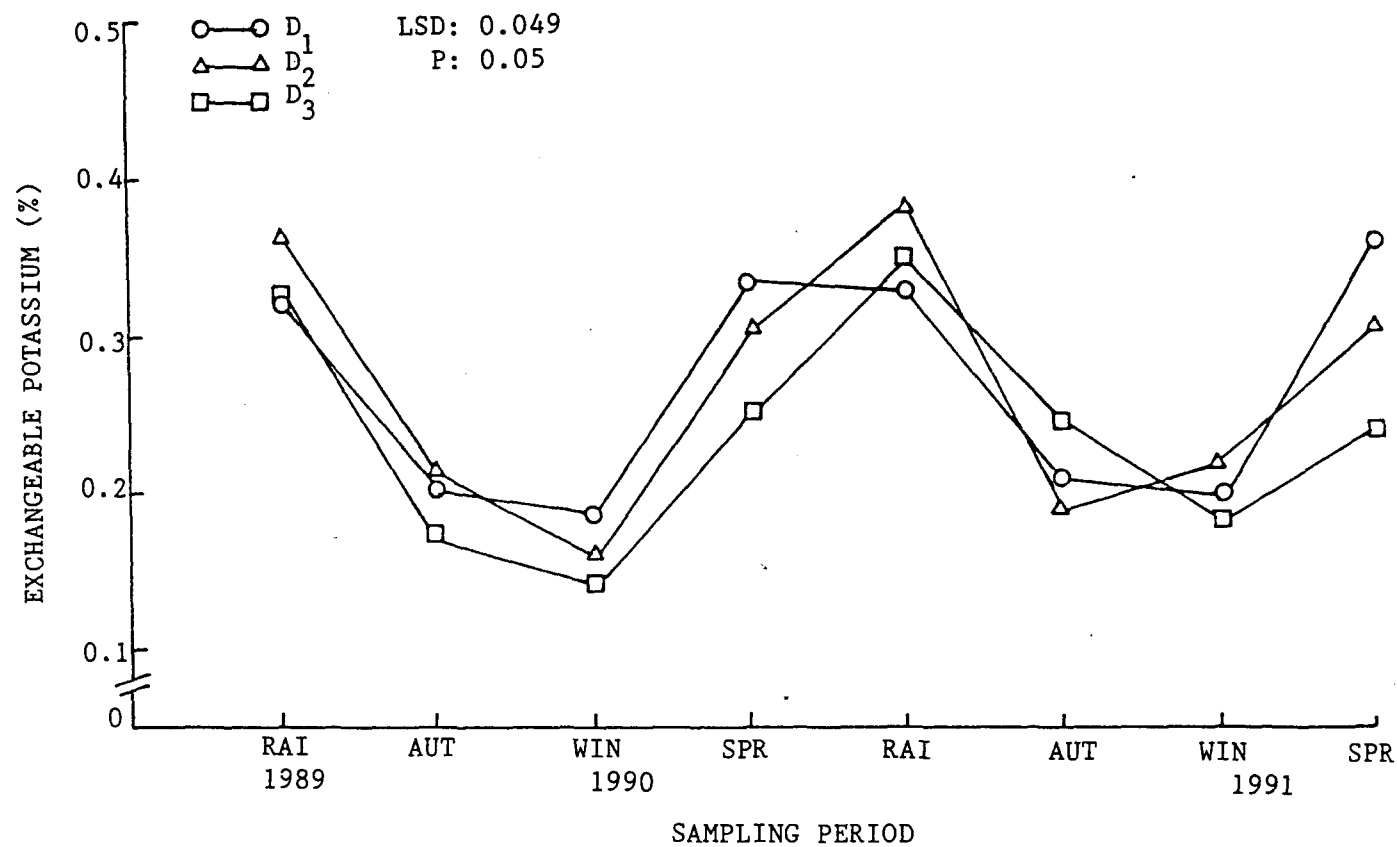


Fig. 8. Seasonal variation in the exchangeable potassium (%) contents at various soil depth at alder forest stand (D₁, 0-15 cm; D₂, 15-30 cm; D₃, 30-45 cm).

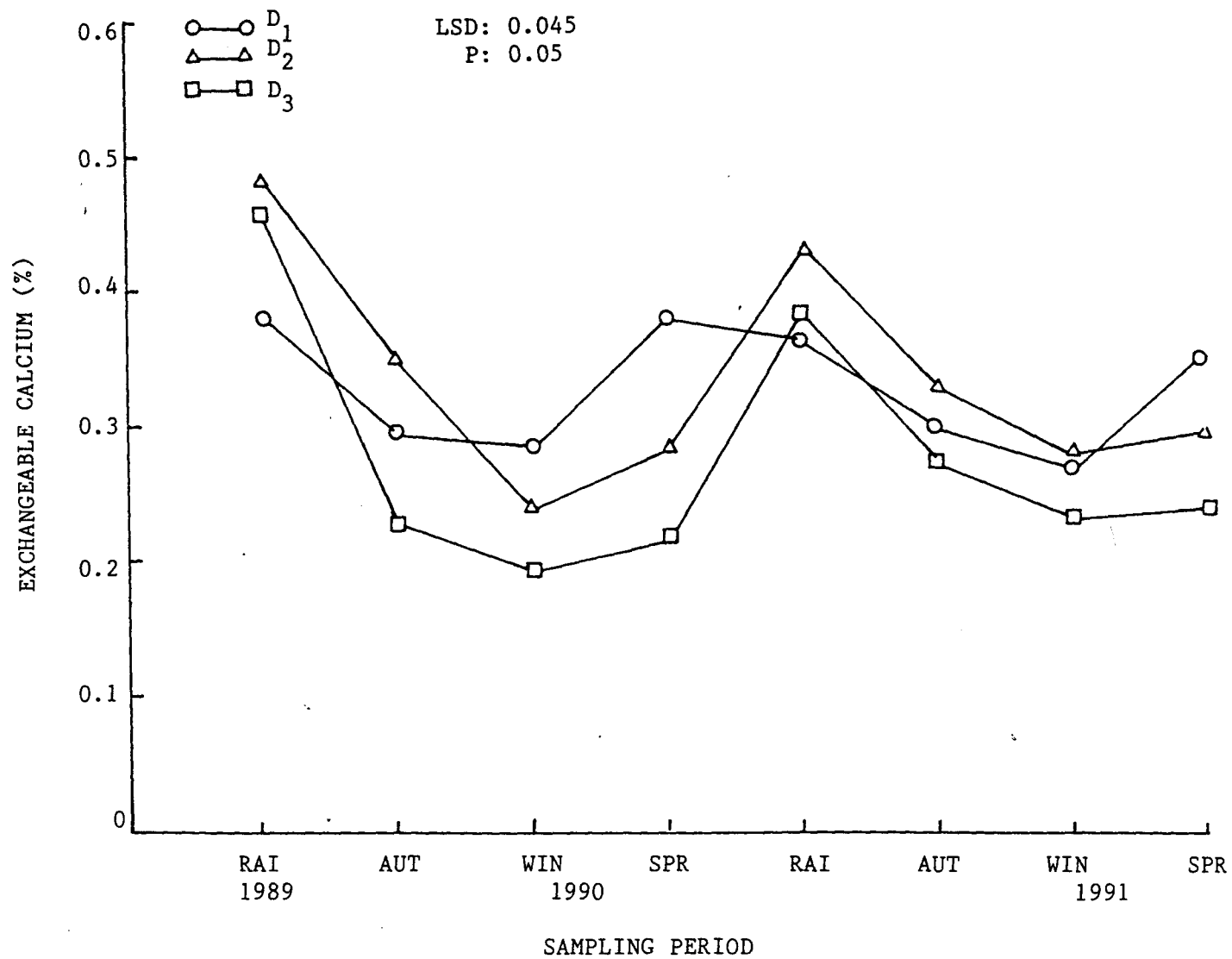


Fig. 9. Seasonal variation in exchangeable calcium content (%) of soil of different depths (D₁, 0-15 cm; D₂, 15-30 cm; D₃, 30-45 cm).

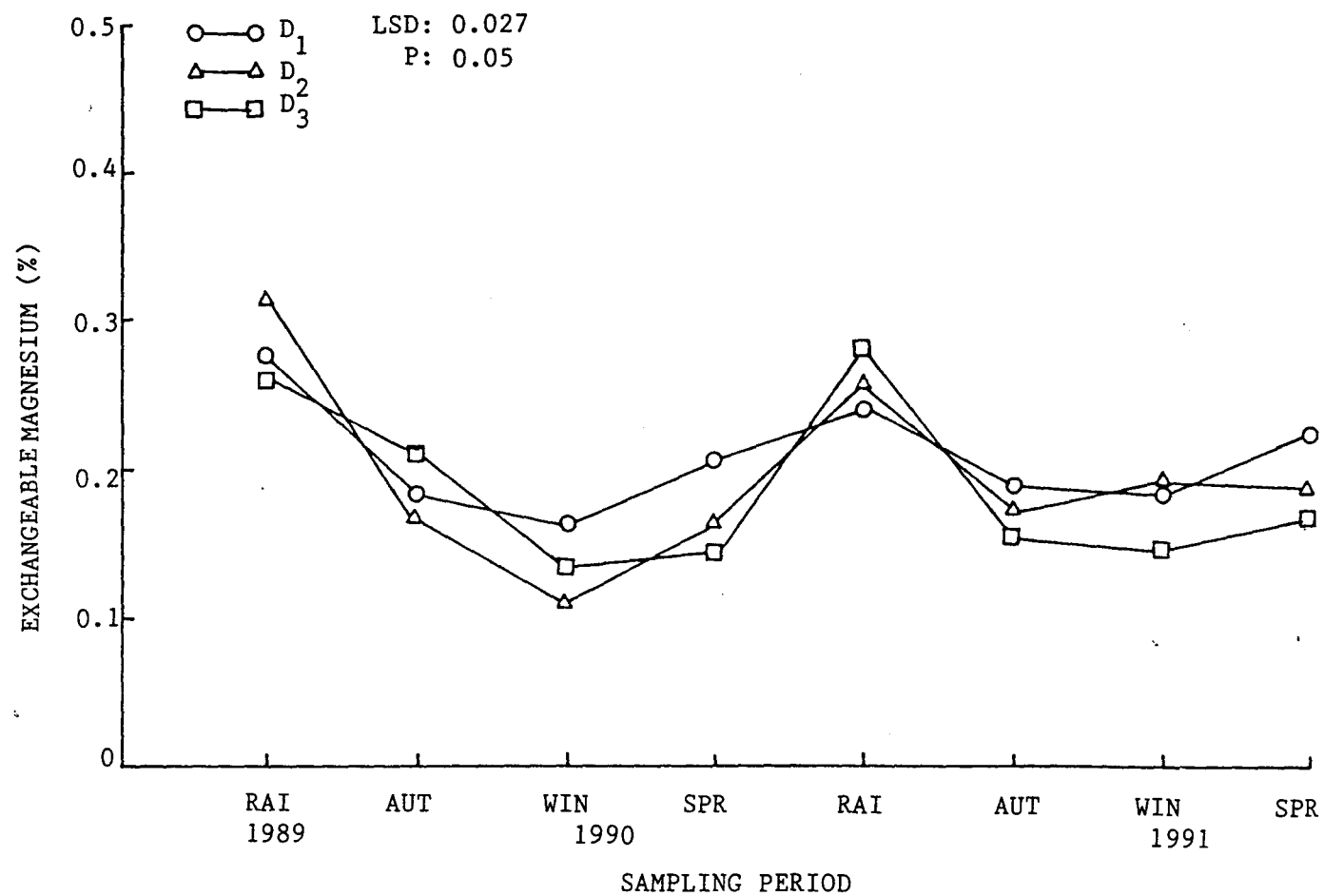


Fig.10. Seasonal variation of exchangeable Magnesium content of soil of different depths (D_1 , 0-15 cm; D_2 , 15-30 cm; D_3 , 30-45 cm).

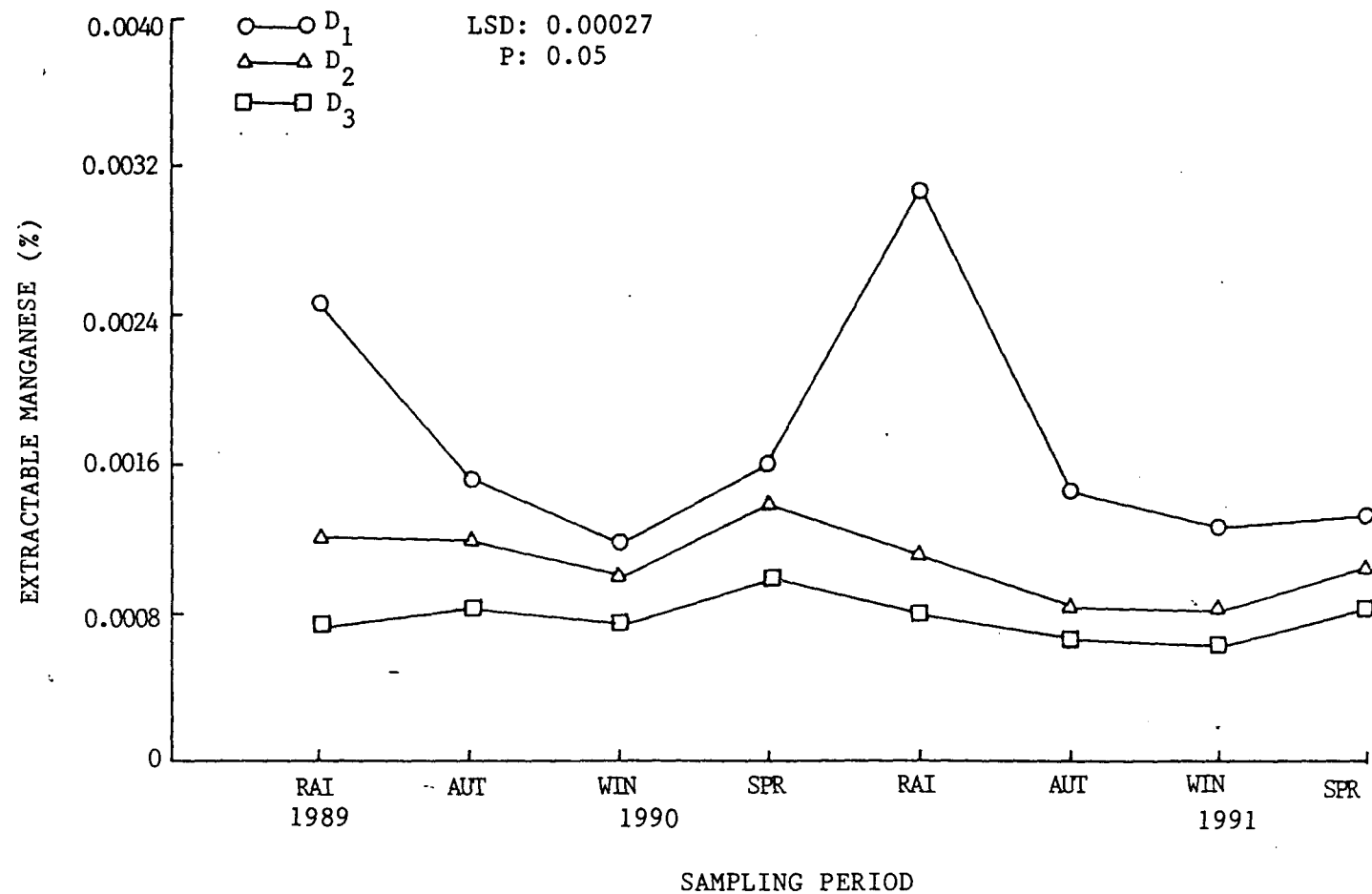


Fig. 11. Seasonal variation in the extractable manganese (%) content of alder forest stand soil (D₁, 0-15 cm; D₂, 15-30 cm; D₃, 30-45 cm).

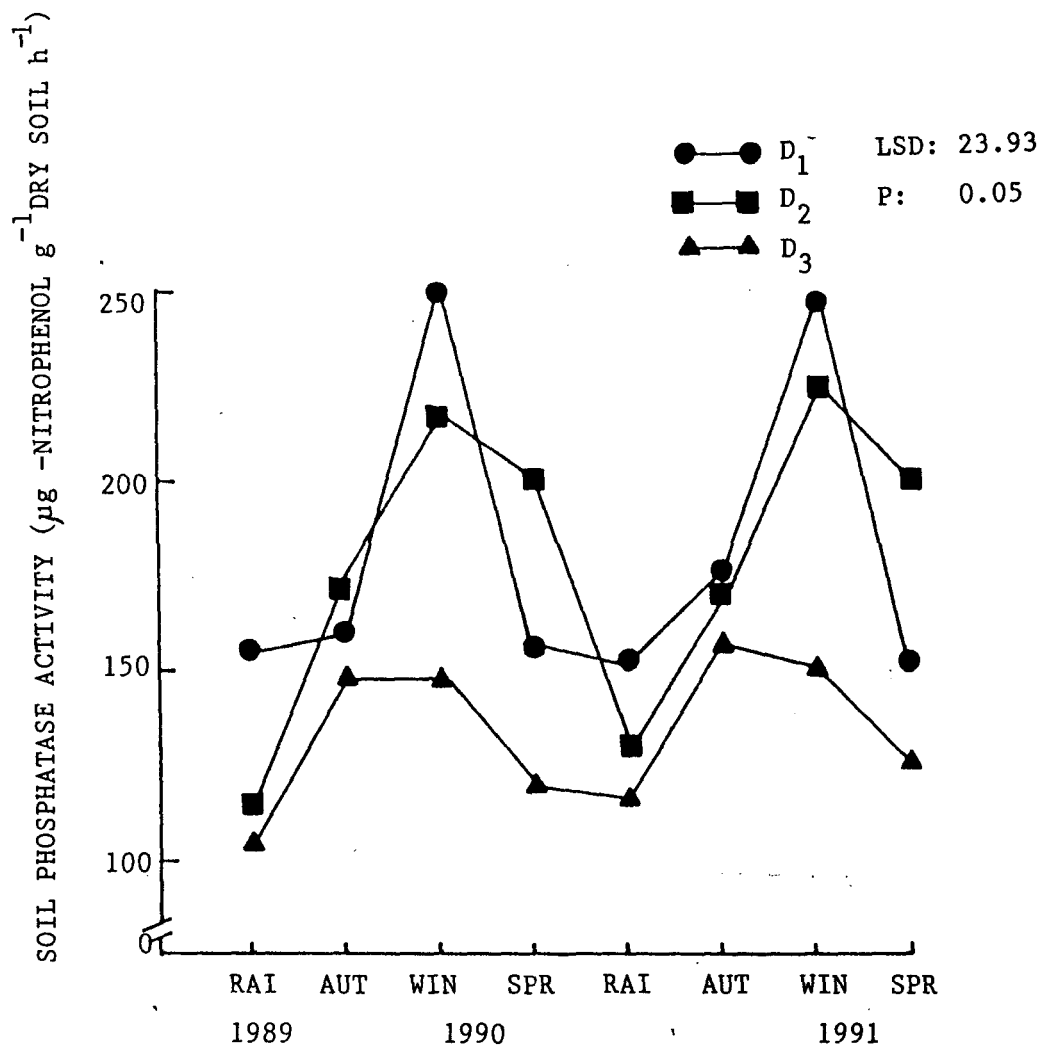


Fig.12. Seasonal variation in soil phosphatase activities of forest supporting A. nepalensis (D_1 , 0-15 cm; D_2 , 15-30 cm; D_3 , 30-45 cm).

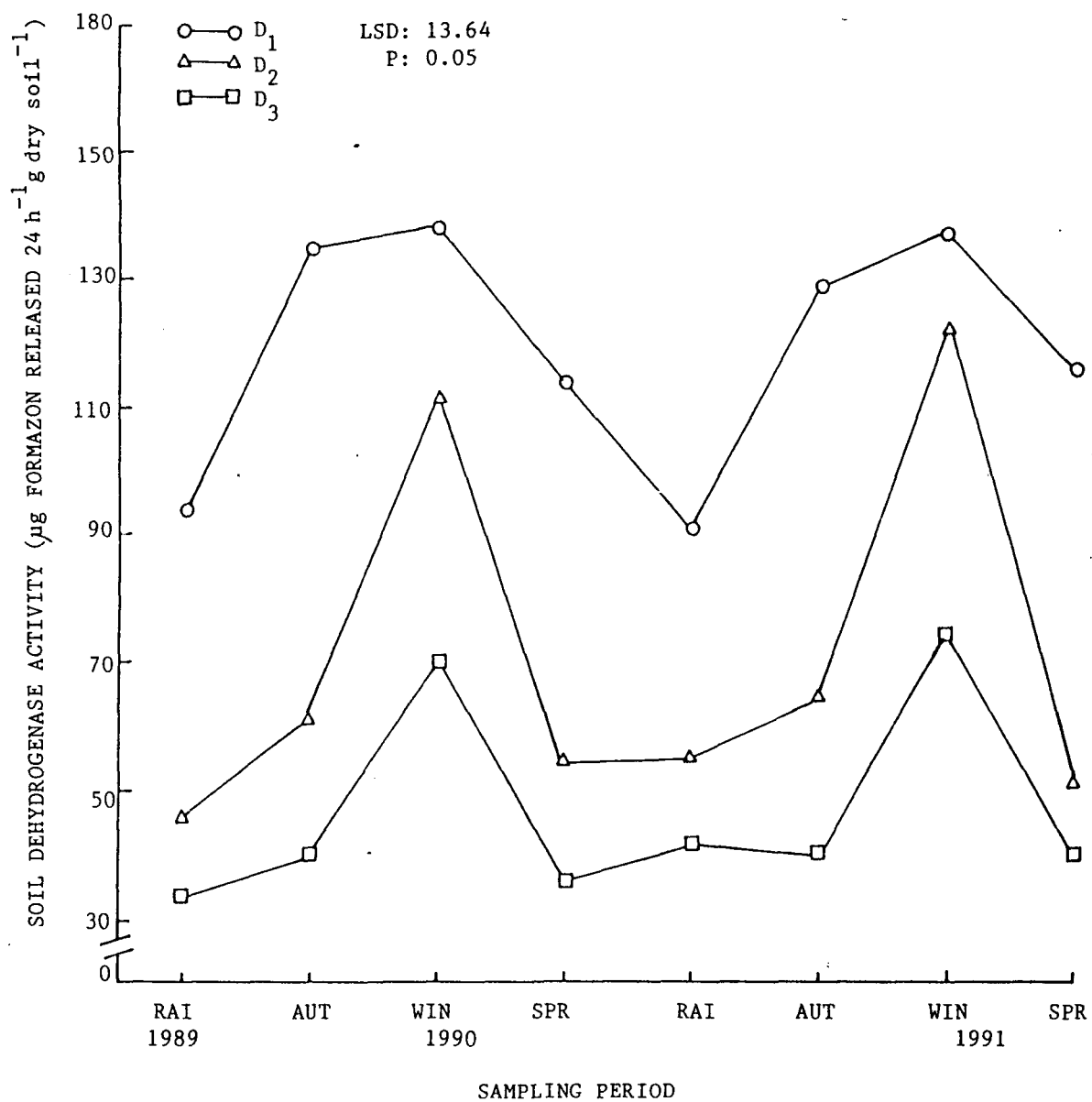


Fig.13. Seasonal variation in the soil dehydrogenase activity (D_1 , 0-15 cm; D_2 , 15-30 cm; D_3 , 30-45 cm).

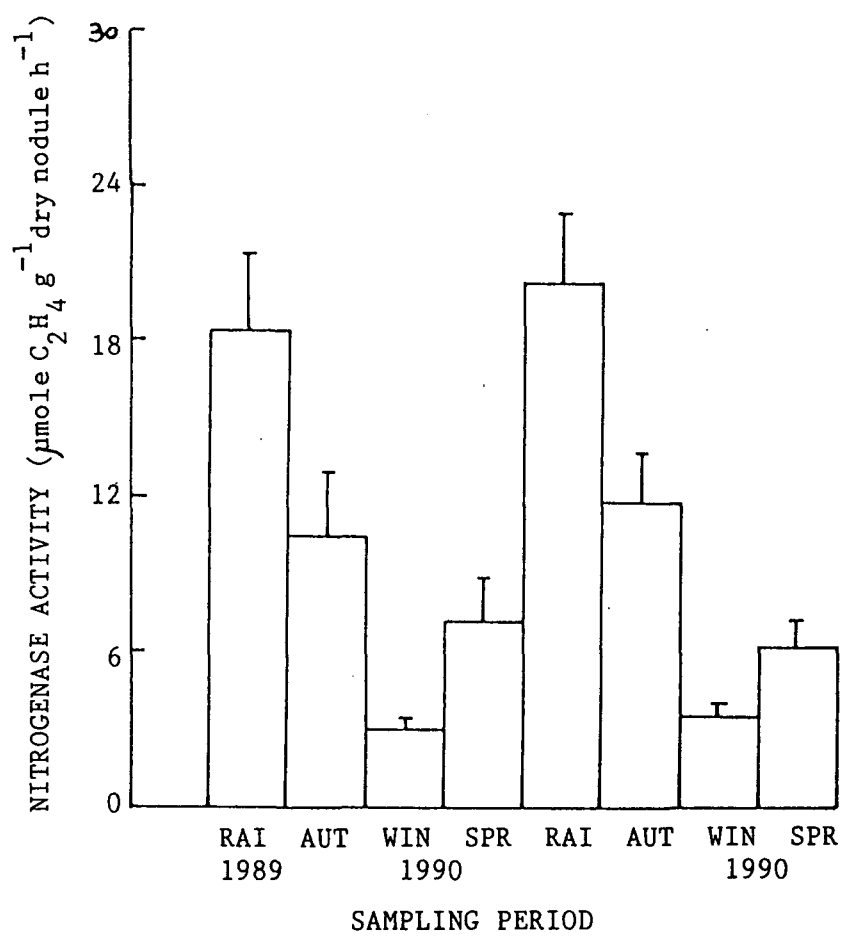


Fig.14. Seasonal variation in nitrogenase activity (Mean $\pm$ SE) of root nodules of A. nepalensis .

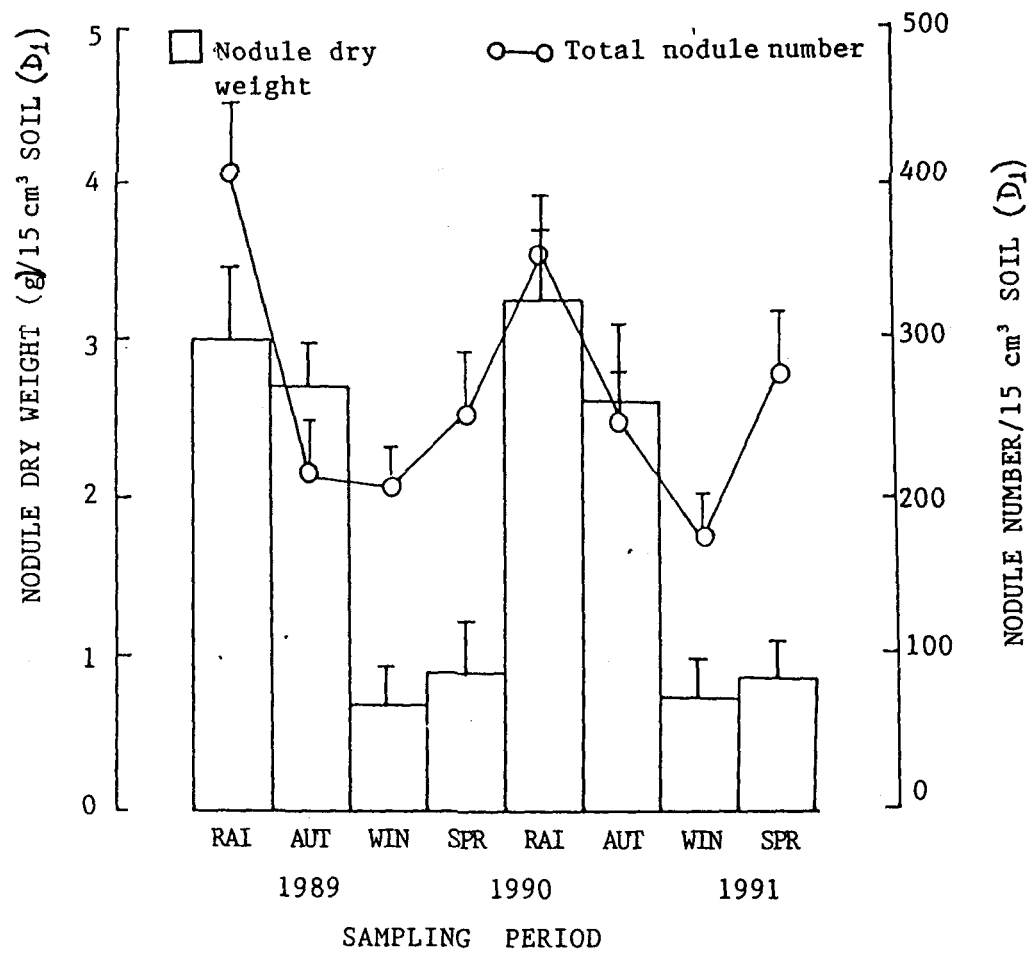


Fig.15. Seasonal variation in nodule dry weight and total nodule number per unit soil volume (D_1) under A. nepalensis stand.

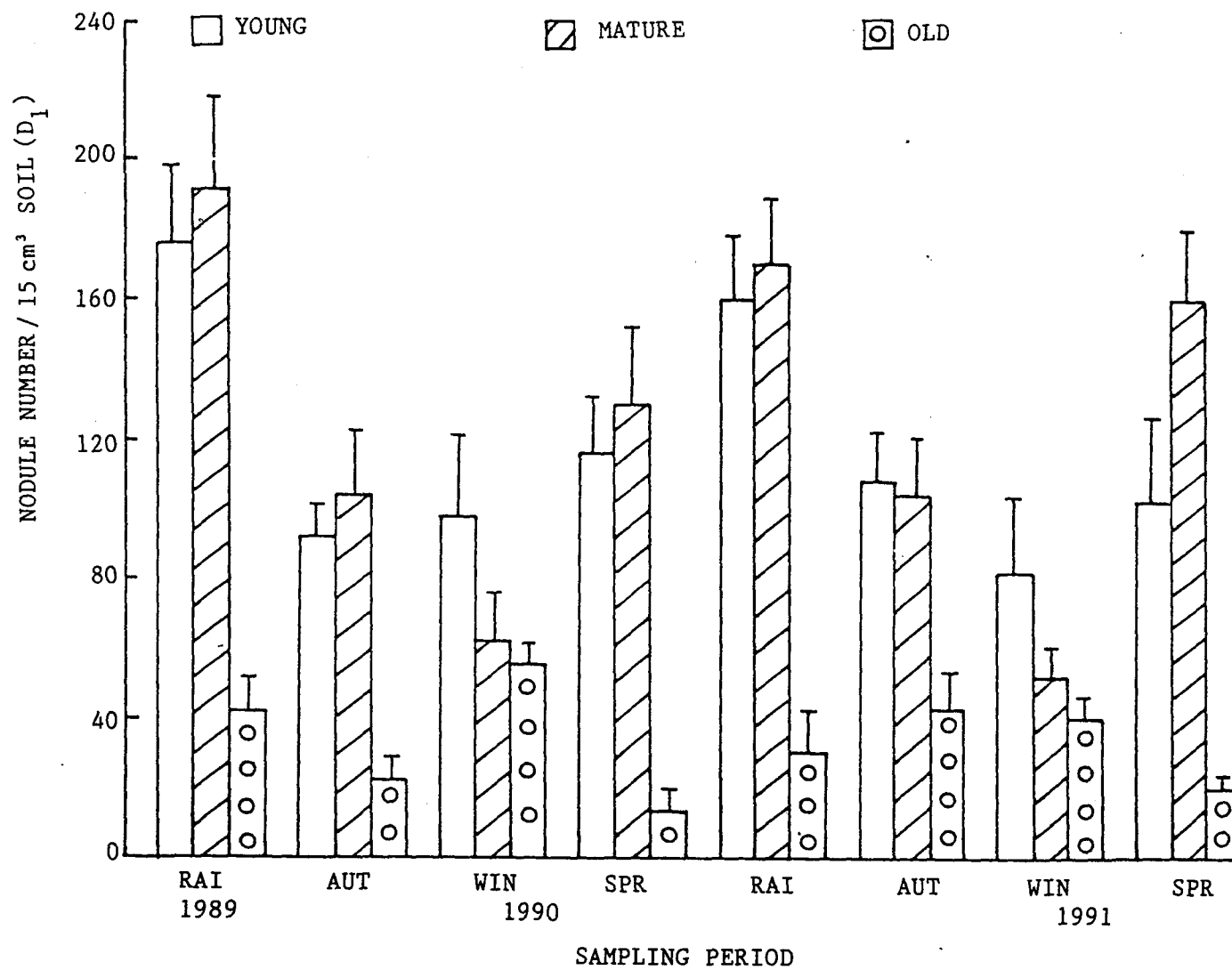


Fig. 16. Seasonal changes in nodule population of different age classes in Alnus nepalensis stand.

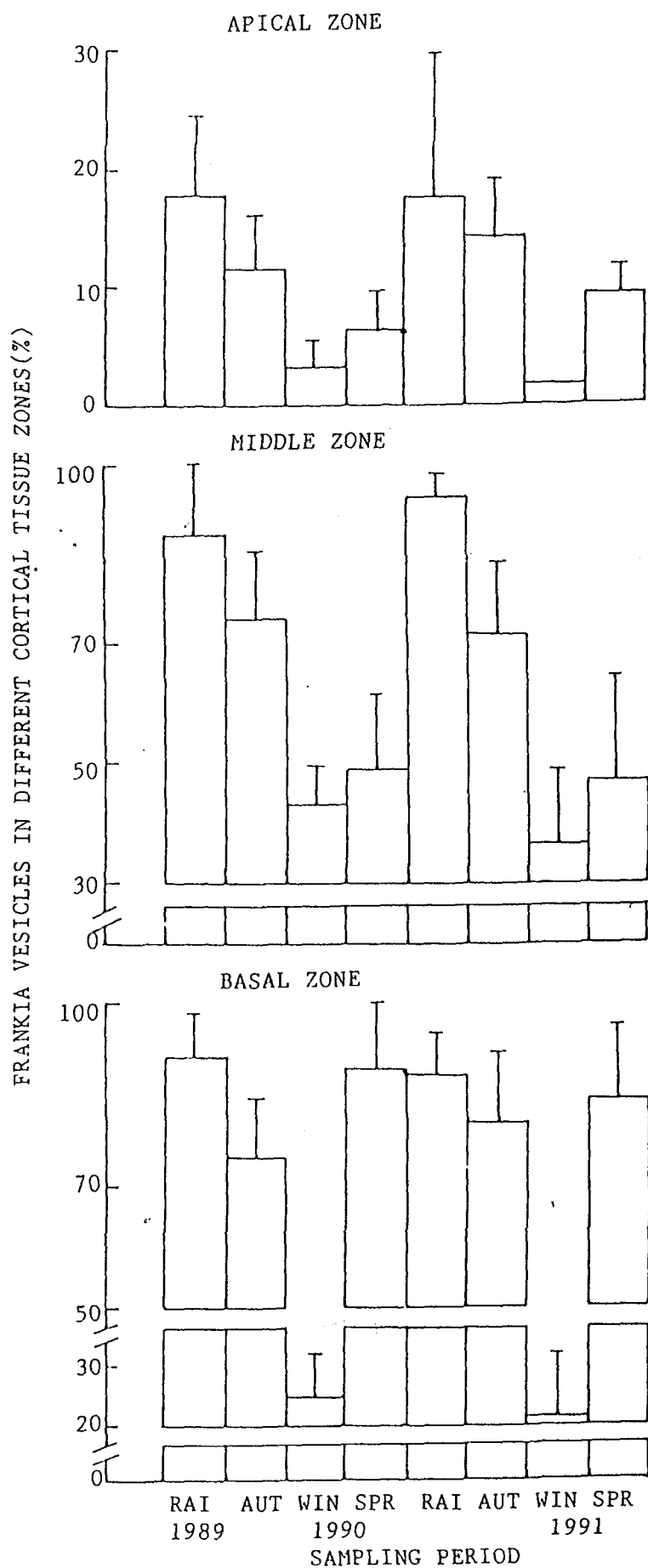


Fig.17. Seasonal changes in Frankia vesicles in different cortical zones (%) of root nodules of A. nepalensis ,

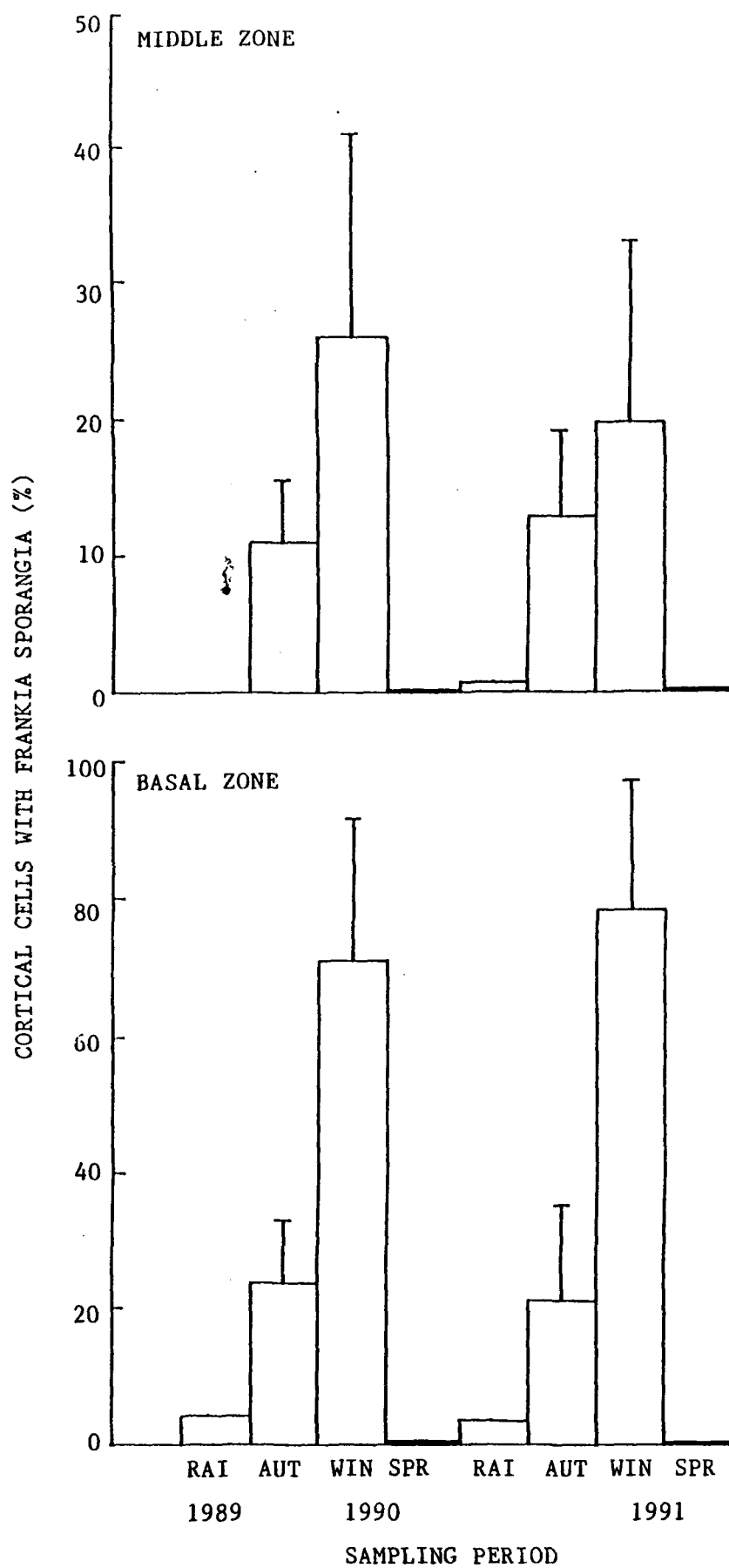


Fig.18. Seasonal dynamics of Frankia sporangia in the cortical cells (%) in various zones of root nodules.

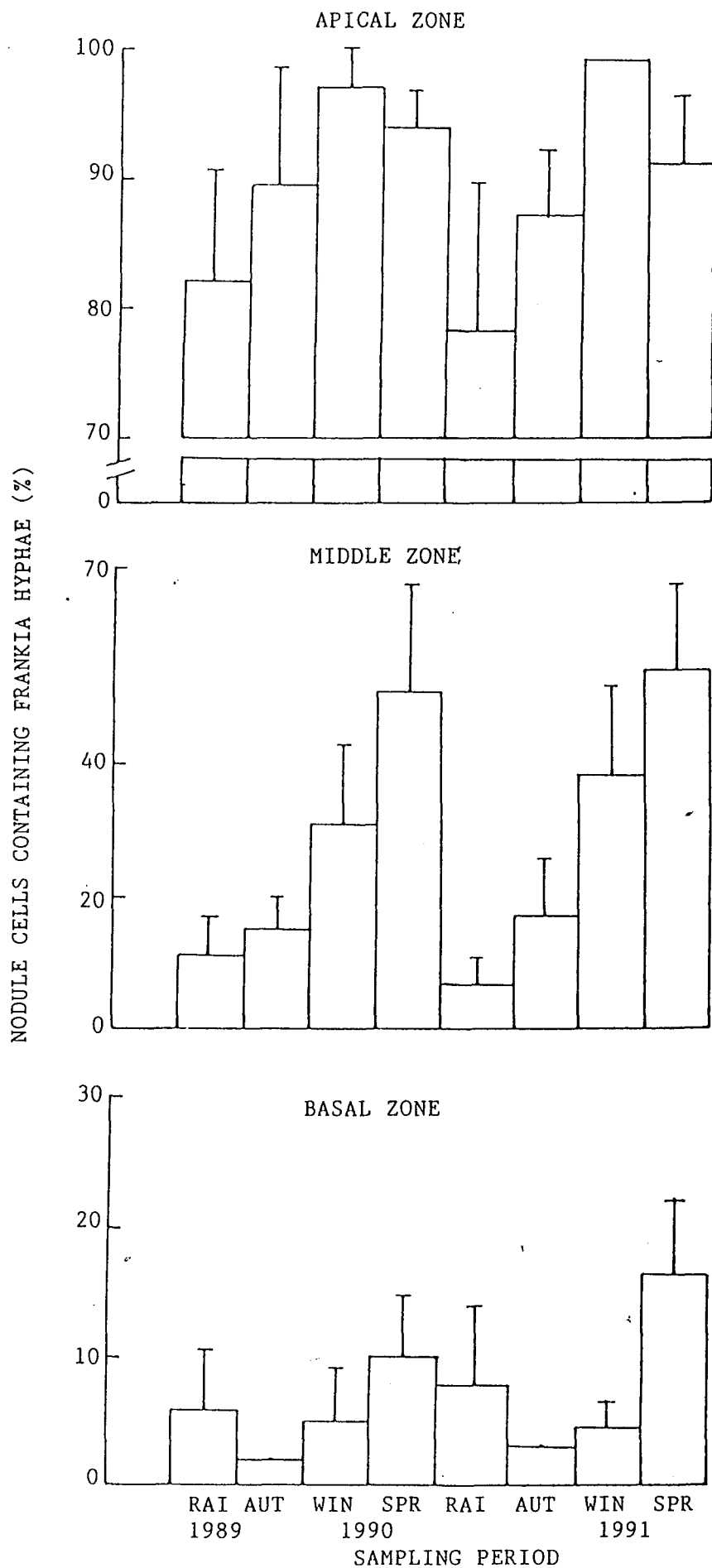


Fig.19. Seasonal dynamics of Frankia hyphae in cortical cells (%) at different zones of the nodule of A. nepalensis.

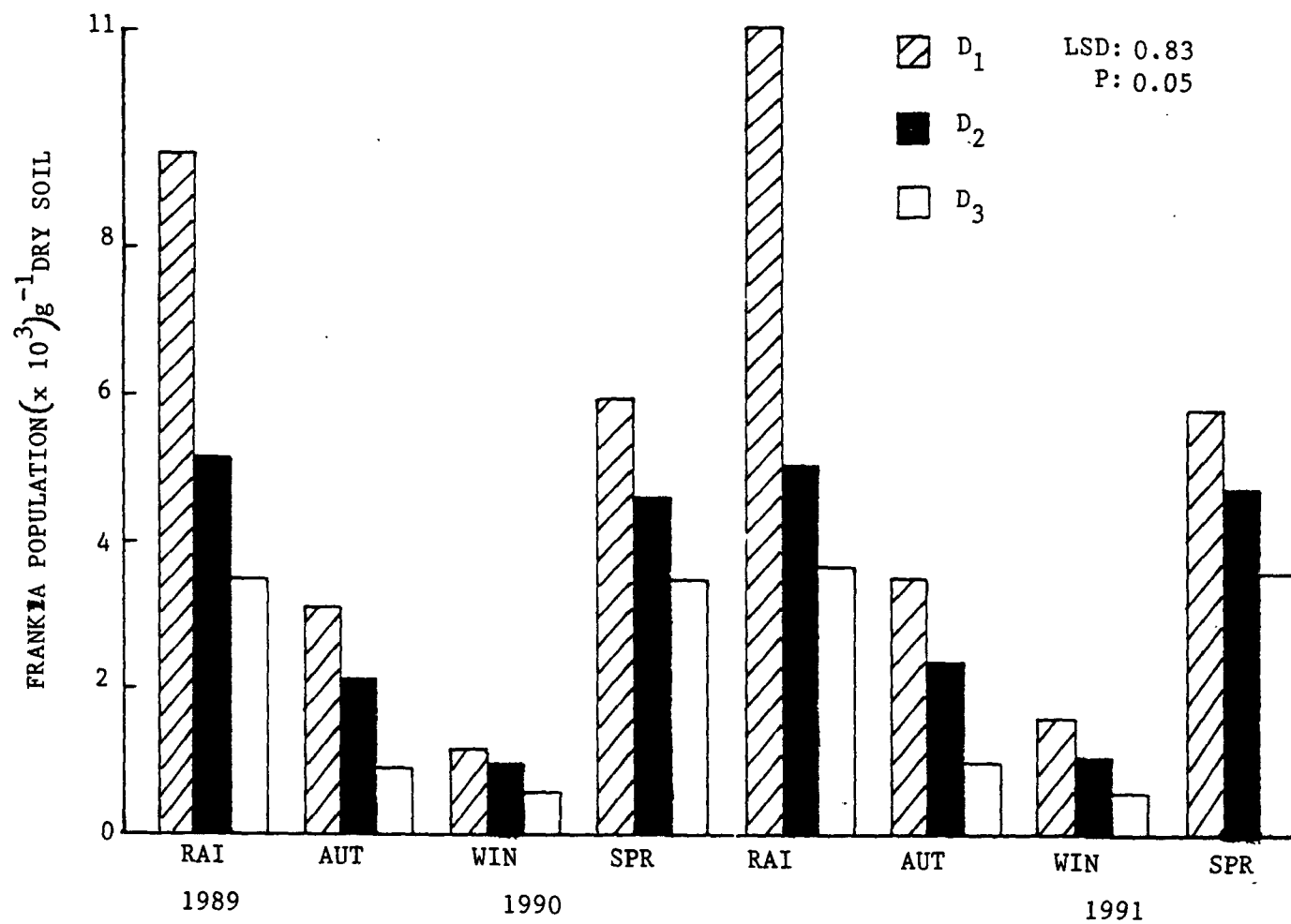


Fig.20. Seasonal & depthwise changes in the population of Frankia in alder forest soil.

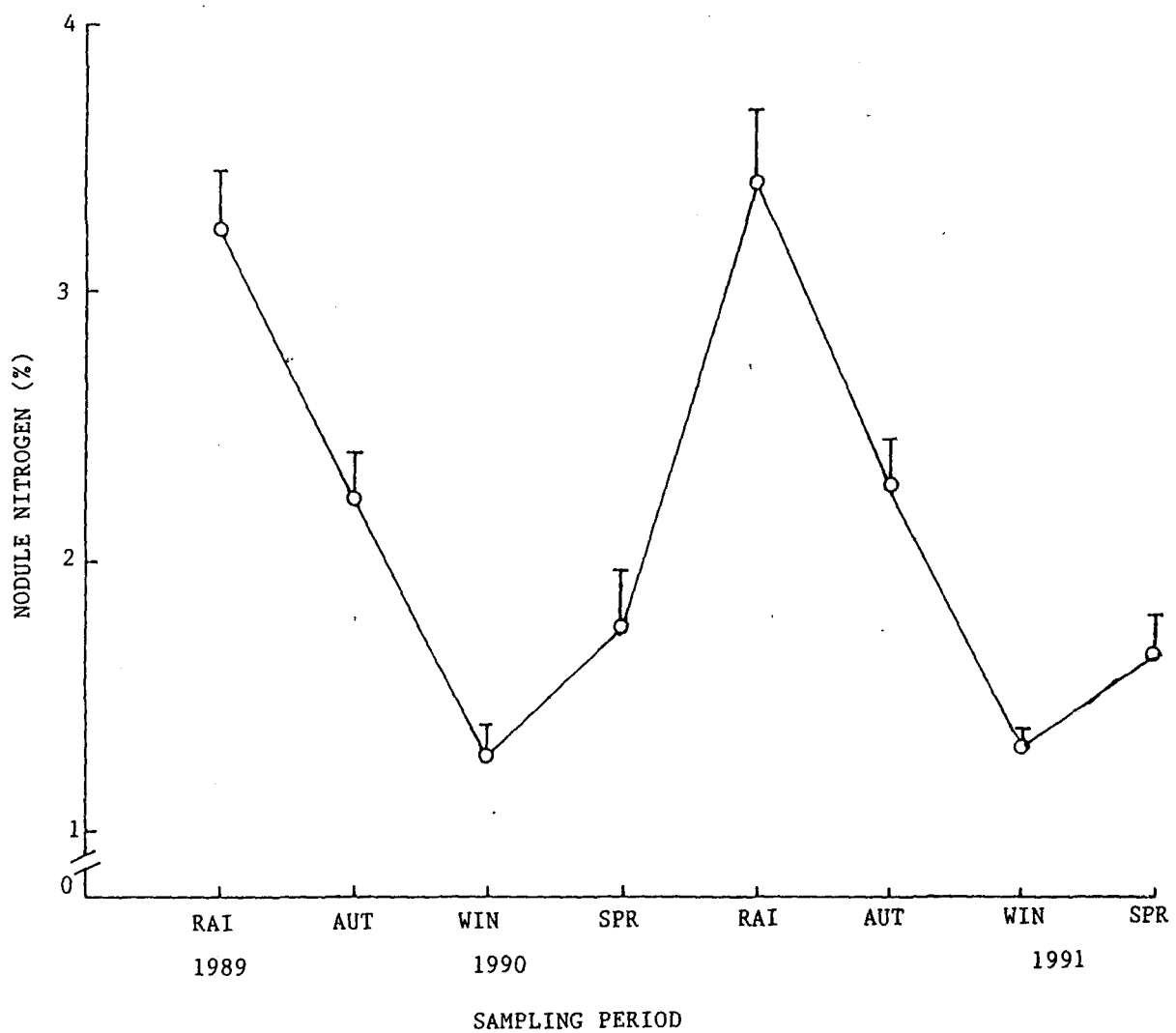


Fig. 21. Seasonal changes in nodule nitrogen content (%) of field collected root nodules of A. nepalensis.

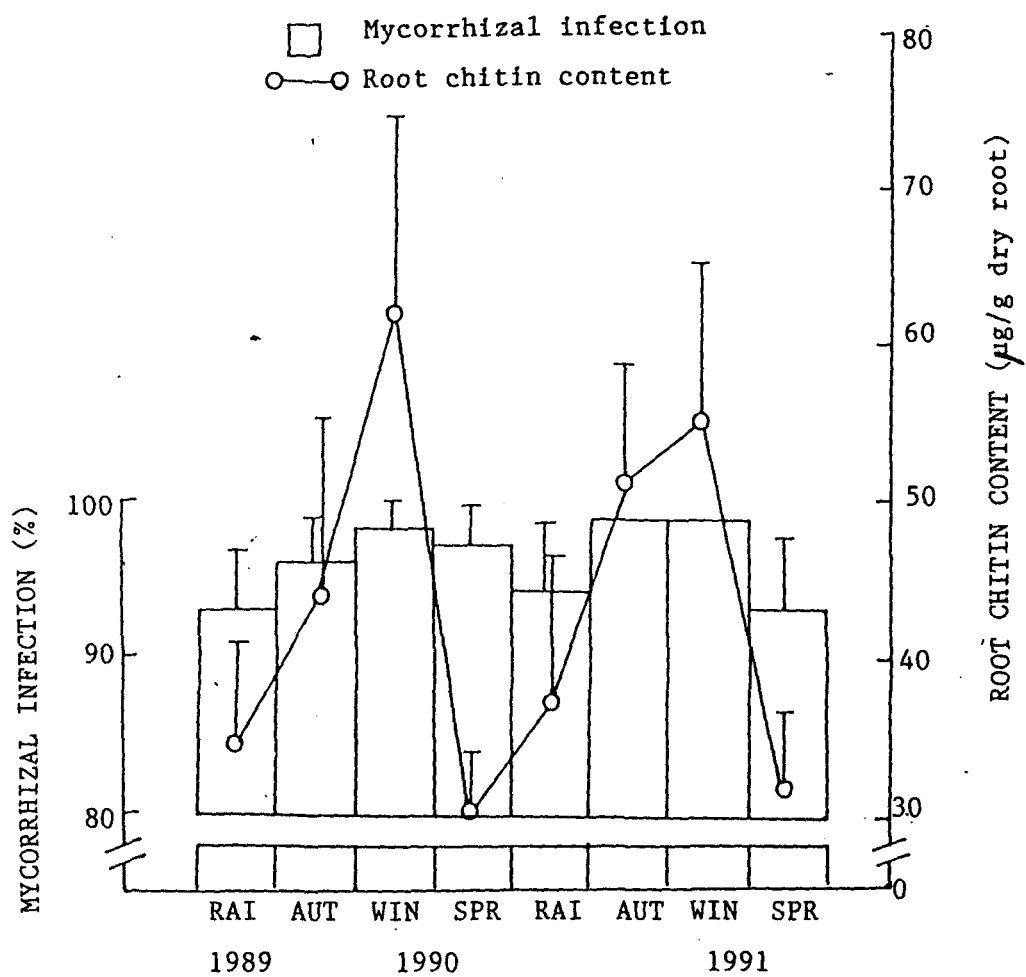


Fig.22. Seasonal fluctuation in vesicular-arbuscular mycorrhizal infection (%) and chitin content ($\mu\text{g/g}$ dry root) in root of A. nepalensis.

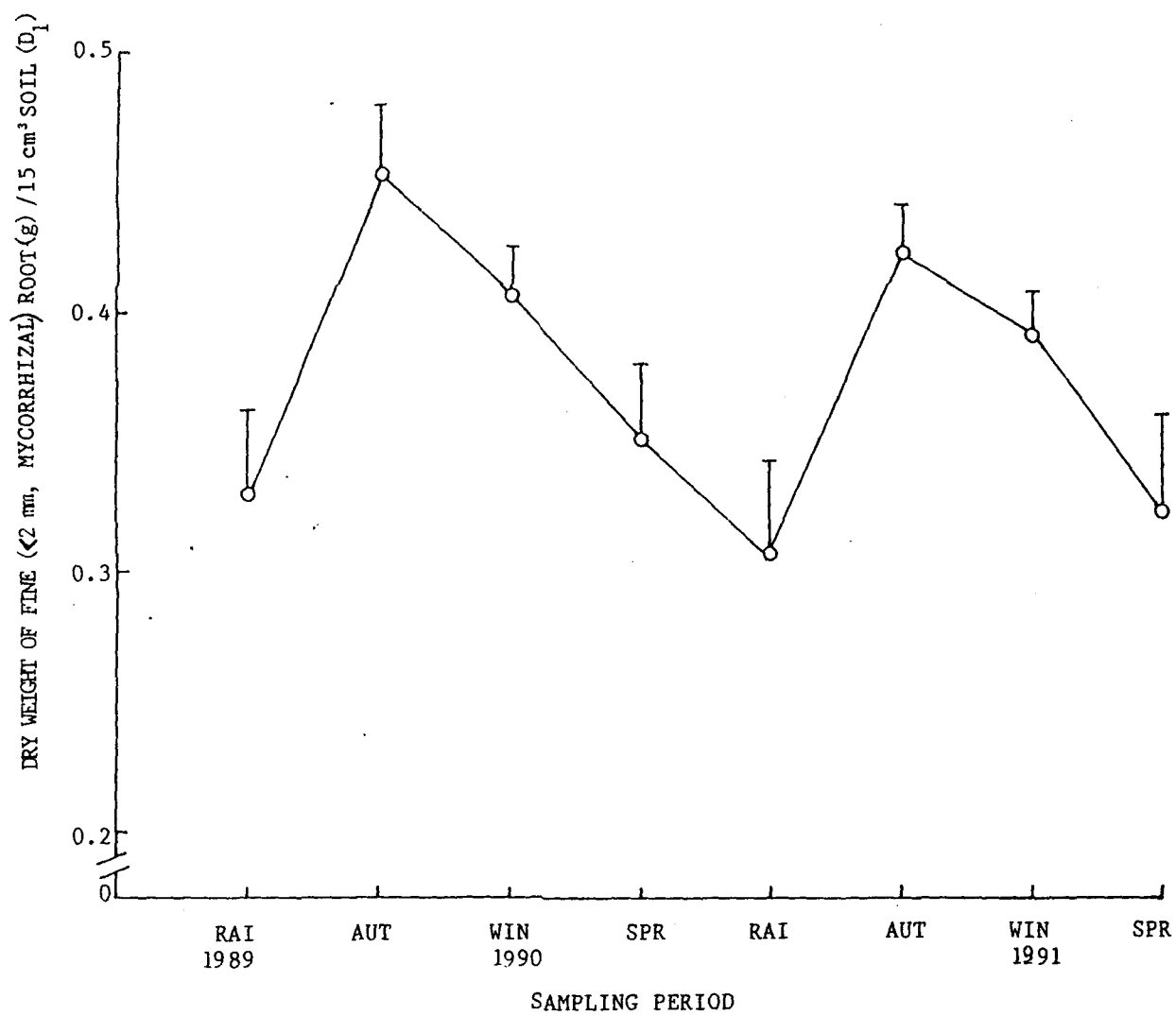


Fig.23. Seasonal changes in biomass of mycorrhizal roots (Mean $\pm$ SE) of *A. nepalensis* under field conditions.

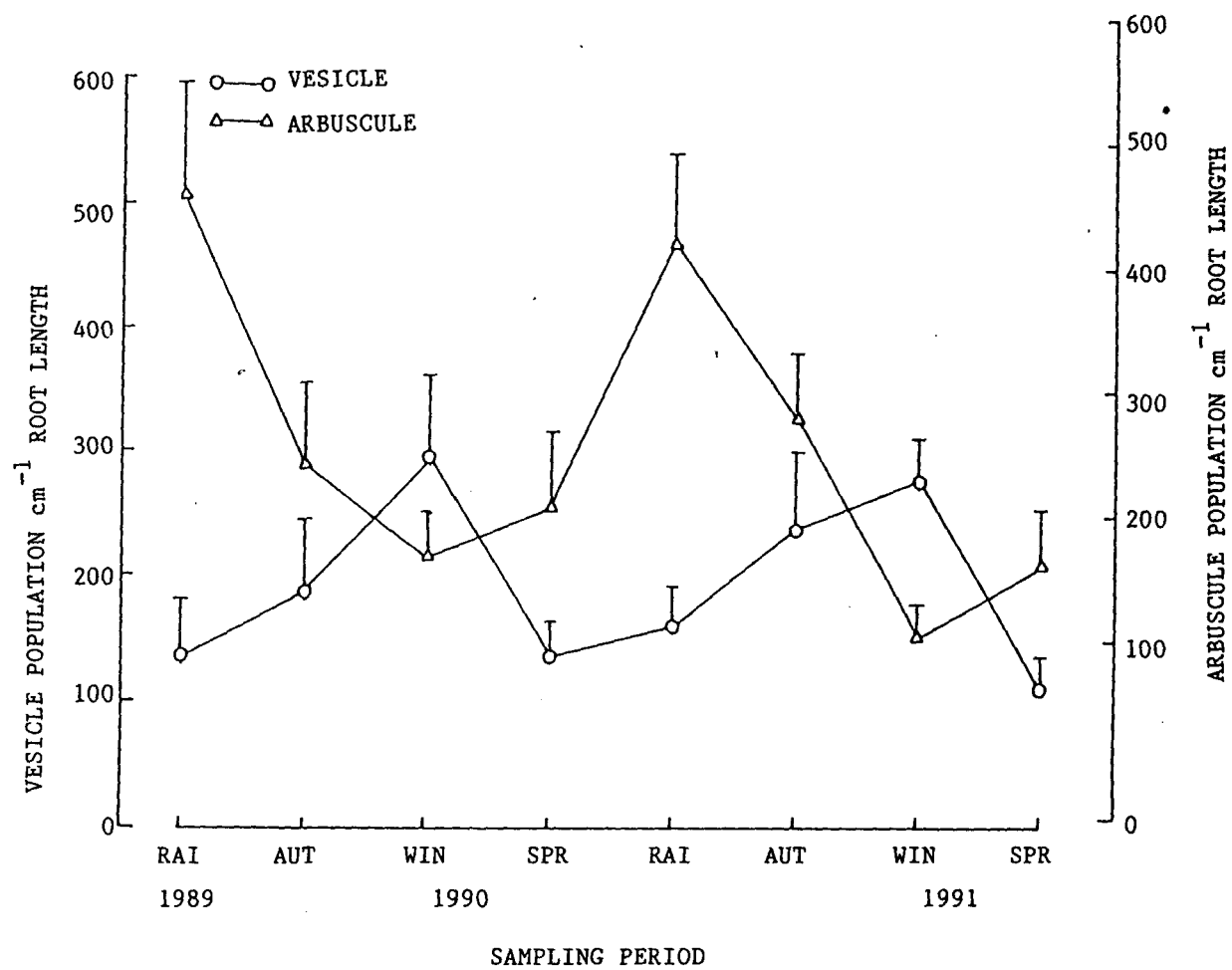


Fig. 24. Seasonal changes in the population of vesicles and arbuscules in mycorrhizal roots of A. nepalensis.

Nitrogenase activity (acetylene reduction assay) was significantly and positively correlated with soil temperature, soil moisture, soil pH, soil organic matter, total N, available P and exchangeable Mg and Mn (Table 3). Similarly significant correlations were established between nodule weight and soil pH, moisture and organic matter (Table 3). Nodule number was positively correlated with most soil characteristics such as temperature, organic matter, total N, available P, Ca, Mg and Mn (Table 3).

Correlation between VAM infection and soil characteristics like soil temperature, pH, moisture, total N, available P, K, Ca, Mg and dehydrogenase activity was significantly and negatively established (Table 4). Chitin content also was significantly and negatively correlated with above soil characteristics except for soil pH, moisture, Mg and Mn (Table 4).

Statistically significant and positive correlation was established between nitrogenase activity and number of cortical cells with vesicles and nodule N, and was correlated negatively with sporangia containing cells (Table 5).

Nitrogenase activity was significantly and

negatively correlated with VAM infection and chitin content (Table 6). Similarly, nodule N was correlated negatively with VAM infection (Table 6).

Discussion :

Seasonal variation in soil temperature and moisture content may be due to changes in the environmental condition. Besides, soil pH exhibited seasonal fluctuations which may be attributed to variation in organic acid content of litter deposited in the preceeding season. Suitable level of soil factors like temperature, moisture and pH during rainy season may be related to higher activity of nitrogenase enzyme in Himalayan alder nodules (Akkermans, 1971; Wheeler, 1971; Sharma and Ambasht, 1984).

High level of soil organic matter and total N during rainy season might have resulted from the decomposition of litter shed during January-February months. This finding is supported by the result of Sharma *et al.* (1986). The available P was found to be high and low during spring and winter respectively. This may be explained by the fact that the nutrients released from that locked up in litters due to microbial decomposition and its absorption by plant roots throughout the growing seasons (April to October)

Table 1. Depthwise and seasonal variation in the endogonaceous spore population in *Alnus nepalensis* forest soil.

Soil depths	RAINY		AUTUMN		WINTER		SPRING	
	1989	1990	1989	1990	1990	1991	1990	1991
D ₁ (0-15 cm)	11	8	54	57	102	96	27	20
D ₂ (15-30 cm)	6	6	56	50	105	97	22	19
D ₃ (30-45 cm)	2	3	21	19	32	20	8	7

Table 2. Seasonal & depthwise variation in relative abundance (%) of VAM fungal spores of *A. nepalensis* forest soil.

VA species	RAINY			AUTUMN			WINTER			SPRING		
	D ₁	D ₂	D ₃	D ₁	D ₂	D ₃	D ₁	D ₂	D ₃	D ₁	D ₂	D ₃
<i>Glomus tortuosum</i>	-	-	-	9	14	5	10	12	23	-	-	-
<i>G. macrocarpus</i>	18	20	21	20	18	24	18	22	18	27	29	42
<i>G. mosseae</i>	23	20	38	20	39	34	33	38	26	25	29	33
<i>G. intraradices</i>	17	16	23	17	20	30	21	16	15	28	27	20
<i>Gigaspora gregaria</i>	19	11	5	15	-	1	-	-	-	4	-	-
<i>Gi. gigantea</i>	10	16	2	9	9	6	7	6	14	-	-	-
<i>Acaulospora scrobiculata</i>	13	17	11	10	-	-	6	6	4	16	9	5

Each value is the mean of 8 replicates of 2 years data.

Table 3. Correlation coefficients (r) for soil pH, moisture, temperature, organic matter, total nitrogen, available phosphorus, exchangeable Potassium, Calcium, Magnesium, Manganese, total phosphatase, dehydrogenase, nodule nitrogenase activity, nodule dry weight and total nodule number.

Sources of variation	Nitrogenase activity (Acetylene Reduction)	Nodule dry weight	Nodule number
Soil pH	0.823 *	0.908 * *	0.210
Soil moisture	0.967 * *	0.908 * *	0.590
Soil temperature	0.879 * *	0.615	0.824 *
Soil organic matter	0.939 * *	0.846 * *	0.838 * *
Soil total N	0.883 * *	0.565	0.904 * *
Available P	0.784 *	0.510	0.906 * *
Exchangeable K	0.407	0.060	0.692
— " Ca	0.600	0.269	0.837 * *
" Mg	0.727 *	0.450	0.952 * *
Extractable Mn	0.914 * *	- 0.068	0.852 * *
Total phosphatase activity	- 0.652	- 0.549	- 0.655
Dehydrogenase activity	- 0.123	- 0.197	- 0.622

*, * * Significant at $P < 0.05$ and 0.01 levels respectively.

Without asterisk statistically insignificant at above levels.

Table 4. Correlation coefficients (r) for soil temperature, pH, moisture, organic matter, total nitrogen, available phosphorus, exchangeable potassium, calcium, magnesium, manganese, total phosphatase, dehydrogenase, mycorrhizal infection and chitin content.

Sources of variation	Mycorrhizal infection	Chitin content
Soil temperature	- 0.795 *	- 0.758 *
Soil pH	- 0.760 *	- 0.018
Soil moisture	- 0.857 * *	- 0.579
Organic matter	0.035	- 0.709 *
Total N	- 0.842 * *	- 0.766 *
Available P	- 0.784 *	- 0.883 * *
Exchangeable K	- 0.798 *	- 0.912 * *
" Ca	- 0.761 *	- 0.913 * *
" Mg	- 0.834 * *	- 0.688
Extractable Mn	- 0.634	- 0.474
Phosphatase activity	0.669	0.715 *
Dehydrogenase activity	0.774 *	0.750 *

*, * * Significant at $P < 0.05$ and 0.01 respectively.

Without asterisk statistically insignificant at these levels.

Table 5. Correlation coefficients (r) for nitrogenase activity, nodule nitrogen, cortical vesicular cells, cortical hyphal cells, cortical sporangial cells and total colonization.

Sources of variation	Nitrogenase activity	Nodule nitrogen	Cortical vesicular cells	Cortical hyphal cells	Cortical sporangial cells	Total colonization
Nitrogenase activity	-	0.986 * *	0.913 * *	- 0.784 *	- 0.652	- 0.200
Cortical vesicular cells	0.913 * *	0.902 * *	-	- 0.579	- 0.863 * *	- 0.393

*, * * Significant at $P < 0.05$ and 0.01 respectively.

Without asterisk statistically insignificant at these levels.

Table 6. Correlation coefficients (r) between mycorrhizal infection, chitin content, root dry weight, arbuscules and vesicle numbers and nitrogenase activity, total nodule number, nodule dry weight and nodule nitrogen.

Sources of variation	Nitrogenase activity	Total nodule number	Nodule dry weight	Nodule nitrogen
Mycorrhizal infection	- 0.840 * *	- 0.563	- 0.713 *	- 0.855 * *
Chitin content	- 0.734 *	- 0.753 *	- 0.261	- 0.499
Mycorrhizal root biomass	- 0.479	- 0.703	- 0.168	- 0.092
Arbuscule number	0.601	0.314	0.564	0.621
Vesicle number	- 0.535	- 0.794 *	- 0.318	- 0.533

*, * * Significant at $P < 0.05$ and 0.01 levels respectively.

Without asterisk statistically insignificant at above levels.

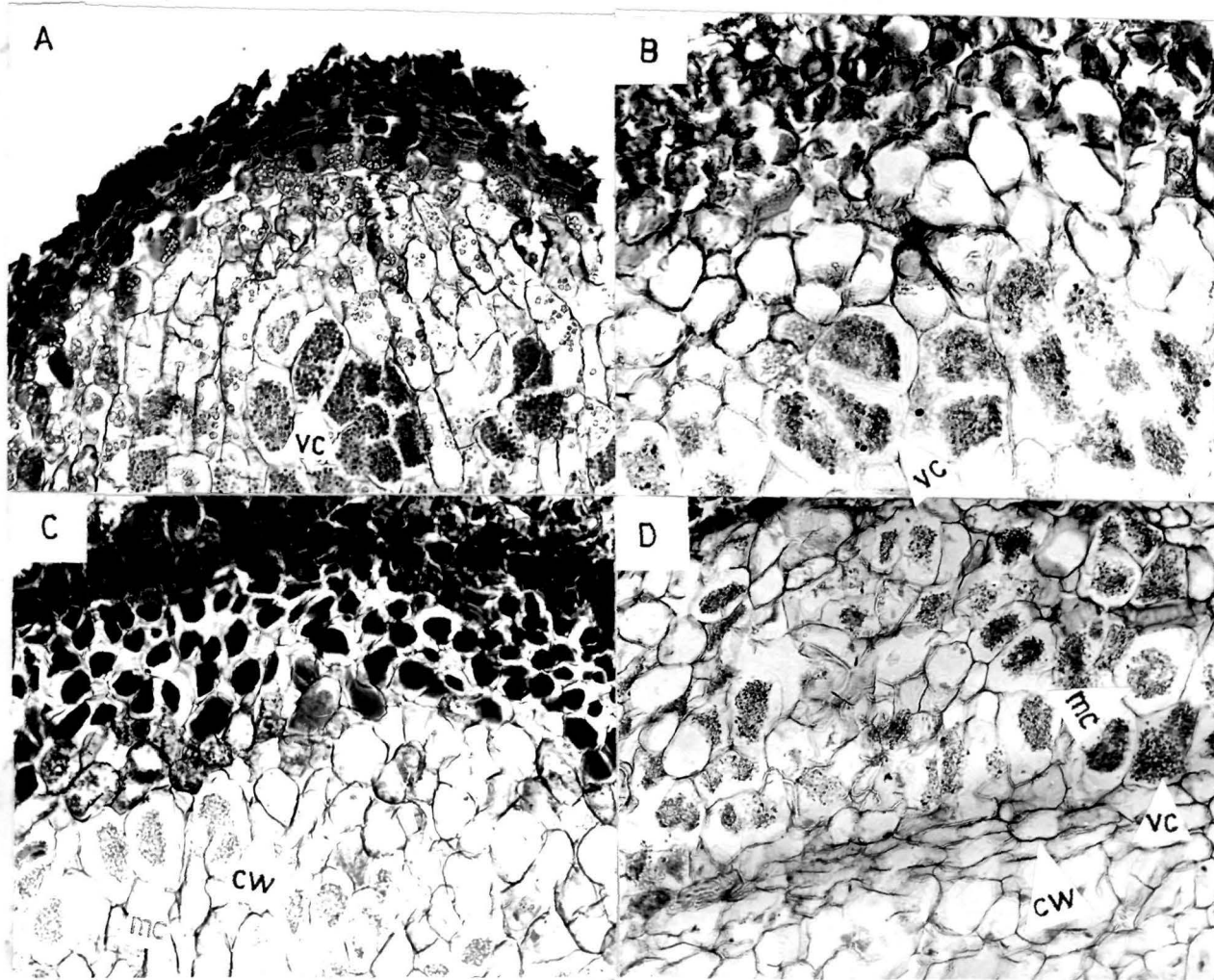


Plate 3. Longitudinal section of apical zone of nodule showing morphology of symbiotic *Frankia*. A = Rainy (x 200); B = Autumn (x 312.5); C = Winter (x 200); D = Spring (x 200); (Vc = vesicle cluster, mc = micro-colony, cw = cell wall).

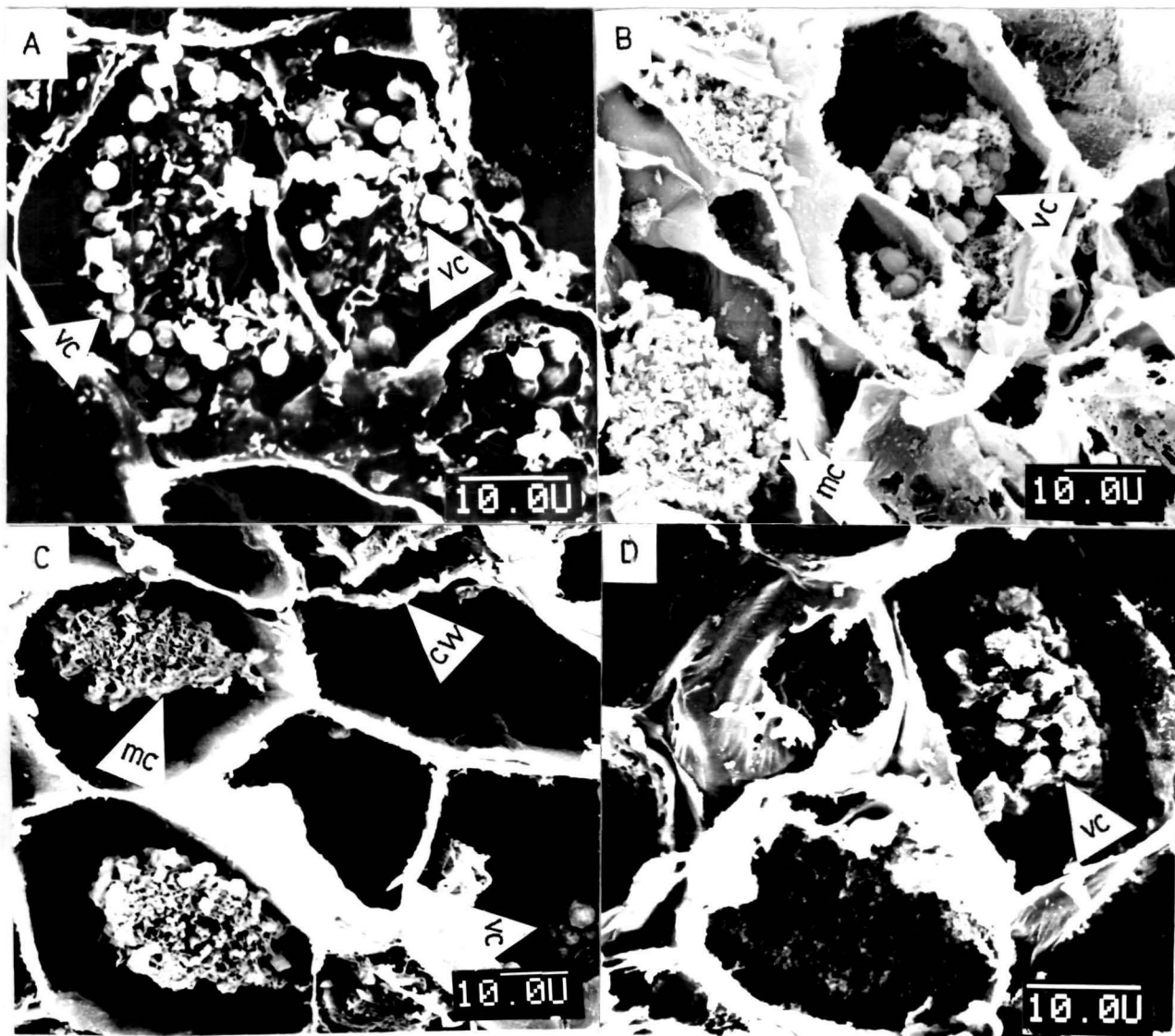


Plate 4. Scanning electron micrographs of L.S. through apical zone of the field collected root nodule of *A. nepalensis*. A = Rainy, bar = 10 um; B = Autumn, bar = 10 um; C = Winter, bar = 10 um; D = Spring, bar = 10 um; (vc = vesicle cluster; mc = micro-colony, cw = cell wall).

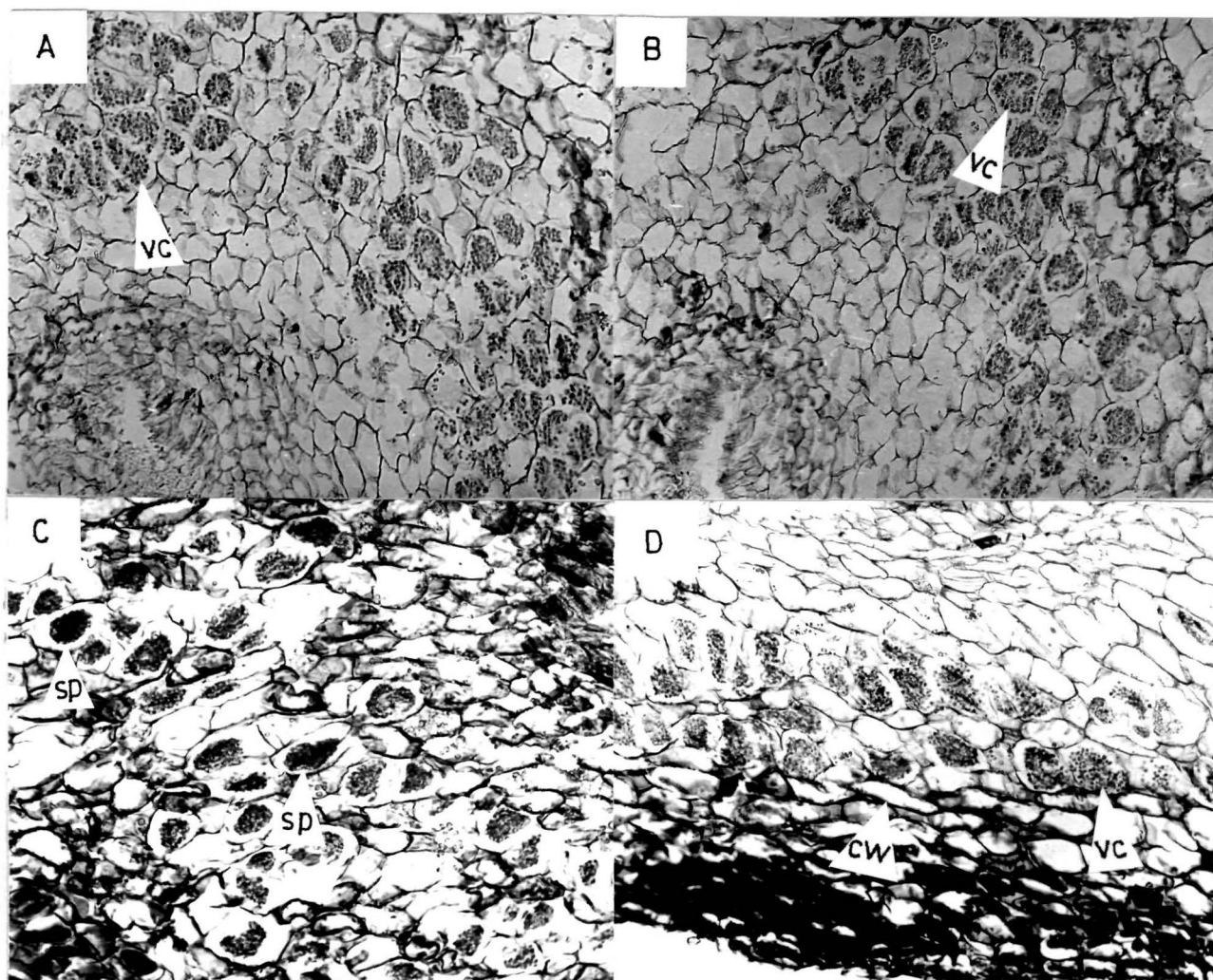


Plate 5. Morphology of *Frankia* in the middle zone of root nodules of *A. nepalensis*. A = Rainy (x 200); B = Autumn (x 200); C = Winter (x 200); D = Spring (x 200); (vc = vesicle cluster, sp = sporangium, cw = cell wall).

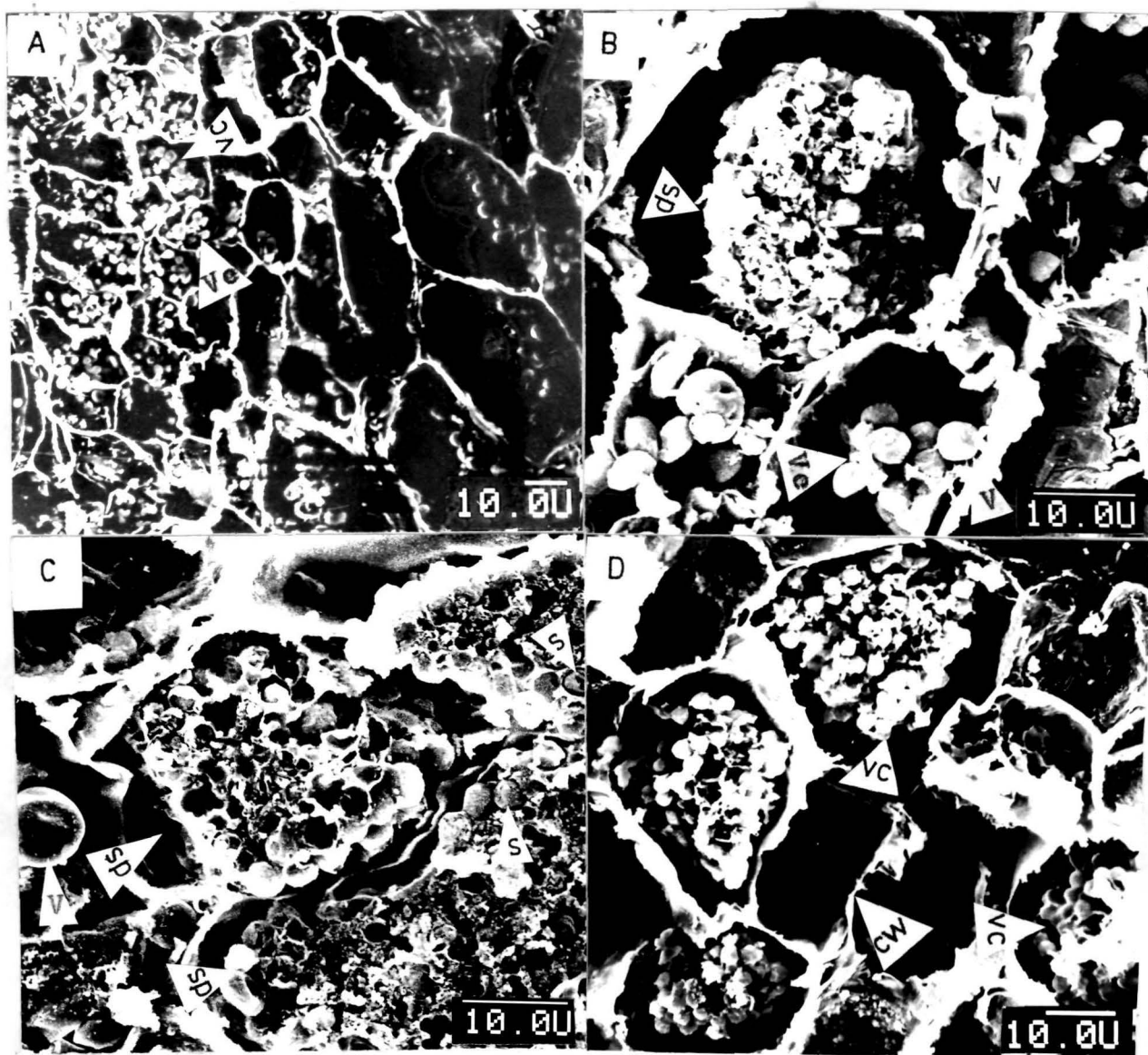


Plate 6. Scanning electron micrographs showing *Frankia* structures in the middle zone of field collected root nodules of *A. nepalensis*. A = Rainy, bar = 10 μ m; B = Autumn, bar = 10 μ m; C = Winter, bar = 10 μ m; D = Spring, bar = 10 μ m; (v = vesicle; vc = vesicle cluster, sp = sporangium, s = spore, cw = cell wall).

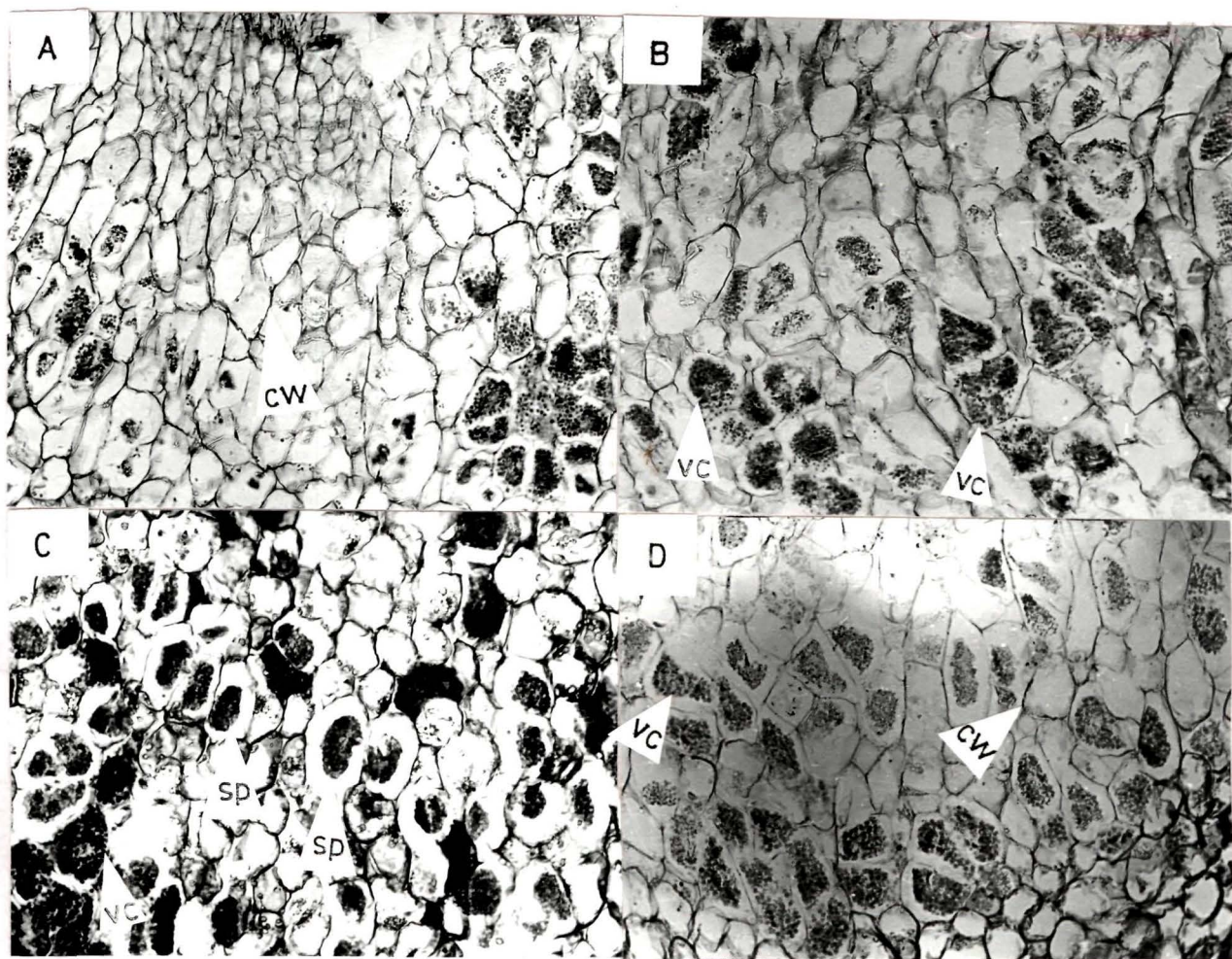


Plate 7. Morphology of *Frankia* in the basal zone of the root nodule of *A. nepalensis* in various seasons. A = Rainy (x 200); B = Autumn (x 200); C = Winter (x 200); D = Spring (x 200) (vc = vesicle cluster, sp = sporangium, cw = cell wall).

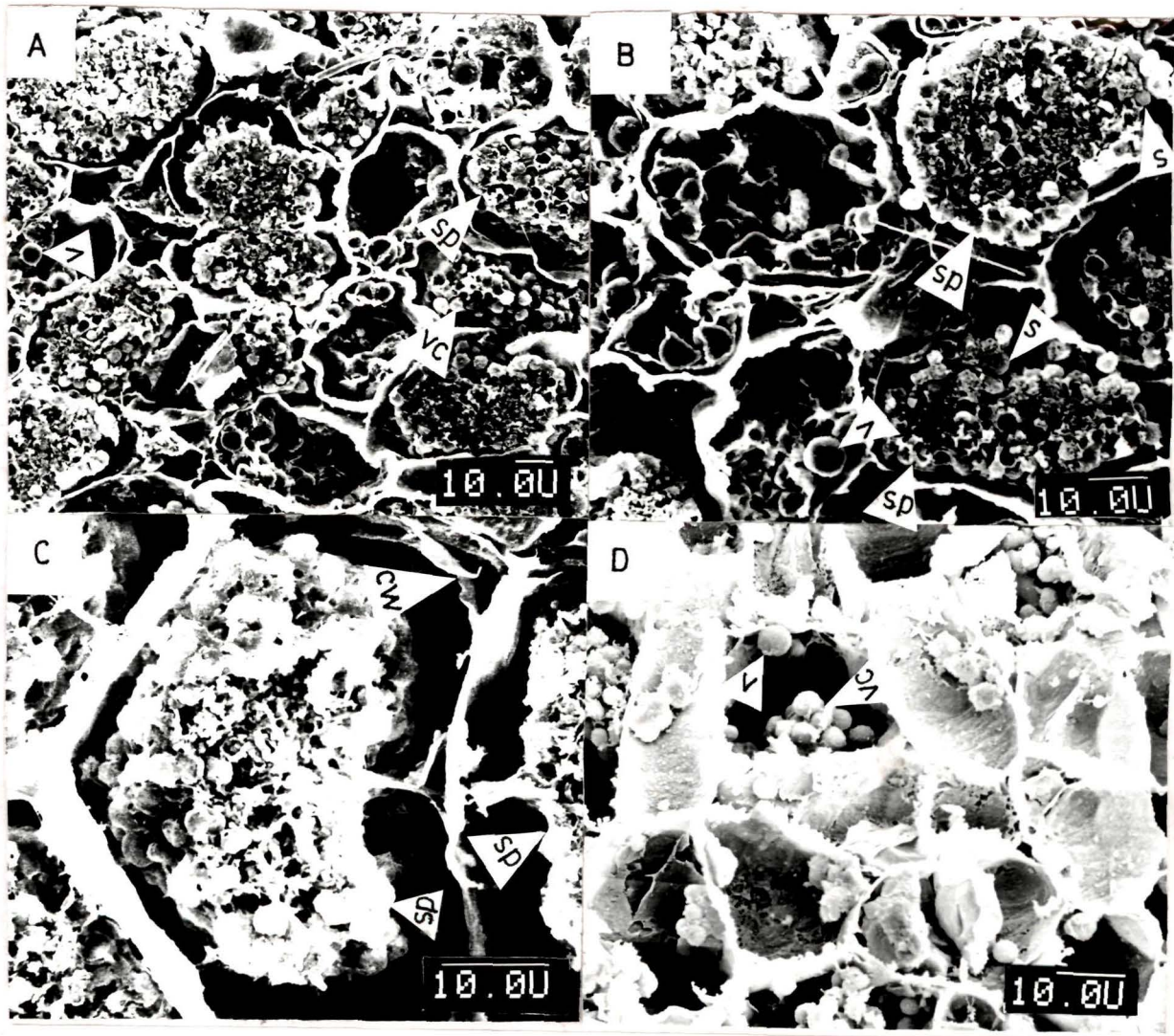


Plate 8. Scanning electron micrographs showing *Frankia* morphology in the basal part of root nodule of *A. nepalensis*. A = Rainy, bar = 10 μ m; B = Autumn, bar = 10 μ m; C = Winter, bar = 10 μ m; D = Spring, bar = 10 μ m; (v = vesicle, vc = vesicle cluster, sp = sporangium, s = spore, cw = cell wall).

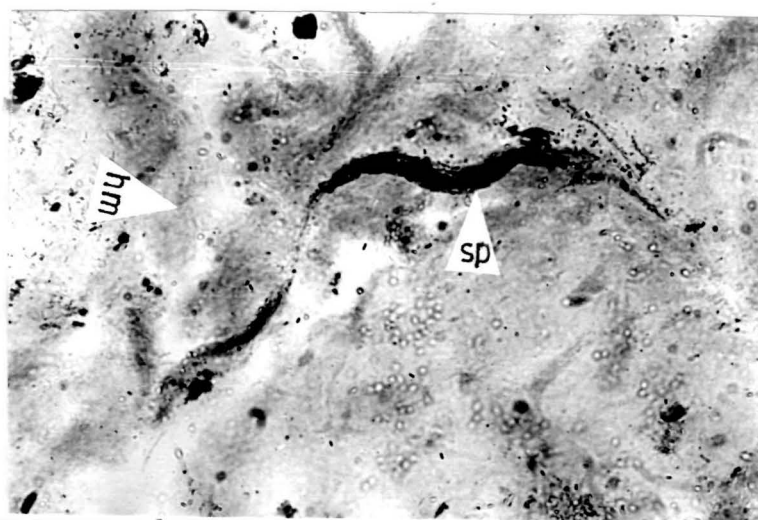


Plate 9. Light micrograph showing morphological features of Frankia isolated from soil supporting A. nepalensis (x 500); (hm = hyphal mat, sp = sporangium).

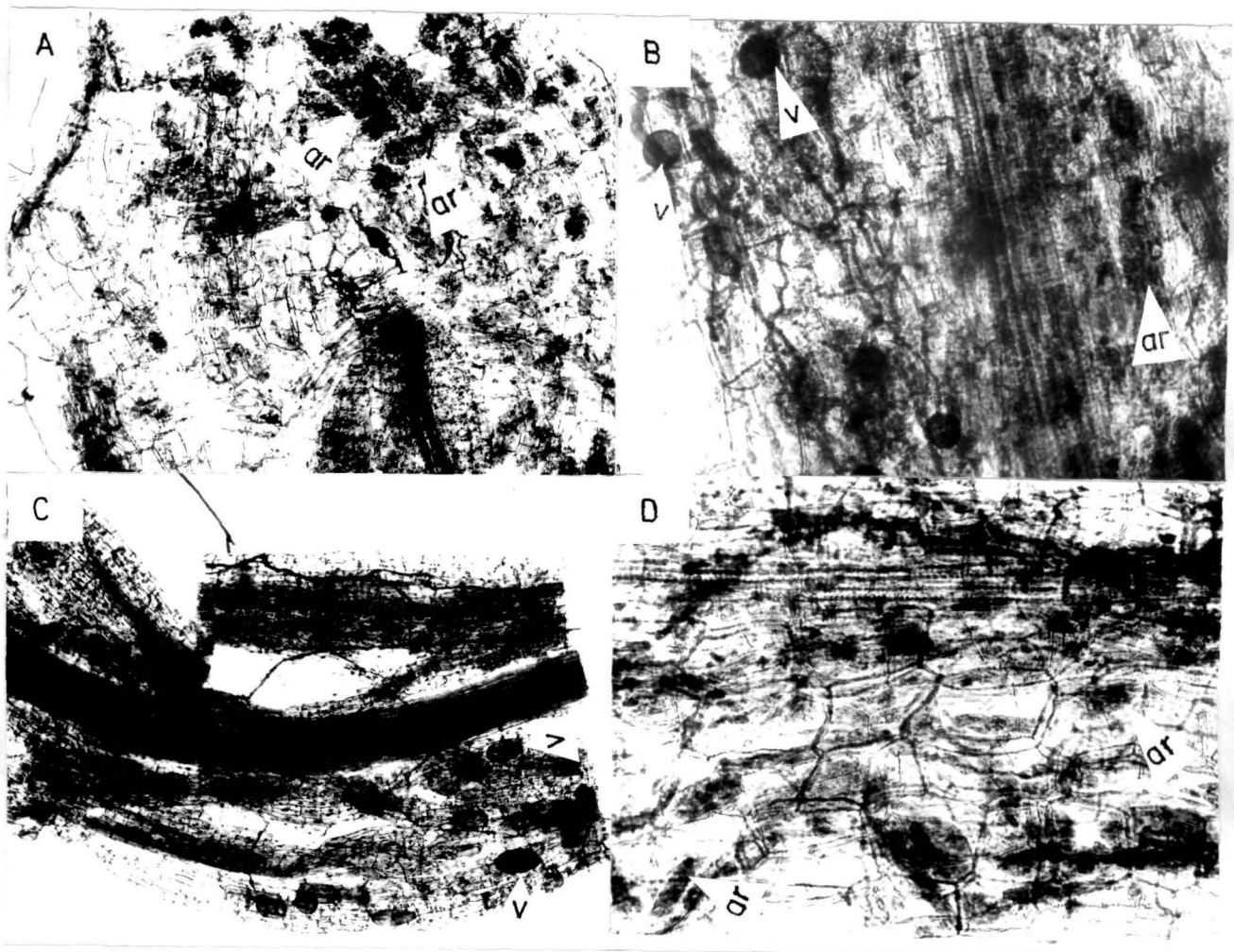


Plate 10. Light micrographs showing in vivo structures of vesicular-arbuscular mycorrhizal fungi of A. nepalensis. A = Rainy (x 200); B = Autumn (x 312.5); C = Winter (x 78.7); D = Spring (x 312.5); (ar = arbuscule, v = vesicle).

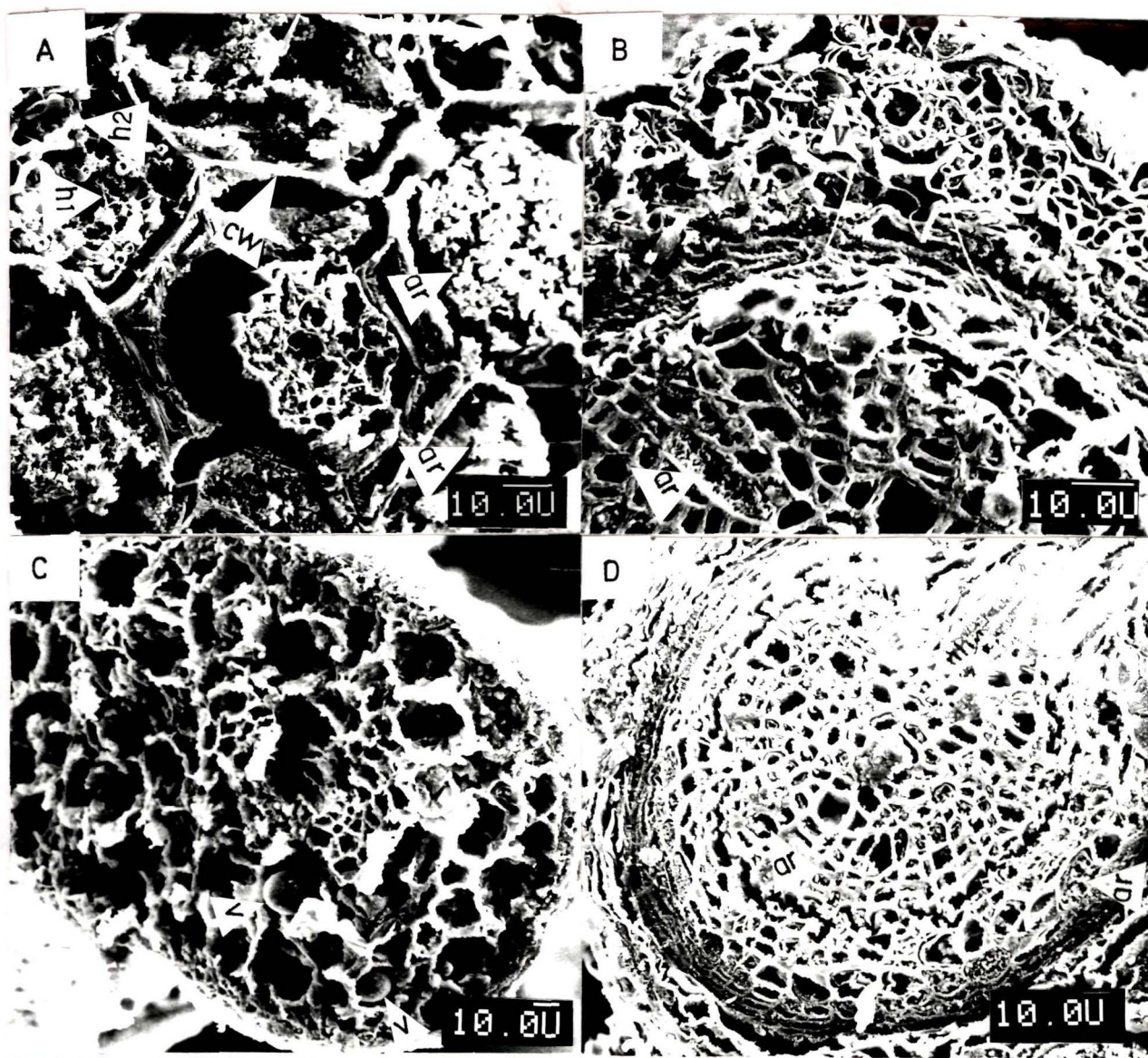


Plate 11. Transverse section through the mycorrhizal root of A. nepalensis showing structures of vesicular-arbuscular mycorrhizal fungi. A = Rainy, bar = 10 μ m; B = Autumn, bar = 10 μ m; C = Winter, bar = 10 μ m; D = Spring, bar = 10 μ m; (ar = arbuscule, v = vesicle, cw = cell wall, h_1 = hyphal type 1, h_2 = hyphal type 2).

leaving aside least in soil. Exchangeable cations which showed same seasonal trend as that of available P may be explained similarly. The leaching of exchangeable cations by heavy torrential rain during rainy season may be responsible for displacement of more cations in lower horizons. Further, decomposition of litter and the release of nutrients also may have had some influence on the seasonal fluctuations of soil micronutrient Mn. The role of soil nutrients such as P, Ca, Mg, Mn and others may be important for symbiotic nitrogen fixation by Himalayan alder as its concentration was positively related to nitrogenase activity. This is supported by the findings of Tisa *et al.* (1981), Dixon and Wheeler (1983), Russo (1989) and Jha *et al.* (1993).

More phosphatase activity during winter, a non-growing season indicates the deficiency of available soil P following its active absorption by plants during growing seasons. Mycorrhizal fungus and other soil microbes may be responsible for the release of extracellular enzyme. Intensity of phosphatase activity in soil may be related to soil pH, N content, organic matter, moisture, exchangeable cations and plant available P (Burangulova and Khaziev, 1965; Hoffman and Elias-Azar, 1965; Ross *et al.*, 1975) and P nutritional status of the roots (Bileski, 1973). It has been shown to vary with soil depth (Harrison, 1979), season (Harrison, 1979; Harrison and Pearce, 1979).

Besides, a spurt in the dehydrogenase activity of the soil during winter may be related to the hostile environmental conditions which may have imposed certain effect on the soil microbes consequently leading to rise in their metabolic activity. Above finding is supported by the work of Lenhard (1956), Casida et al. (1964) and Jha et al. (1989). A separation of peak activity in time between soil phosphatase, dehydrogenase and nitrogenase may be related to environmental factors which selectively affects and root microendophytes.

A marked seasonal fluctuation in nodule nitrogenase activity was observed. This aligns with the findings of Sharma and Ambasht (1984). The seasonality in enzyme activity may be attributed to factors like soil temperature, number of hour of illumination and photosynthesis by host (Wheeler, 1971; Wheeler and Lawrie, 1976; Huss-Danell, 1978) and metabolic state of microsymbiont.

The restriction of root nodule to upper soil depth may signify the role of oxygen in nodulation of Himalayan alder as observed by Dalton and Zobel (1977).

Higher nodule biomass recorded during rainy

season may be attributed to the favourable climatic and edaphic factors. Rate of nodule production in rainy season was higher than in winter. Sharma and Ambasht (1984) also noted a high nodule production during rainy season. A fall in nodule production may be explained by the arrival of unfavourable climatic and soil condition like low P (Kant and Narayana, 1978), soil temperature (Redell *et al.*, 1985; Sharma, 1986), pH (Bond, 1957) moisture (Kant and Narayana, 1978) deficiency of nutrients (Robson and Loneragan, 1978) and low inoculum potential of infective *Frankia*.

Total number of nodules produced by Himalayan alder during rainy season exceeded than in any other season. The proportion of young and mature (active) nodules was highest during rainy season which indicates that production of young nodules and their transition into mature and later into old ones may be equivalent. This may be considered as a ecological strategy to maintain nodules of various age classes by host plant.

The characteristic morphology of symbiotic *Frankia* in various seasons may have some ecological implications. The dominance of vesicles in nodule cortical cells during rainy season which coincided with the rate of nitrogenase activity indicates that vesicles are the site of

enzyme activity. The findings of Mian and Bond (1978), Schwintzer et al. (1982) corroborated above. Besides, the cells in basal and middle zone of nodular tissue harboured abundant *Frankia* sporangia during mid winter. This indicates a termination of active growth and nitrogen fixation by nodule endophyte which coincided with the onset of unfavourable and hostile conditions. Data of Torrey (1978), Akkermans and Van Dijk (1981) and VandenBosch and Torrey (1985) substantiate above findings. The ecological implication in the formation of sporangia by endophyte also may be related to the maintenance of soil *Frankia* by its dispersal after nodule decay (Van Dijk, 1979) and to tide over the hostile environmental condition so as to allow endophyte to infect new root sites in the following growing season (Akkermans and Van Dijk, 1981).

A variation in nitrogen content of nodule was a characteristic feature of the season. N content of nodule tissue was correlated positively with nitrogenase activity which agrees with the findings of Domenach et al. (1989).

Frankia strains isolated from soils supporting *A. nepalensis* exhibited a branched, septate, 1 μ m diameter and cushion-shaped hyphae, electron dense vesicles borne on short stalk and multi-locular sporangia which resembled those

obtained from root nodules. Baker and O'Keefe (1984) also isolated *Frankia* directly from the soil.

The population of soil *Frankia* was markedly influenced by different seasons of the year. Reduction in population during winter may be attributed, to unfavourable physico-chemical properties of soil. Soil pH, moisture, aeration, and nutrients may thus probably affect *Frankia* abundance. Rodrigues-Barrueco (1968) and Akkermans and Houwers (1979) found that soils devoid of detectable *Frankia* were acidic. In addition Smolander and Sundman (1987) and Smolander et al. (1988) revealed positive correlation between the number of nodulation units of *Frankia* and soil pH. Further, the *Frankia* population was maximum during rainy season which may be due to suitable environmental and soil conditions for their rapid growth and development. Smolander et al. (1988) and Smolander (1990) revealed that available cations have significant effect on nodulation capacity. *Frankia* have been shown to proliferate in the rhizospheric region of host (Lalonde et al., 1981; Diem et al., 1982; Vergnaud et al., 1985). The role of host root exudates certainly ^{is} ~~be~~ important in its proliferation (Dewadar and Mansour, 1992) when a part of photosynthetically fixed carbon may be secreted by host plant in the form of root exudates.

In addition, restriction of *Frankia* propagules to soil depth D_1 may be related to absorption of cells to clay as noted for *Rhizobium* (Marshall, 1969). Presence of nodule endophyte in the lower horizons may be related to its dispersal by mass water movements (Hamdi, 1974; Worrall and Roughley, 1991) and earthworms (Reddell and Spain, 1991). Moreover, aeration, light, nutrients and other unidentified factors may restrict extranodular growth of *Frankia* in deeper soil horizons.

Mycorrhizal infection was high throughout the seasons of the year although highest infection was recorded during winter season. This indicated that the host *Alnus nepalensis* is highly mycotrophic which may be attributed to low soil P level and its high P requirement for nodulation and nitrogen fixation which may eventually be fulfilled by mycorrhizal endophyte under P deficient field conditions. This is in line with the findings of Asai (1944). However, in rainy season mycorrhizal infection was lower than other corresponding seasons which may be attributed to the regulatory influence of soil moisture conditions on VA mycorrhizal development. Khan (1978) also found that high soil moisture content resulted in reduction of root infection by symbiotic fungus. In addition, higher concentration of available P and N in the soil and plant are often related to

reduction of mycorrhizal development (Hayman, 1970; Chambers *et al.*, 1980; Subba Rao and Krishna, 1988).

The nitrogenase activity of nodules was negatively correlated with VAM infection, which suggests that under field conditions the microsymbionts have tendency to behave differently in response to prevailing environmental conditions and may have implication in their varying tolerance to environmental factors. Further, mutual understanding between microendophytes itself may be involved in order to avoid competition for the host photosynthate.

The biomass of mycorrhizal roots was recorded low during rainy as compared to winter season. This may also be related to the decreased production of fine mycorrhizal root by host in response to high moisture level.

Chitin assay showed a seasonal variation in its content which was generally correlated with mycorrhizal infection and this supports ~~with~~ the findings of Bhattarai (1983) and Peterson *et al.* (1991).

A seasonal variation in the morphology of VAM fungi *in vivo* was correlated to the environmental changes in

different seasons. High number of arbuscule/ during rainy season may indicate that nutrient transfer between host-fungus is ~~more~~ active. A relationship between population of arbuscules and nitrogenase activity signifies the role of former in the exchange of nutrients especially P between host and fungus (Cox et al., 1975) which is essential for nodulation and nitrogen fixation. Decrease in ratio between arbuscules and vesicles during winter was also observed by Doud and Chaney (1986). This increase in vesicle population during non - growing (dormant season) may be explained as it acts as a storage organ and fungal inoculum as well (Gerde mann and Trappe, 1974; Biermann and Linderman, 1983) which are related to host root infection and supply of nutrients for nodulation and nitrogen fixation when limiting.

Further, the mixed infection of host root by two or more endophytes as evidenced by the presence of morphologically distinct fungi agrees with the findings of McGonigle and Fitter (1990) and Rosendahl et al. (1989) who noted infection of host roots by several species of mycorrhizal fungi under field condition .

Population of endogone spores in soil also showed a distinct seasonal fluctuation. Fungal spore count was high during winter and low during rainy^{season}. Advent of

unfavourable climatic condition may have induced spore formation by VAM fungi, which may be better adapted to tolerate harsh soil and climatic conditions. Further, it may also be related to the fact that production of more and more VA spore may ensure more infection of host root thereby ensuring its survival. A decreased spore abundance with increasing soil depth in alder stand agrees with the findings of Schwab and Reeves (1981) and Allen et al., (1984). Quantity of endogonaceous spores affected by the soil (Hayman, 1970; Khan, 1971) and host factors like root exudates. Their density may be correlated with infection level (Daft and Nicolson, 1972).

The present finding of occurrence of more than one endogonaceous spore types from field conditions agrees with the findings of Crush (1973), Hayman et al. (1975), Abbott and Robson (1977) ^{and Ragupathy et al. (1988)} who also observed spores of more than one species of mycorrhizal fungi in the rhizosphere soils. The dominance of *Glomus mosseae* in most seasons indicated its competitive nature and may in fact be selectively preferred by host. This agrees with the findings of Mosse (1973) and Schenck and Kinlock (1980).

The significance of negative correlation between nitrogenase activity and VAM characteristics such

as mycorrhizal infection, chitin assay and endogone spore abundance may be ecologically important and indicates that despite mutual synergistic effects (Visser *et al.*, 1991) they behave differently in their growth and development in response to climatic factors. In addition, it may also signify that this could be their mutual ecological strategy to avoid inter-symbiont competition under field conditions where several limiting factors are operative.

Chapter	:	Isolation, cultivation and characterization of
II	:	<i>Frankia</i> from root nodules of <i>Alnus nepalensis</i>
	:	D. Don growing in field condition .

INTRODUCTION

The group of microorganisms that are able to induce nitrogen-fixing root nodules in non-leguminous (actinorrhizal) plants has been broadly classified under the genus "*Frankia*". Till date, more than 200 species of non-leguminous plant species belonging to 24 genera have been recorded to form actinorrhizal associations (Dixon and Wheeler, 1986). After the initial hurdle before 1978, the first successful isolation of nodule endophyte in axenic culture followed by demonstration of nodulation of its original host was made by Callahan et al. (1978). Since then hundreds of *Frankia* isolates have been obtained from root nodules of different *Alnus* spp. (Baker and Torrey, 1979; Wheeler et al., 1986; Baker, 1989) and other actinorrhizal plants (Diem and Domergues, 1982; Diem et al., 1983; Zhang et al., 1984; Luis and Lalonde, 1986; Hiyoshi et al., 1988; Racette and Torrey 1988; Favre-Raynaud et al., 1990).

Apart from the obvious interest in mechanism of symbiotic association with plant roots and ability to fix

atmospheric nitrogen, members of *Frankia* initiate curiosity mainly by virtue of their characteristic morphologically diverse structures, infectivity and effectivity with original host and other hosts and compatibility in terms of biomass production by its hosts.

A significant variation in efficiency of nitrogen fixation by *Frankia* both between strains from the same plant species (Wheeler et al., 1986; Hooker and Wheeler, 1987; Normande and Lalonde, 1987) and between strains from different plant species (Dawson and Sun 1981; Dillon and Baker 1982; Weber et al., 1987) has been documented. In addition, the nodulation capacity of *Frankia* isolates is known to differ remarkably with various host species which in turn may be controlled by both endophyte and host plant (Nesme et al., 1985). Besides, instances that *Frankia* strains are unable to nodulate original host but other host species have also been demonstrated (Zhang et al., 1984).

Studies pertaining to growth of microendophyte on various C sources have demonstrated that a wide variety of sugars, sugar alcohols and organic acids serve as C sources. Marked differences in the pattern of C and N utilization are shown to occur from strain to strain (Akkermans et al., 1983; Shipton and Burggraaf 1983 a; Shipton and Burggraaf 1983 b; Tisa et al., 1983).

Attempts in the recent years to classify existing

Frankia isolates by micromorphology (Baker et al., 1981), physiology (Lechevalier and Ruan, 1984; Lechevalier et al., 1983; Horriere, 1984), cellular proteins (Benson and Hanna, 1983), cell composition (Mort et al., 1983) infectivity and effectivity (Normande and Lalonde, 1982; Burggraaf and Valstar, 1984; Zhang et al., 1984), Chemotaxonomy (Lechevalier and Lechevalier, 1980; Lechevalier et al., 1982; Chaboud and Lalonde, 1983), cytogenetical (Normand et al., 1983; Simonet et al., 1984; Parson et al., 1985) and genetical (An et al., 1985; Simonet et al., 1989; Sylvie et al., 1989; Bloom et al., 1989; Dobritsa and Stupar 1989; Simonet et al., 1990; Nazeret et al., 1991) aspects have led to the cleavage of nodule endophytes into several large groups (Baker et al., 1981; Baker, 1982; Lechevalier et al., 1982). However, lack of phenotypic characteristics correlating with DNA-DNA hybridization data still prevents them giving species status (Lechevalier and Lechevalier 1989; Lechevalier and Lechevelier, 1990).

The ability to fix atmospheric nitrogen symbiotically depends on both host plant genotype (Gordon and Wheeler 1978; Huss-Danell 1980; Palmgren et al., 1985; and Hahn et al., 1988) and the *Frankia* strain. Efficiency of the nitrogenase activity also has been shown to vary between different host-endophyte combinations (Dawson and Sun, 1981; Dillon and Baker, 1982; Normand and Lalonde, 1982; Sellstedt et al., 1986; Hooker and Wheeler, 1987; Weber et al., 1987).

Ecological importance of actinorrhizal plant species is tremendous especially in afforestation, land reclamation, soil melioration and short term forestry . *Alnus nepalensis* D. Don , a fast-growing tree species is considered to be a useful nitrogen fixing plant which colonizes the nutrient- deficient and hostile habitats of Eastern Himalayas (Sharma and Ambasht, 1984) and also grow naturally or in plantations in higher altitudes of Khasi Hills (India).

Keeping in view the crisis of present time, especially to reduce the investment in nitrogenous fertilizers, in increasing plant productivity and reclamation of denuded lands as created by human interference, the isolation, characterization and screening of efficient *Frankia* strains in terms of its compatibility and effectivity with host is most demanding. Unfortunately little is known about the occurrence of *Frankia* in the Indian subcontinent (Hafeez et al., 1984; Choudhary and Mirza, 1987) and North eastern part of India, harbouring different actinorrhizal plants . Therefore, the present study was conducted to isolate and characterize endophytic *Frankia* from the root nodules of *A. nepalensis* growing at Upper Shillong area (Study site).

MATERIALS AND METHODS :

1. Isolation of *Frankia* from root nodules of *A. nepalensis* :

The root nodules of *A. nepalensis* collected from

Upper Shillong were washed thoroughly under a jet of tap water. The healthy nodules (pale-white in colour) were then sorted out and washed several times in tap water followed by breaking up nodules manually into individual nodule lobes. The cleaned nodules after Tween 20 treatments, were then transferred separately to conical flask containing 2 % v/v glutaraldehyde, 2 % v/v sodium hypochlorite (NaOCl_2), 5% w/v bleaching powder (CaOCl_2), 1 % w/v osmium tetroxide (OsO_4) and 30 % v/v hydrogen peroxide (H_2O_2) and the intervals of contact were 15, 20, 20, 2 and 10 min respectively.

Solid media

technique A :

After thorough washing with sterile distilled water, nodules were homogenized in a sterile mortar and pestle using little amount of sterile water. Then 2 ml of filtered nodule extract was layered upon 60, 45 and 30 % steam sterilized sucrose solution in sterile graduated centrifuge tubes and was capped with heat sterilized aluminium foil. The tubes were centrifuged at 2,500 g for 20 min and the solution lying in the interface of 60 and 45% sucrose solution was used to inoculate media using pour plate technique (1:100) coupled with double agar layer. The media tested were defined proportionate minimal (DPM) media, Q mod, BAP M⁶B and DPM/N (DPM plus 8mM NH_4Cl). A thin layer of medium was poured over the solidified inoculum. All the media were supplemented with

micronutrients solution (Lalonde and Calvert, 1979), 400 mg/l cycloheximide and 15 mg/l of nystatin. The pH of media was adjusted to 6.7 - 6.8 $\pm$ 0.05 before autoclaving. The composition of various isolating media used in this study was as follows:

a. Defined propionate minimal (DPM) media (Baker and O'Keefe, 1984)

As described in chapter I

b. Qmod (Lalonde and Calvert, 1979) :

Component	Concentration (mg/l)
K ₂ HPO ₄	300
NaH ₂ PO ₄	200
MgSO ₄ · 7H ₂ O	200
KCl	200
Yeast extract	500
Bactopeptone	5 g
Glucose	10 g
Ferric citrate (1% solution)	1 ml
1-a Lecithin	50

Minor Salts

According to Lalonde and Calvert (1979).

Agar 10 g

c. BAP medium (Murray et al., 1984) :

Component	Concentration (g/l)
K ₂ HPO ₄	0.591

KH_2PO_4	0.952
NH_4Cl	0.267
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.095
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.010
FeNa-EDTA	0.010
Sodium propionate	0.480

Micronutrients

According to Lalonde and Calvert, (1979).

Vitamins (mg/l)

Thiamine H_2O : 10	1 ml
Nicotinic acid : 50	
Pyridoxine H_2O : 50	
Folic acid : 10	
Calcium <u>pantothenate</u> : 10	
and Riboflavin : 10	

C

C

?

Agar : 10 g

d. M⁶B (modified from Baker and Torrey, 1979) :

Component	Concentration (g/l)
Yeast extract	5
Dextrose	10
Casamino acids	5
KH_2PO_4	1
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1

CaCl ₂ .2H ₂ O	0.01
CoCl ₂	0.001
FeNa-EDTA	0.01

Trace elements :

According to Lalonde and Calvert (1979).

Vitamin solution :

	As described in BAP	
Agar		10g

e. DPM/N (Norridge and Benson, 1986)

Component	Concentration (g/l)
KH ₂ PO ₄	1.0
MgSO ₄ .7H ₂ O	0.1
CaCl ₂	0.01
Sodium propionate	1.2
Ammonium chloride	8 mM

Micronutrients :

According to Lalonde and Calvert (1979).

Agar	10g

Technique B :

After surface sterilization, the nodule lobes

were washed thoroughly in sterilized distilled water and the nodule epidermal layer was peeled off using sterile razor under the laminar flow chamber. The nodule cubes were washed 8 - 10 times in sterile distilled water and were aseptically placed on the solidified medium followed by pouring a layer of medium so as to cover the nodule lobe in the Petri dishes.

Technique C :

The surface sterilized nodule lobes were washed thoroughly to remove the surface sterilant and were crushed in sterile mortar and pestle. The homogenate was filtered aseptically through sterile cheese cloth (pore size 20 μ m and 50 μ m) using a vacuum pump. The filtrate was collected in the sterile Erhlenmayer flasks and was inoculated onto various media employing pour plate method (dilution 1:100) coupled with double agar layer technique.

Technique D :

The nodule lobes after surface sterilization were microdissected and two nodule slices were placed on the solidified nutrient enriched Qmod medium in the Petri dishes at $28 \pm 1^\circ\text{C}$ for 14 days in dark. The microbe-free nodule lobes were homogenized using a tissue homogenizer with little amount of sterile water. The filtered nodule homogenate was inoculated in Petriplates containing solidified media.

All above procedures starting from surface

sterilization of nodule lobes to inoculation were carried out in a sterile laminar flow chamber. The inoculated Petridishes were incubated at $28 \pm 1^{\circ}\text{C}$ for 60 days in dark.

Liquid media

Technique E :

The surface sterilized nodule lobes were washed 8-10 times with sterile distilled water, aseptically transferred to Erhlenmayer flasks containing 20 - 30 ml liquid media (minus agar) , crushed with sterile glass rod and cotton plugged.

Technique F :

A variant of technique D in which the culturally sterilized one nodule lobe was placed aseptically in the Erhlenmayer flask containing liquid media and crushed into finer pieces with sterile glass rod and capped with sterilized cotton plugs.

Technique G :

The micro-dissected nodule lobes as described in technique B were placed in liquid media and crushed into finer fragments using a sterile glass rod and plugged with sterilized cotton plugs.

Technique H :

One ml of nodule homogenate obtained as in technique A was diluted to 100 ml with different media and 1 ml inoculum was used to inoculate Erhlenmayer flasks containing 20 ml media.

After inoculation all above Erhlenmayer flasks were then transferred to BOD incubator maintained at $28 \pm 1^{\circ}\text{C}$ and kept for 60 days in dark.

Morphology of Nodule endophyte :

The slow-growing actinomycete colonies appearing after 60 days of inoculation with less than 1mm diameter were harvested aseptically with the help of micropipette and placed on clean glass slides, stained with 0.1% toluidine blue in 1% boric acid and mounted on 5% acidic glycerol. The slides were visualized under the phase contrast microscope (Leitz DX 20). The dimensions of the *Frankia* hyphae, vesicles and sporangia were recorded.

Cultivation (subculture) :

Isolated *Frankia* strains were subcultured at 4 to 8 week intervals. The inoculum harvested, either by pasteur pipette (solid medium) or centrifugating at 2,000 g (liquid medium), washed thrice with sterile distilled water diluted and homogenized by repeated passage through a sterile 0.8 mm needle. They were inoculated into 50 ml ~~capacity~~ Erhlenmayer flask containing 20 ml sterile DPM medium under

aseptic laminar flow chamber and inoculated at $28 \pm 1^{\circ}\text{C}$.

Utilization of carbon and nitrogen sources :

Frankia isolates from *A.nepalensis* varying in colony morphology, pigmentation and micromorphology were harvested separately with Pasteur pipette or centrifugation at 2,000 g, washed 3-4 times in sterile distilled water and ^a little amount of sterile water was added. *Frankia* inoculum was then homogenized using 0.2 mm diameter syringe by passing through it 15-20 times. The protein content of inoculum was estimated following the method of Bradford (1976). Each inoculum was treated with 1 ml NaOH (1N) and was heated at 90°C for 30 min, followed by addition of 5 ml of 0.5% alkaline CuSO_4 reagent. The solution was mixed thoroughly and 1 ml of Folin Ciocateau reagent (1:1; Folin : water) was added and stirred to allow simultaneous mixing of reagents. After 30 min, absorbance of the solution was read at 600 nm in a spectrophotometer. The protein content of the *Frankia* isolates was determined using the standard curve of bovine serum albumin (Fraction IV). Thereafter 0.5 ml of the standardized *Frankia* inoculum was added separately to 50 ml Erhlenmayer flasks containing 15 ml steam sterilized basal medium (DPM) with various C sources. Standardized *Frankia* isolates were inoculated similarly in the basal medium containing various N sources.

The composition of basal medium and concentration

of various C and N sources used were as follows :

Basal medium

Components	Concentration (g/l)
$\text{KH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$	1.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1
CaCO_3	0.01

Micronutrient solution

According to Lalonde and Calvert (1979)

pH was adjusted to 6.8 ± 0.05

Carbon (C) sources	Concentration used (g/L)
Glucose	0.5
Fructose	''
Starch	''
Sodium propionate	''
Sodium pyruvate	''
Tween 80	''
Sodium acetate	''
Succinic acid	''

Nitrogen (N) sources	Concentration used (g/L)
Ammonium chloride	0.2
Potassium nitrate	''
Glutamic acid	''
Thymine	''

Five flasks were used for each *Frankia* strain in each C and N source. The *Frankia* inoculum thus added to medium containing single C or N source was cotton plugged and incubated at $28^{\circ} \pm 1^{\circ}\text{C}$ in dark. After the expiry of each experimental period i.e. 7, 14, 21 and 28 days, three flasks were withdrawn from each treatment and *Frankia* was harvested aseptically by centrifugation. After washing with distilled water for 3-4 times the protein content of it was estimated following the method suggested by Bradford (1976) and expressed in $\mu\text{g protein ml}^{-1}$.

Growth on various Temperature regime/:

Growth of *Frankia* isolates under various temperature conditions was performed using same basal medium as above for growth with C or N sources with sodium propionate as sole C source. The temperature regimes tested were 15, 20, 25, 28, 30, 35 and 40°C . The growth response of *Frankia* isolates on different temperature was estimated by determining protein content (Bradford, 1976).

Growth of *Frankia* on different pH regimes :

In experiment specifically designed to assess influence of pH on *Frankia* isolates, the growth medium used composed of basal medium as described earlier with the inclusion of a C source i.e. sodium propionate and was buffered with either PIPES or phosphate buffer (Burggraaf and Shipton,

1982). One ml standardised *Frankia* inocula were aseptically added and the growth of *Frankia* under various pH regime after 21 days of incubation at 28°C was determined as described earlier (Bradford, 1976).

Assessment of nitrogenase activity of *Frankia* in vitro :

Nitrogenase activity of different *Frankia* strains was assessed by a gas chromatograph . The apparatus was fitted with a hydrogen flame ionization detector and 2.13 m stainless steel with 0.32 mm diameter column packed with 80-100 Poropack R. The temperature programme was isothermal; injector 64°C, column 54°C and detector 100°C. All gases used were purity grade in which the following rates were maintained, H₂ - 25 ml min⁻¹, O₂ - 100 ml min⁻¹ and N₂ - 25 ml min⁻¹.

Culture tubes filled with 5 ml sterilized liquid DPM medium (plus propionate or plus ammonium chloride) was inoculated with 1 ml standardized *Frankia* strains and maintained at 28^{±1}°C. On each occasion after the termination of experimental period, i.e, 7, 14, 21 and 28 days, three tubes were randomly withdrawn from each treatment and fitted with air tight suba seals. Further experimental process and assessment of nitrogenase activity were performed in the same manner as stated in Chapter I. The activity, however, was expressed in nmole C₂H₄ 5 ml⁻¹ h⁻¹.

Nodulation test and *Frankia* strain reisolation :

Five 1 month old seedlings of *Alnus nepalensis* obtained by growing NaOCl_2 (5 % w/v) sterilized seeds on 2% agar, were washed several times in sterile distilled water and transferred aseptically to the 500 ml capacity Erhlenmayer flasks containing solidified agar and moistened with 1/4 strength of N-free Hoagland solution (Hoagland and Arnon, 1968). After one week of transfer, seedlings were inoculated with 1 ml homogenized 12-13 weeks old *Frankia* isolate. Inoculations of seedlings with *Frankia* isolates were also carried out in sterilized sand kept in polyethylene jars. The inoculated seedlings in the Erhlenmayer flasks and jars were kept in growth chamber maintained at 25/18°C day/night temperature and 14/10 h day/night photoperiod. Inoculation of *Frankia* was performed again after 2 weeks as described above. Each inoculation experiment was repeated at least twice and the nodulation was assessed after 2, 4, 6, 8, 10 and 12 weeks. Nodule was considered to be formed only if root swelling was conspicuous under stereomicroscope.

Nodules induced by *Frankia* strains on axenically grown seedlings were recovered, washed thoroughly and disinfected with 30% H_2O_2 for 10 min in dark. After thorough rinsing, the nodule lobes were singly transferred to Erhlenmayer flasks containing 15 ml sterilized DPM medium, crushed to finer fragments and incubated for 60 days at 28[±]1°C in dark.

Assessment of nitrogenase activity of root nodule :

After each harvest, the root system of nodulated plants was washed thoroughly in running tap water, excised with sterile razor and was treated with 0.1% (v/v) Tween 20 for 5 min. They were washed thoroughly with distilled water. The root systems were transferred to 10 ml capacity serum tubes and fitted with suba seals. Further determination of nitrogenase activity was carried out in the same manner as described in Chapter I. The nitrogenase activity was expressed as $\text{nmole C}_2\text{H}_4 \text{ h}^{-1} \text{ seedlings}^{-1}$.

RESULTS

Techniques of isolation of *Frankia* :

Among various techniques applied for the isolation of *Frankia*, microdissection coupled with double agar layer (Technique B) and sucrose density fractionation with pour plate coupled with double agar layer (technique A) in solid media and modified crushed nodule method (technique E) in liquid media, proved to be most successful.

Surface sterilizing agent :

Surface sterilant like H_2O_2 and OsO_4 were evaluated to be more effective ones in eliminating surface contaminants amongst various chemical agents tested.

Isolation media :

For the isolation of symbiotic *Frankia* from the root nodules of *A. nepalensis* into axenic culture, an array of media ranging from simple N-free DPM medium to very complex media including Qmod M⁶B, BAP, whose constituents included α -lecithin and various vitamins were used. Only DPM and Q mod media supported the growth of different isolates of *Frankia* (Table 7).

Morphology of *Frankia* :

All the *Frankia* strains showed a characteristic cushion-like, branched, septate hyphae ranging from 0.5 - 1.2 μ m (Table 8). Spherical to ovoid vesicles were produced, terminally borne on short parental hyphae ranging from 2-6 μ m in diameter. Sporangia were either intercalary or terminal, whose frequency increased with the increasing age of *Frankia* in culture. Variation in the dimension of sporangia was most pronounced amongst *Frankia* isolates (Plates 9a, 9b & 9c).

Three strains of *Frankia* i.e. AnF₁, AnF₂ and AnF₃ isolated from the root nodules of *A. nepalensis* occurred in the same locality (Upper Shillong). Strains AnF₁ and AnF₂ were isolated solely on DPM (solid) while AnF₃ was obtained with DPM (liquid) and Qmod (liquid) media. Colonies of all the *Frankia* isolates did not exceed 3 mm in diameter on DPM agar medium after two months of growth (Table 7). Production of pigments was characteristic for the *Frankia* isolate AnF₁ and AnF₂ which produced dark-brown and yellow-brown pigment respectively.

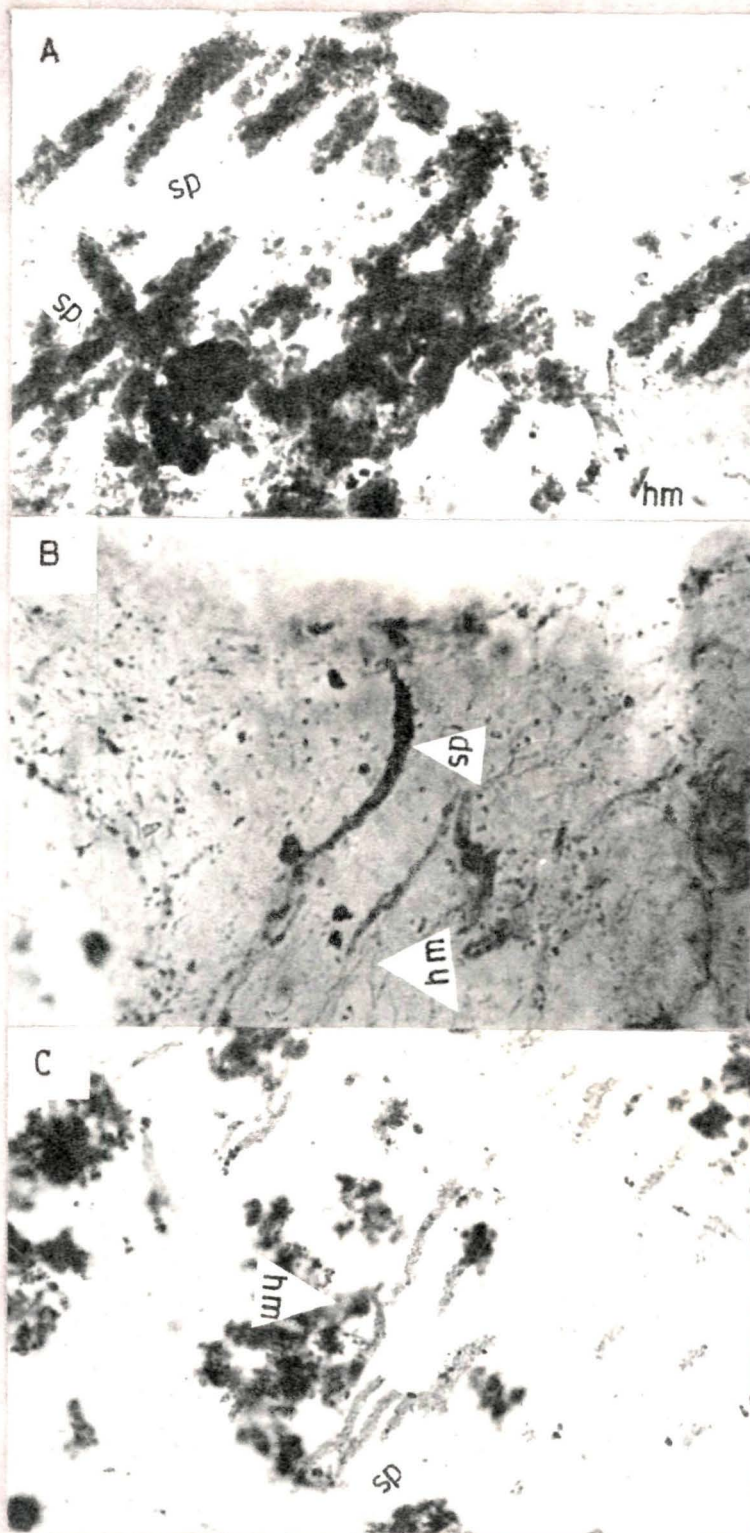


Plate 12. Morphology of in vitro cultured Frankia strains isolated from root nodules of A. nepalensis. A = Frankia AnF₁ (x 312.5); B = F. AnF₂ (x 312.5); C = F. AnF₃ (x 200);¹(hm = hyphal mat; sp = sporangium).

Frankia strain AnF₃ however, produced no pigment (Table 7). The colonies were transparent on periphery and dense in the centre and grew submerged in the agar layer or at the bottom of liquid medium.

Production of vesicles and sporangia in all the *Frankia* isolates tested depended on the composition of growing medium (Table 11). DPM medium was most effective in the induction of vesicles or sporangia for all the isolates whereas, they produced less vesicles and sporangia on Qmod medium. Vesicles were generally repressed on BAP medium but it was effective for AnF₂ and efficiently induced sporangia production. On DPM/N medium, vesicle formation was repressed in AnF₁ and AnF₂ but not in AnF₃. However, it did not increase sporangia development in the two strains.

Growth of *Frankia* on various carbon sources:

The growth of *Frankia* isolates on various C sources after 7, 14, 21 and 28 days is summarized in Table 9. Some interesting comparisons in terms of the ability to use C sources by *Frankia* isolates emerged. Isolate AnF₁ exhibited strong growth on propionate and pyruvate but showed poor utilization of glucose, fructose, starch, Tween 80, yeast extract and Succinic acid. Its growth on sodium acetate was better than the above C sources. *Frankia* isolate AnF₂ showed greater versality on glucose, fructose, starch, propionate, pyruvate, sodium acetate and succinic acid. However, it did

not perform well on Tween 80.

C sources like sodium propionate, pyruvate, glucose and fructose, succinic acid were utilized by AnF₃ but lesser versatility on starch and sodium acetate sources was noted. However, the strain grew poorly on Tween 80 and yeast extract amended media.

Utilization of nitrogen sources :

The growth performance of three *Frankia* isolates differs variously on various N sources (Table 10). Isolate AnF₁ exhibited better growth on NH₄Cl than other N sources. However, ammonium chloride and aspartate were somewhat better utilized than other N sources by AnF₂ strain. Growth response of AnF₃ was generally similar to AnF₁ except that former showed better growth on casamino acid amended medium.

Effect of temperature on *Frankia* growth :

Effect of temperature on the growth of three *Frankia* isolates was markedly distinct. The optimum temperature requirement for the growth was 28 - 30°C for all the three isolates. Further increase or decrease in temperature proved detrimental for their growth. No net gain in total protein yield was observed at 40°C. Growth at 35°C however, was marginally better for most *Frankia* isolates than at 40°C. *Frankia* AnF₁ was most sensitive to decrease in temperature followed by AnF₂ except for AnF₃ which showed

higher growth at same temperature range (Fig. 25).

Effect of pH on *Frankia* growth :

The growth response of *Frankia* strains AnF₁, AnF₂ and AnF₃ to various pH regimes differed ~~variously~~. No isolate grew at pH 4.0. The optimal pH requirement favouring growth of *Frankia* isolates AnF₁ and AnF₂ was between 6 and 7 and for AnF₃ around 5 (Fig. 26). With increase in pH over 7.0 - 8.0, the growth of *Frankia* isolates was affected to some extent. Generally the growth of *Frankia* isolates was ^{more} ~~superior~~ in medium buffered with phosphate than buffered with PIPES (Fig. 26).

Nitrogen fixation by *Frankia* strains in vitro :

The rate of nitrogen fixation by various *Frankia* isolates on propionate and ammonium chloride amended media showed interesting features with the lapse of time. Propionate generally induced higher activity of nitrogenase than ammonium chloride. However, with increase in time, the difference in nitrogenase activity of *Frankia* grown on two energy sources narrowed (Fig. 27). However, unlike propionate, generally the activity of ammonium chloride grown *Frankia* increased linearly with time.

Nodulation and reisolation :

The nodulation time for each *Frankia* isolate varied significantly (Fig. 28). Strains AnF₁ and AnF₂ were more

effective in nodulating original host i.e. *A. nepalensis* within 30 days of inoculation. However, the isolate AnF₃ could induce nodules only after 30 days of inoculation. Generally the morphology of reisolated strains of *Frankia* from root-nodules of its host resembled original ones (Table 11).

Nitrogen fixation by *Frankia* in vivo :

The efficiency to fix atmospheric nitrogen by root nodules induced by various *Frankia* strains on its host varied markedly (Fig.29). The nodules produced by AnF₁ showed higher nitrogenase activity than those induced by AnF₂ and AnF₃.

Dominance of *Frankia* :

It is evident from Table 12 that strain AnF₁ was most dominant *Frankia* in root nodules of *A. nepalensis* over various seasons and strain AnF₃, the least one.

Discussion:

The success in the isolation of endophytic *Frankia* in axenic culture using microdissection in combination with double agar layer may be related to efficient elimination of contaminating organisms associated with nodule epidermal layer. The advantage in the use of double agar layer in solid medium is two-fold i.e. in the maintenance of microaerophilic conditions as required for the growth of *Frankia* strains as well as in spatially separating the contaminants as also noted

Table 7. Colony characteristics of three *Frankia* isolates obtained from root nodules of *Alnus nepalensis*. 5/

<i>Frankia</i> Strains	Colony size (mm)	Colony shape	Pigmentation	Mode of growth	Obtained on
AnF ₁	1-3	diffuse irregular	diffusible brown to dark brown	growing submerged in agar or bottom of liquid media	DFM (Solid and liquid)
AnF ₂	1-3	diffuse irregular	diffusible light brown	growing submerged in agar or bottom of liquid media	Gmod/DFM (Solid and liquid)
AnF ₃	< 1	minute irregular	-	growing at the bottom of liquid media	DFM (Liquid)

Table 8. Measurements of hyphae, vesicles, sporangia and spores of *Frankia* strains isolated from *Alnus nepalensis*.

<i>Frankia</i> Strains	Hyphae (um)	Vesicles (um)	Sporangia (um)	Spores (um)
AnF ₁	0.5 - 1.0	2 - 5	20 - 60	1.5 - 2.0
AnF ₂	0.5 - 1.2	3 - 6	20 - 40	1.0 - 2.0
AnF ₃	0.8 - 1.0	3 - 6	10 - 25	< 1.0

Table 9. Growth performance (μg protein /15 ml medium) of three *Frankia* strains on different Carbon sources after various experimental period.

Growth of <i>Frankia</i> (μg protein /15 ml) mean $\pm$ S.E						
Carbon sources	Harvesting period (days)					
	7			14		
	AnF ₁	AnF ₂	AnF ₃	AnF ₁	AnF ₂	AnF ₃
Glucose	8 $\pm$ 2.1	16 $\pm$ 7.1	72 $\pm$ 5.8	9 $\pm$ 3.5	12 $\pm$ 2.8	49 $\pm$ 10.5
Fructose	7 $\pm$ 1.7	22 $\pm$ 5.1	51 $\pm$ 6.5	5 $\pm$ 1.1	10 $\pm$ 2.5	35 $\pm$ 13.8
Starch	10 $\pm$ 2.8	15 $\pm$ 6.2	16 $\pm$ 2.2	8 $\pm$ 3.1	15 $\pm$ 7.2	19 $\pm$ 5
Sodium Propionate	206 $\pm$ 40.1	58 $\pm$ 8.9	154 $\pm$ 61	271 $\pm$ 22	61 $\pm$ 15.2	113 $\pm$ 42.2
Sodium Pyruvate	171 $\pm$ 29.5	40 $\pm$ 6.2	129 $\pm$ 17	156 $\pm$ 16.2	25 $\pm$ 10.4	121 $\pm$ 31.2
Tween-80	4 $\pm$ 1.1	12 $\pm$ 1.81	11 $\pm$ 1.0	2 $\pm$ 0.8	8 $\pm$ 1.6	15 $\pm$ 2.8
Sodium acetate	16 $\pm$ 5.2	25 $\pm$ 3.2	34 $\pm$ 15.4	14 $\pm$ 3.6	21 $\pm$ 5.8	29 $\pm$ 6.8
Succinic acid	5 $\pm$ 1.0	17 $\pm$ 5.5	23 $\pm$ 5.8	5 $\pm$ 2.9	15 $\pm$ 6.2	11 $\pm$ 6.3

Table 9. (Contd.)

Growth of <i>Frankia</i> (μg protein /15 ml) mean $\pm$ S.E						
Carbon sources	Harvesting period (days)					
	21			28		
	AnF ₁	AnF ₂	AnF ₃	AnF ₁	AnF ₂	AnF ₃
Glucose	6 $\pm$ 1.5	5 $\pm$ 2.2	31 $\pm$ 21.2	1 $\pm$ 0.6	2 $\pm$ 1	16 $\pm$ 5.9
Fructose	3 $\pm$ 2.1	6 $\pm$ 2.8	17 $\pm$ 6.4	1 $\pm$ 0.3	1 $\pm$ 0.2	5 $\pm$ 1.5
Starch	4 $\pm$ 1.5	9 $\pm$ 3.5	10 $\pm$ 3.1	3 $\pm$ 1.1	3 $\pm$ 2.1	2 $\pm$ 1
Sodium Propionate	131 $\pm$ 28.6	31 $\pm$ 12.1	45 $\pm$ 20.1	104 $\pm$ 15.8	19 $\pm$ 5.8	18 $\pm$ 7.1
Sodium Pyruvate	102 $\pm$ 41.5	16 $\pm$ 5.5	36 $\pm$ 12.1	96 $\pm$ 25.5	8 $\pm$ 2.7	11 $\pm$ 3
Tween-80	00	00	6 $\pm$ 3.0	00	00	00
Sodium acetate	9 $\pm$ 2.2	13 $\pm$ 6.8	15 $\pm$ 6.1	4 $\pm$ 1	5 $\pm$ 1.2	7 $\pm$ 2
Succinic acid	3 $\pm$ 0.7	6 $\pm$ 1.2	5 $\pm$ 1.4	2 $\pm$ 0.8	2 $\pm$ 0.6	00

Table 10. Growth response of three *Frankia* isolates on different nitrogen sources (μg protein 15 ml^{-1}) after various experimental period.

Growth of <i>Frankia</i> (μg protein 15 ml^{-1}) $\pm$ S.E												
Nitrogen sources	Harvesting period (days)											
	7			14			21			28		
	AnF ₁	AnF ₂	AnF ₃	AnF ₁	AnF ₂	AnF ₃	AnF ₁	AnF ₂	AnF ₃	AnF ₁	AnF ₂	AnF ₃
Inorganic sources :												
Ammonium chloride	23 ± 5.0	16 ± 5.5	14 ± 2.8	18 ± 10.5	15 ± 1.3	10 ± 4.3	7 ± 2.8	6 ± 2.1	5 ± 1.5	3 ± 1.2	2 ± 1.0	3 ± 1.2
Potassium nitrate	6 ± 1.1	8 ± 3.1	19 ± 3.9	8 ± 4.2	11 ± 3.2	14 ± 4.6	4 ± 1.1	5 ± 0.6	4 ± 0.3	1 ± 0.6	1 ± 0.4	2 ± 0.8
Organic sources :												
Glutamic acid	28 ± 11.9	5 ± 1.4	5 ± 2.1	21 ± 5.8	7 ± 2.8	6 ± 1.3	8 ± 1.7	3 ± 0.6	4 ± 1.5	4 ± 1.1	00	1 ± 0.2
Casamino acid	7 ± 0.9	8 ± 0.9	10 ± 3.1	9 ± 1.8	4 ± 1.3	7 ± 2.0	6 ± 0.7	3 ± 1.0	5 ± 3.1	2 ± 0.6	2 ± 0.7	1 ± 0.1
Aspartic acid	8 ± 2.1	20 ± 5.7	5 ± 1.5	8 ± 1.1	17 ± 6.7	3 ± 0.9	4 ± 2.4	10 ± 2.9	1 ± 0.2	00	2 ± 0.5	00

Table 11. Production of vesicles and sporangia by three *Frankia* isolates on different growth media.

Growth media	AnF ₁		AnF ₂		AnF ₃	
	Vesicles	Sporangia	Vesicles	Sporangia	Vesicles	Sporangia
DPM	+	+	+	+	+	*
Gmod	-	+	*	*	-	*
BAP	*	+	-	*	-	+
M ⁶ B	-	*	+	+	-	+
DPM/N	-	*	-	+	*	*

+ = present, - = absent and * = seldom present.

Table 12. Isolation of *Frankia* strains from Himalayan alder nodules collected in different seasons.

Growth media	Rainy			Autumn			Winter			Spring		
	AnF ₁	AnF ₂	AnF ₃	AnF ₁	AnF ₂	AnF ₃	AnF ₁	AnF ₂	AnF ₃	AnF ₁	AnF ₂	AnF ₃
DFM	+	+	-	+	+	+	+	-	+	+	+	-
Gmcd	+	+	-	+	+	-	+	+	+	+	-	-

Data given above are condensed from two years study.

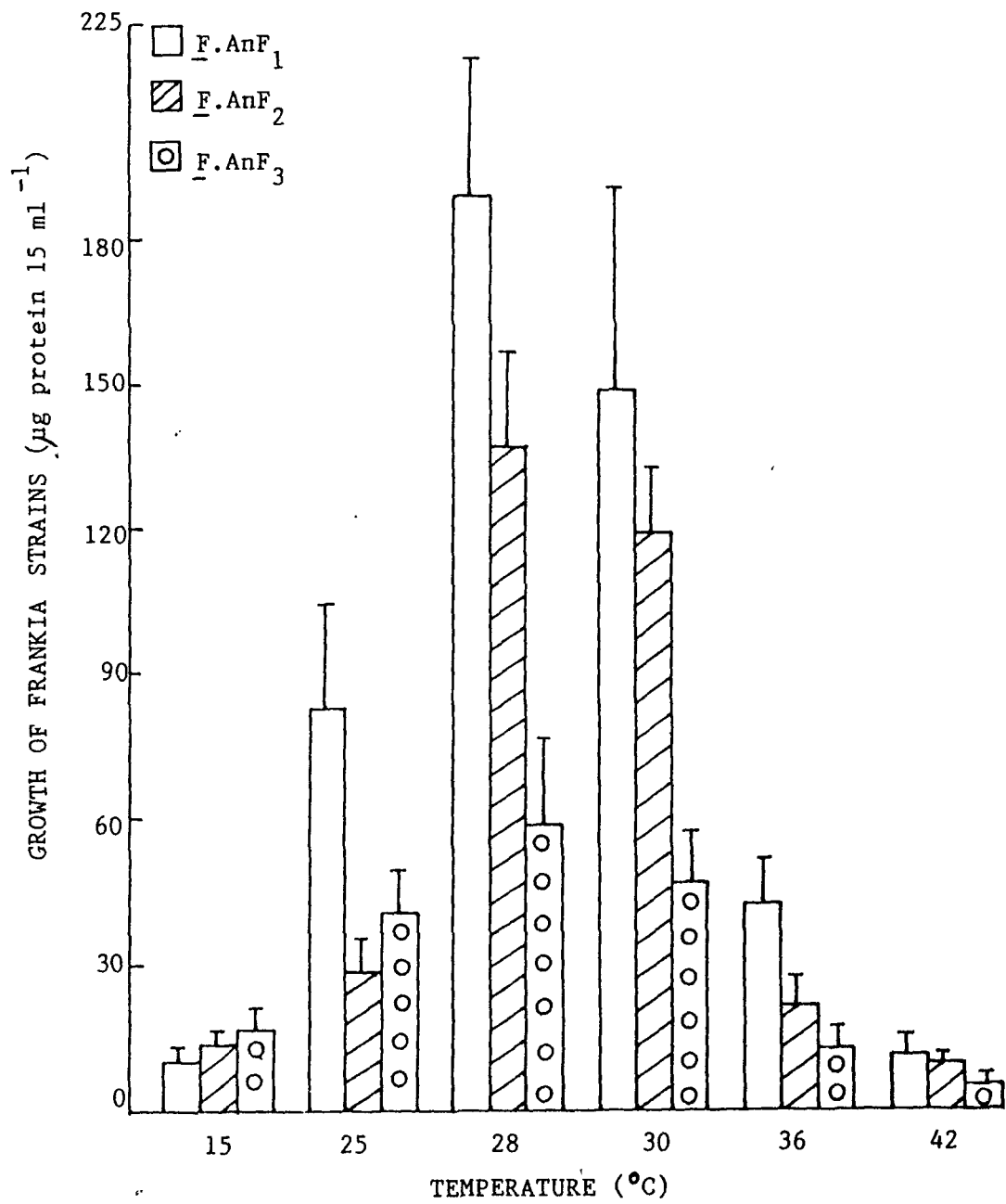


Fig. 25. Effect of temperature on the growth of different strains of Frankia in vitro.

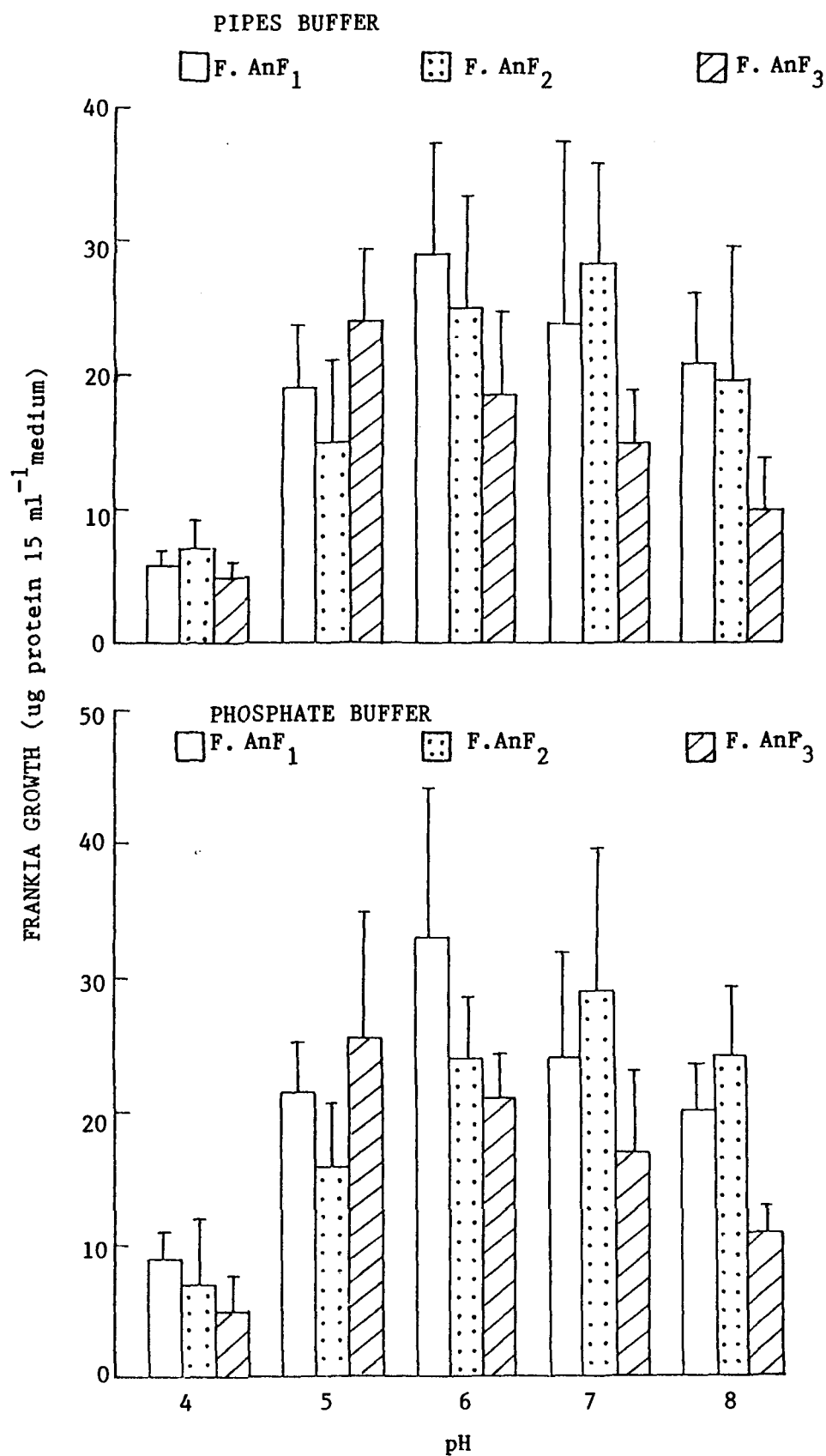


Fig. 26. Growth performance of three Frankia isolates (after 21 days) on growth media adjusted to various pH with PIPES and phosphate buffers.

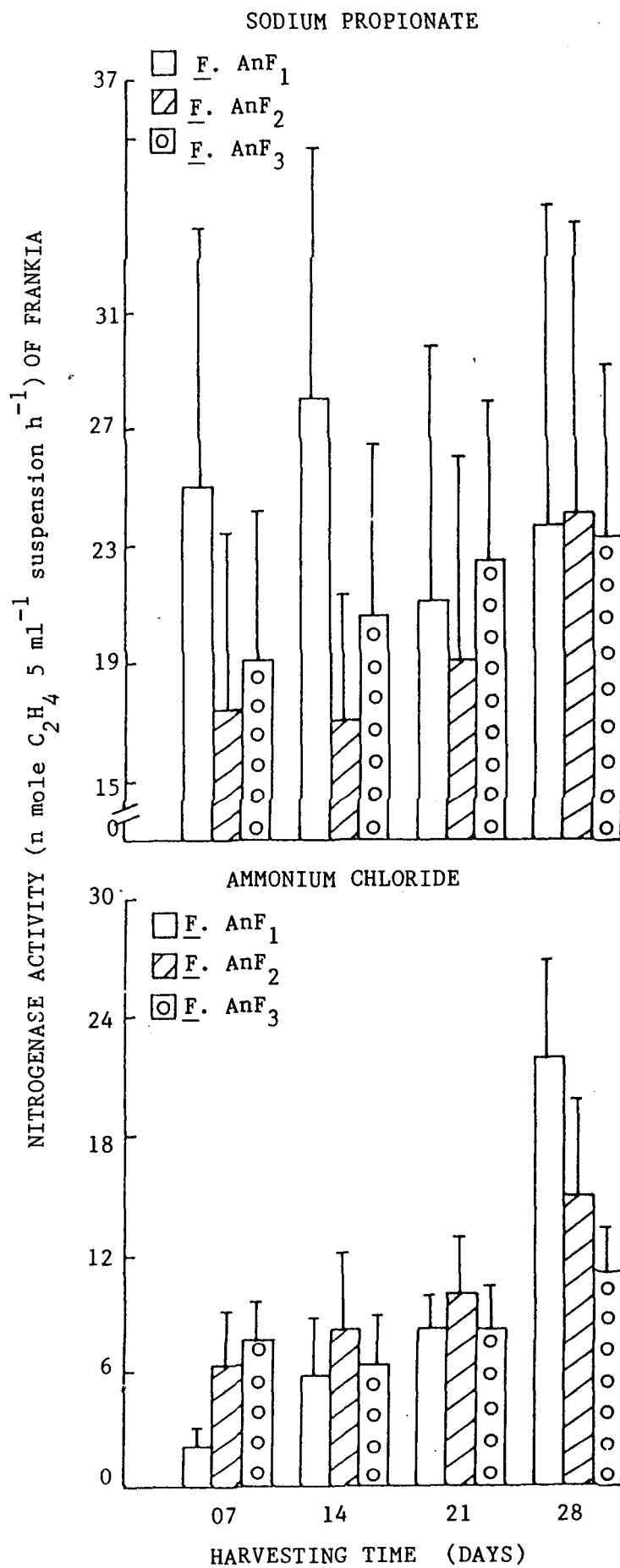


Fig.27. Nitrogenase activity of different Frankia isolates on DPM medium supplemented with sodium propionate and ammonium chloride.

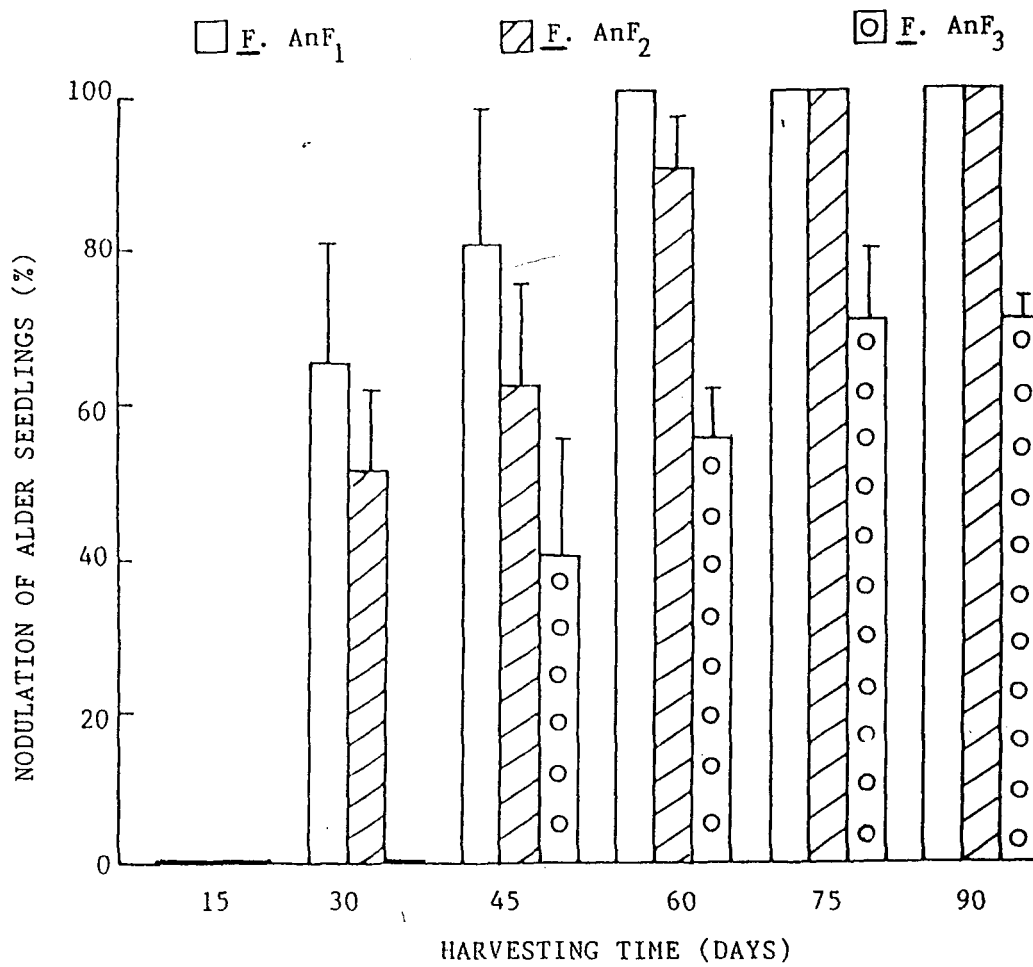


Fig. 28. Nodulation of alder (*Alnus nepalensis*) seedlings with different strains of *Frankia*.

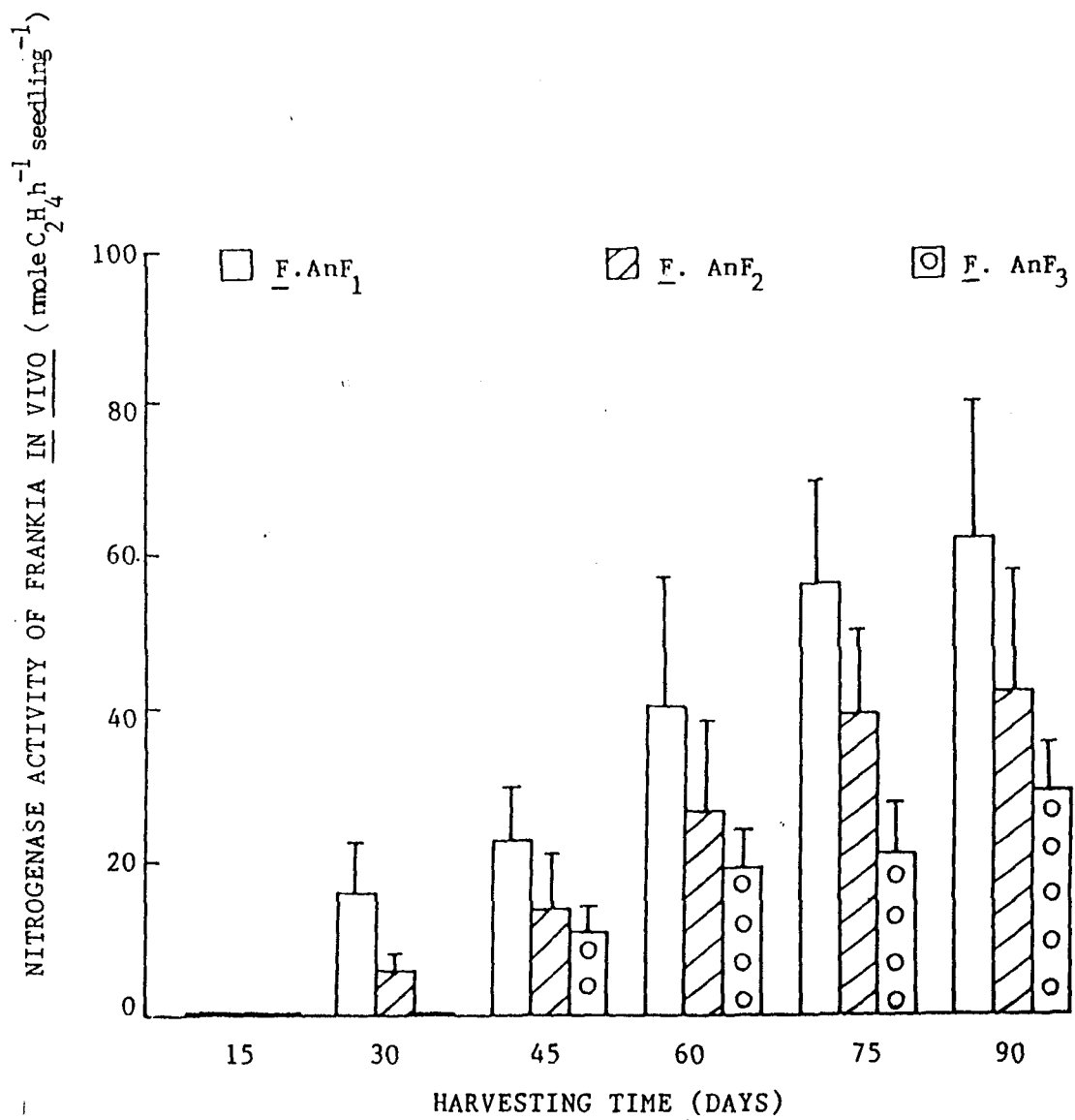


Fig.29. Nitrogenase activity of different strains of Frankia in vivo.

by Diem and Dommergues (1982). In solid culture, the efficiency of sucrose density gradient technique coupled with double agar layer mainly be attributed to higher precision in isolating *Frankia* to a particular sucrose band and microaerophilic nature of *Frankia*. In liquid culture, modified nodule crushing method proved beneficial in the isolation of *Frankia*, which renders effective disintegration of endophyte containing nodule cells ultimately leading to dispersal of infective/viable *Frankia* propagules directly in contact with nutrient medium.

The role of hydrogen peroxide and osmium tetroxide as potential surface sterilizing agents which are basically strong oxidants has also been noted by Berry and Torrey (1979) and Lalonde et al. (1981) respectively. These sterilants are efficient in eliminating surface contaminants from nodule lobes without causing a harmful effect on cortical cells containing symbiotic *Frankia*.

Various media employed for the isolation of *Frankia* are complex and are supplemented with yeast extract and various nutrients which are believed to promote *Frankia* growth in culture media i.e. lecithin (Lalonde and Calvert, 1979), root lipid extract (Quispel et al., 1983) and various vitamins (Baker and Torrey, 1979). However, in the present study, successful isolation of *Frankia* from Himalayan alder nodules was achieved using simple DPM medium (Baker and O'Keefe,

1984), its variations and slightly complex Qmod (-lecthin) (Lalonde and Calvert, 1979). Baker and O'Keefe (1984) formulated and successfully used DPM medium to selectively isolate *Frankia* from *Alnus*, *Casuarina*, *Myrica*, *Purshia* and other actinorhizal plants and rhizospheric soil. However, advantages in using nodule lobes or nodule homogenate may also be attributed to the fact that active compounds (Qispel and Tak, 1978) necessary for the growth of symbiont in axenic culture may be provided by nodule itself.

In vitro morphology of *Frankia* isolates resembled other *Frankia* strains isolated from various species of alder (Berry and Torrey, 1979; Benson, 1982; Normande and Lalonde, 1982; Baker, 1989). Vesicles and sporangia were produced by all three *Frankia* strains, though at different degrees. The isolates producing abundant sporangia formed compact flocks and grew at slower rate. Formation of sporangia was dependent on constituents of media (Berry and Torrey, 1979) and may also be determined by genetics of organisms involved (Hafeez et al., 1984). The variability in the size of sporangia of different strains align with the findings of Berry and Torrey (1979). The decrease in frequency of vesicles when supplemented with ammonium N sources may be related to suppression of vesicle formation in response to exogenous N supply.

The observations on temperature requirement

for the *in vitro* growth of *Frankia* isolates showed that all the three isolates grew optimally in the range of 28-30°C, beyond which growth was hampered. Shipton and Burggraaf (1983) also found that optimal temperature for the growth of most *Frankia* isolates was around 30°C. In addition, better growth response of AnF₁ over AnF₂ and AnF₃ at 28 - 30 ° C temperature range may afford additional advantages for its symbiotic growth with *A. nepalensis* in the growing season (March - October) when the soil temperature increases from 15°C in March to 21°C through July to October. However, better growth performance of *Frankia* AnF₃ at 15°C may provide advantage over coisolates AnF₁ and AnF₂ during relatively shorter colder part of the year i.e. Nov - Jan for growth and symbiosis with its host.

The higher growth response of *Frankia* isolates, in the present study when buffered with phosphate buffer was supported by the data of Burggraaf and Shipton (1982). Hampered growth of all *Frankia* isolates at pH 4.0 may be explained by detrimental effect of acidity on it which was supported by findings of Bond (1951), Bond *et al.*, (1957) and Frej and Huss-Danell, (1987). An optimal pH range for AnF₁ and AnF₂ was between 6 and 7, however, in natural conditions, the soil pH of Himalayan alder stand ranged from 5.5 to 5.65 during rainy season and thus may not be favourable for infection and growth by them. However, their higher dominance over most seasons of the year may indicate that pH optima for *in vitro* (soil) growth may be different than that during symbiosis with

host. Besides, the competition between the isolates AnF₁ and AnF₃ may be less expected especially during winter season with low soil pH as the optimal pH of two isolates lie between 6-7 and around 5 respectively. Result of Kurdali et al. (1990) substantiates above findings.

The different utilization capabilities shown by three *Frankia* isolates in terms of C and N substrates for energy sources is particularly interesting and indicated that a considerable diversity related to ecological relationships of these coisolates may occur. However, possibilities of other factors like inoculum density, age and pregrowth conditions may also influence the adaptations of *Frankia* strains to various substrates so that in short term they do not appear to be utilized.

The limited growth response of AnF₁ on glucose and starch may not represent an ability to cleave glucosidic bonds. However, the possibility may be that heat degradation took place or that growth was supported by traces of fatty materials and proteins which may be found in starch. Lack of growth of AnF₁ on tricarboxylic acid possibly indicates the absence of suitable uptake system rather than a deficiency of tricarboxylic acid cycle enzymes. Total absence of growth of *Frankia* isolates in most C sources after 21 days of growth may be thus attributed to autolysis of *Frankia* cells, which is supported by the findings of Berry and Torrey (1979) and Murry

et al. (1984). Autolysis may be an important feature in symbiosis since hyphal and vesicles disintegration is observed in ageing nodules (Newcomb et al., 1978) as well as carbon-rich synthetic media (Murry et al., 1984). Further lack of growth of *Frankia* strains AnF₁ and AnF₂ on medium supplemented with Tween 80 could be related to lack/repression of uptake system or glyoxylate cycle enzymes (Blom, 1982; Blom and Harkink, 1983).

There appears to be no evidence to speculate that these *Frankia* can act as primary colonizer of organic debris. It is however, feasible that they could act as secondary colonizer utilizing simple breakdown products released through the activities of other microbes. many organic acids are commonly found in the aerobic and anaerobic soils. Root exudates may contain a range of organic acids or amino acids which include those most commonly found to be utilizable by them (Rovira, 1965) suggesting that substrates for the growth of *Frankia* are commonly present in soils. All of these traits are characteristics of organisms equipped for a symbiotic and /or rhizospheric existence.

Frankia isolates AnF₁ and AnF₂ preferred ammonium chloride than potassium nitrate which may be ~~better~~ explained by better assimilation of NH₄⁺ and utilizing^{ed} it for growth. Preference of *Frankia* isolates to ammonium chloride as the sole N source has previously been documented (Shipton and Burggraaf, 1982; Lechevalier et al., 1983; Shipton and

Burggraaf, 1983). Besides, better growth yield of AnF₁ and AnF₂ on aspartate and glutamate may be due to suitable assimilation activities of the organisms. Out of all combined N sources, yeast extract seemed to be least effective in stimulating growth of most of *Frankia* isolates, which may be due to the absence of uptake systems.

Nitrogenase induction was influenced by the nature of nutritional factors. Depression in nitrogenase activity in the NH₄⁺Cl grown *Frankia* may be related to the suppression in the induction of vesicles and nitrogenase enzyme in response to exogenous N supply. The support comes from the study of Murry et al., (1984). In contrast stimulation of nitrogenase in propionate may have resulted from availability of suitable C source for the rapid growth of *Frankia* thus leading to enhanced activity to fulfil nitrogen requirement in its absence in environment. After 21 days of growth of *Frankia* on NH₄Cl, the nitrogenase activity increased. It indicated that *Frankia* strains have used up nitrogen supplied externally and fixed nitrogen under stressed condition. The acetylene reduction by all the isolates confirmed that that these strains are true nitrogen fixers like other *Frankia* strains (Gauthier et al., 1981; Diem et al., 1982).

The difference in the rate of nodulation of *Frankia* isolates may be due to variation in compatibilities between host and *Frankia* strains. Most of them could nodulate

their host after 4 weeks. Baker et al. (1979) and Rosbrook (1990) also noted that *Frankia* inoculated plants developed nodules after 4 weeks of inoculation. However, Nesme et al. (1985) showed that nodulation by *Frankia* isolates varied from 7 to 16 days. A variation in the nitrogen fixing activity of nodules formed by different *Frankia* isolates signify the existence of host-endophyte compatibility (Dillon and Baker, 1982).

Based on the above criteria, *Frankia* isolates obtained from root nodules of *A. nepalensis* growing in the same locality, may be grouped into three different "ecological niche" groups. Besides, it also signifies the diversity of *Frankia* strains in the same alder stand as evidenced by Benson and Hanna (1983), Hafeez et al. (1984) and Burggraaf and Valstar (1984).

Dominance of AnF₁ strain in most part of the year indicated its broad ecological amplitude adopted to different climatic and edaphic conditions over other coendophytes. Burggraaf and Valstar (1984) and Faure-Raynaud et al., (1992)^{also} concluded that host genotype exerts preference to endophyte strain from the populations of *Frankia* in soil which host occupies.

Chapter	:	Isolation and characterization of vesicular-
III	:	arbuscular mycorrhizal(VAM) endophytes from <i>Alnus</i>
	:	<i>nepalensis</i> D. Don forest stand.

Introduction :

In nature more than 200 species belonging to 24 genera of nonleguminous plants bearing *Frankia* nodules (Dixon and Wheeler, 1986) are economically important for symbiotic nitrogen fixation in the tropical and temperate regions of the world. In addition to *Frankia* symbiosis, many of them harbour VA mycorrhizal symbionts in their root system thus forming a formidable tripartite symbioses which consequently aid in the plant nutrition and growth. Several reports may be cited demonstrating the occurrence of VAM symbiosis in majority of actinorhizal plants (Williams and Aldon, 1976; Daft and Hacskeylo, 1976; Williams, 1979; Rose, 1980; Rose and Trappe, 1980; Clelland et al., 1983; Gardner, 1986; and Sidhu et al., 1990). In fact, such dually infected plant species are the true colonizers of bare and infertile habitats unsuitable for other plants lacking symbionts (Daft and Hacskeylo, 1976). Besides, such plants may be regarded to be of utmost importance in the improvement of infertile lands.

Majority of fungi involved in VA mycorrhizal associations generally produce spores which mainly is evaluated on the basis of morphological characteristics (Mosse and Bowen, 1968; Hall and Fish, 1979; Jibaji-Hare et al., 1986). However, characterizations of VAM fungi based on biochemical (Sen and Hepper, 1986), immunological (Wilson et al., 1983; Wright et al., 1987; Williams et al., 1987) and genetical aspects (Simon et al., 1992) have also proved useful. The microscopic characters which include the finer details complicate the identification of different fungal species. As a result, the taxonomical studies on endogonaceous members have been fragmentary, though, they play an important role in plant nutrition.

In North-East India a vast array of wild plants flourish many of which are actinorhizal. Later groups of plants form symbiotic association with VAM fungi. Little is known about VA symbiont of forest soils of the region (Sharma et al., 1986). Considering their universal occurrence and their role in plant nutrition, an investigation on VAM fungi of actinorhizal hosts could add to the present knowledge of range of mycorrhizal associations and their implications on afforestation programmes in the near future.

The present investigation was undertaken to access mycorrhizal association of actinorhizal *Alnus nepalensis* D. Don growing in monocultural condition (Study site). Endogonaceous

spores have been indentified and described in this chapter.

Isolation of Endogonaceous fungi :

The spores of endogonaceous fungi were isolated from the soils of Himalayan alder (*A. nepalensis*) forest following wet sieving and decanting method of Gerdemann and Nicolson (1963) as described previously (Chapter I). The spores were separated from soil debris and were transferred to watch glasses containing water. The identical spores were separated and stored in glass vials containing Ringer's solution. The different types of spores were mounted in polyvinyl lactophenol (PVL) on glass slides for microscopic observations. Ringer's solution and PVL medium were prepared as described in Chapter I.

Establishment of culture of VA mycorrhizal fungi :

Isolated spores of VAM fungi were surface sterilized following the method of MacDonald (1981). The spores were transferred by micropipette to 45 mm diameter filter paper disc, which was covered by identical disc thus forming filter paper sandwich and was placed in a plastic filter holder. They were sterilised at 121°C for 15 min before addition of spores. Then the surface sterilant solution, 2 % w/v chloramine-T with 0.02 % w/v streptomycin sulphate plus a trace of Teepol (Mosse, 1959) was injected from sterile syringe through 0.22 µm porosity membrane filter until excess liquid

emerged. A total of 20 ml sterilant was used for sterilizing 200 spores and the contact time being 20 min. The sterilant was removed from spores by passing 200 ml sterile distilled water. The sterilized spores were stored between damp filter paper in Petridishes at 4°C. The sterilized suspension of isolated spores of VA endophytes was inoculated on sterilized potted garden soil 2 cm below the soil surface. The germinated seeds of maize, obtained from surface-sterilized seeds, were placed on the VAM treated soil and covered with soil. The host plant was grown for a period of 2 months and was allowed to dry.

Description of endogonaceous fungi :

Seven species of VA fungi were identified from the soils of Himalayan alder forest. The description of each of them is as follows:

***Glomus* :**

Four species of *Glomus* were identified i.e. *Glomus mosseae*, *G. macrocarpus*, *G. intraradices* and *G. tortuosum*.

1. *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe

Sporocarp unknown, chlamydospores yellow to brown, globose to ovoid ranging from 90-110 µm in diameter and the thickness of spore wall 7 µm (Plate 13 A).

2. *G. geosporum*

(Nicol. and Gerd.) Walker.

Chlamydospores formed singly in soil, sporocarp unknown, globose or sub-globose, 110 - 150 x 110 - 143 μ m smooth, dark brown to black, spore wall 10-15 μ m thick, perforated and hyphal attachment 12 - 15 μ m in diameter (Plate 13 B).

This species was also described in agricultural fields by Bhattacharjee et al. (1980).

3. *G. intraradices* Schenck and Smith

Sporocarp unknown, chlamydospores forms singly or in clusters in the root, chlamydospores predominantly globose to subglobose 93-120 x 112-130 μ m diameter. spore wall 3-15 μ m thick, yellow to grey brown. Spore content globular, yellow to light brown, wall of spore extending into the hyphal attachment forming an tubaeform flare at the junction of hyphal attachment, 9-33 μ m wide (Plate 13 c).

The occurrence of this species from the soils of Florida, USA was first noted by Schenck and Smith (1982).

4. *G. tortuosum* Schenck & Smith

Sporocarp unknown, chlamydospores formed singly in soil but occasionally adhering in pairs. Spores yellow to dull grey-brown with a mantle of sinuous hyphae closely appressed.

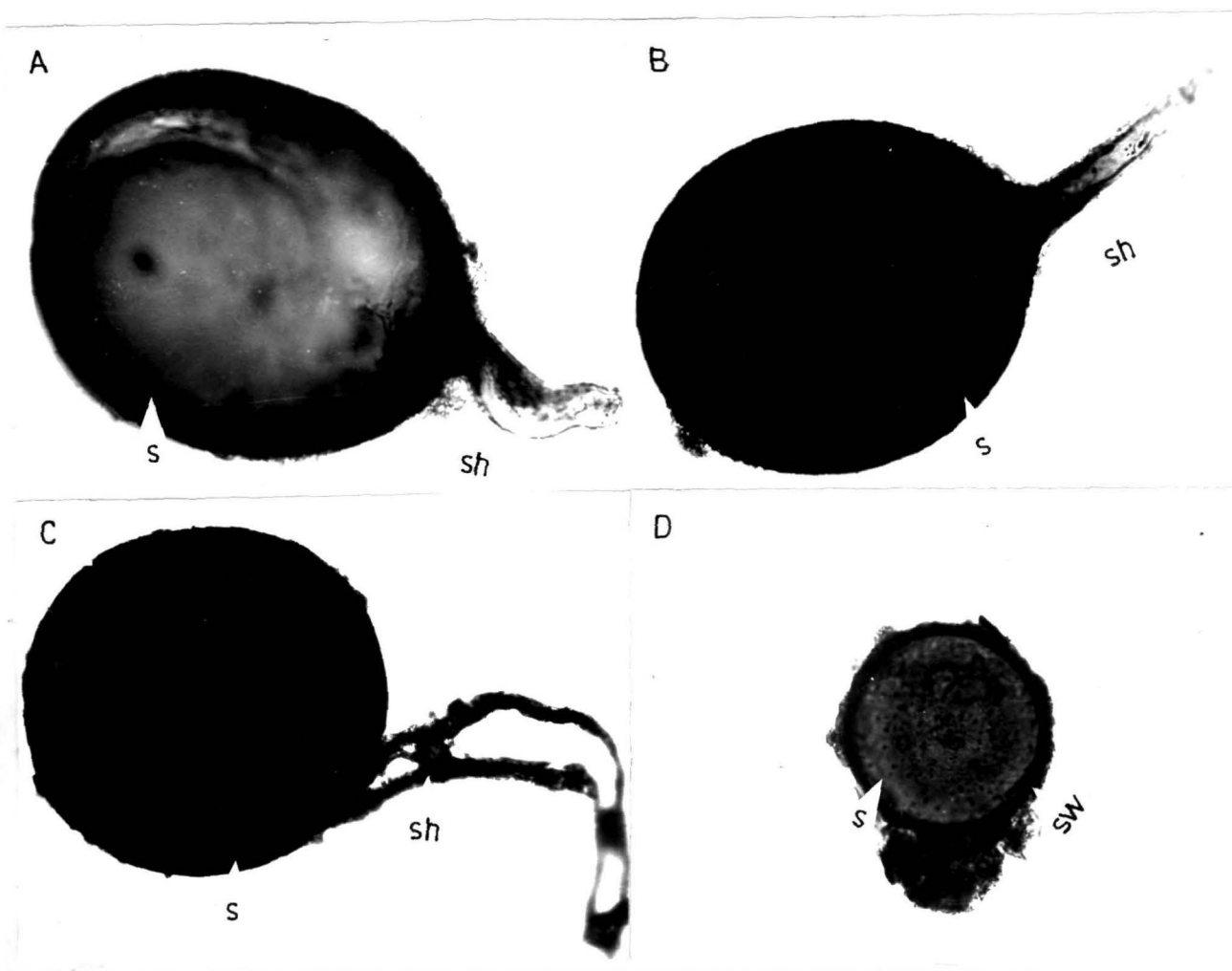


Plate 13. Spores of *Glomus* species isolated from soils of Himalayan alder forest. A = *G. mosseae* (x 200); B = *G. macrocarpus* (x 200); C = *G. intraradices* (x 200); D = *G. tortuosum* (x 78.7); (sh = subtending hypha; sw = spore wall; s = spore).

Chlamydospores largely globose to subglobose 94-180 x 112-230 um diameter. Spore content globular but occasionally obscured by the mantle. Width of hyphal attachment at the spore base 9-20 um (Plate 13 D).

This species was first described by Schenck & Smith (1982) from Florida, USA.

***Gigaspora* :**

Two species of *Gigaspora* were identified i.e. *Gigaspora gigantea* and *Gi. gregaria*.

1. ***Gi. gigantea* (Nicol. and Berd.) Gerdemann and Trappe**

Azygospores formed singly in soil, 260 - 370 x 260 - 350 um globose, greenish yellow, spore wall 9 - 16 um, suspensor-like cell bulbous, 42 -48 um in diameter, giving rise to a suspensor hyphae which projects to the spore, vesicles not observed.

This form was also reported from the mixed woodland of Dehradun by Bakshi (1974) (Plate 14 A).

1. ***Gigaspora gregaria* Nicol. and Sch.**

Azygospores formed singly in soil, 200 - 300 um in diameter, globose, dark brown, spore wall 12 - 25 um thick suspensor-like cell 50 um in diameter concolrous with spores and giving rise to a slender hyphae projecting into the spore surface.

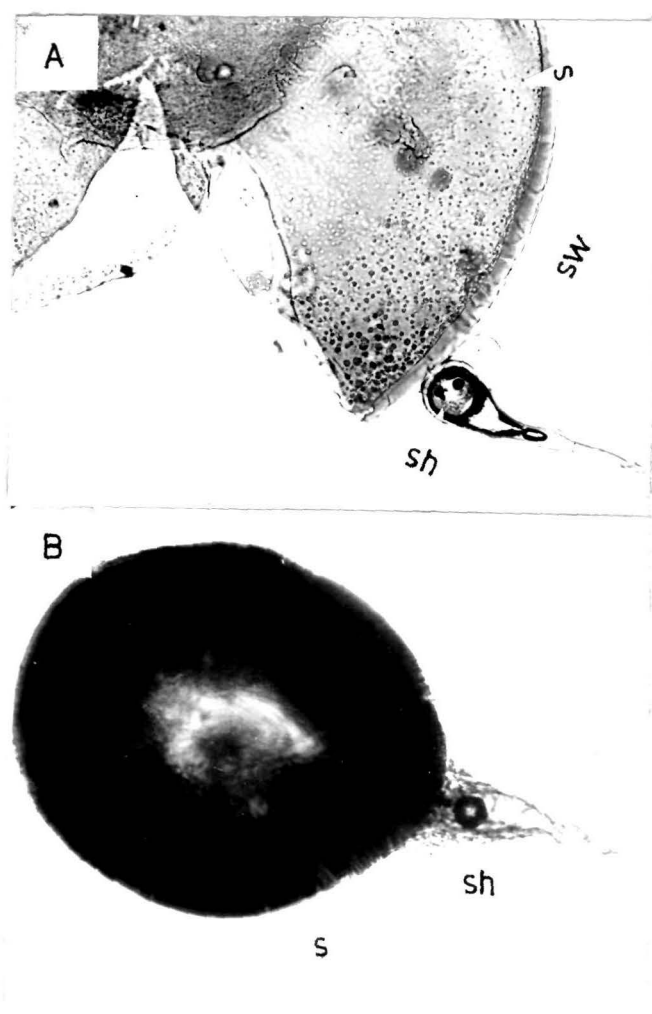


Plate 14. Spores of *Gigaspora* species isolated from Himalayan alder forest soil. A = *Gi. gigantea* (x 200); B = *Gi. gregaria* (x 200); (sh = subtending hypha, sw = spore wall, s = spore).

This species was earlier described by Sharma et al. (1986) from rhizosphere of *Alnus nepalensis* (Plate 14 B).

***Acaulospora* :**

One species of *Acaulospora* is reported in the present investigation namely *A. scrobiculata*.

1. *Acaulospora scrobiculata* Trappe.

Sporocarp unknown, spores formed singly in soil, sessile, borne laterally on thin-walled hyaline hyphae that terminate in a thin walled vesicle, spore shape varying from globose to ellipsoid, 100 - 200 um, olive to light brown, spore surface evenly pitted (Plate 15).

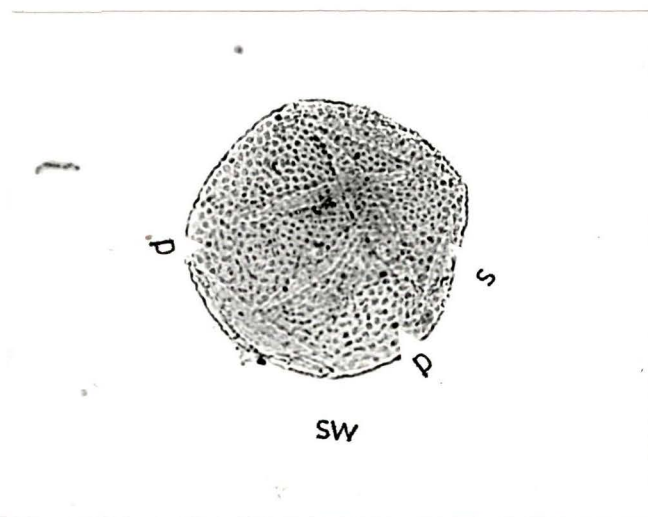


Plate 15. Spore of Acaulospora scrobiculata (x 150); (sw = spore wall; p = pit; s = spore).

Chapter	:	Interaction between <i>Frankia</i> and <i>Glomus mosseae</i> on
IV	:	P uptake, nitrogen fixation and seedling growth of
	:	<i>Alnus nepalensis</i> under various soil pH regimes.

Introduction:

Soil reaction affects plant growth and survival by mediating the availability of nutrients. Differential tolerance by various plant species may be an important ecological strategy to occupy and establish in varying soil conditions. *Alnus nepalensis* D. Don (Himalayan alder), a pioneer actinorhizal plant species colonizes the hostile nutrient-deficient soil conditions in Eastern Himalaya (Sharma and Ambhast, 1986) and flourishes well under moderately acidic conditions as other alder species (Fergusson and Bond, 1953; Wheeler *et al.*, 1986). This preference of host plant in particular soil pH may also determine the growth of endophyte (Graw, 1979; Lambert *et al.*, 1980).

Survival and effectivity of symbiotic *Frankia* and VAM fungi which form tripartite symbiotic association with non-legumes may be influenced by soil reaction. Susceptibility and

consequential death of *Frankia* in highly acidic soils (Burggraaf and Shipton, 1982; Faure-Raynaud *et al.*, 1986; Smolander and Sundman, 1987; Smolander *et al.*, 1988) and ameliorative proliferation and growth at higher pH (Burggraaf and Shipton, 1982) than its host may affect symbiosis, its effectivity and fate of both host and endophyte. Soil reaction has been shown to have profound effect on nodulation (Fergusson and Bond, 1953; Seilor and McCormick, 1982; Smolander *et al.*, 1988) and nitrogen fixation by symbiotic *Frankia* (Reddell, 1986).

In addition, influence of soil reaction on VAM fungal abundance (Kwarkelman, 1973), spore germination (Green *et al.*, 1986), infection (Skipper and Smith, 1979; Vourdwijk and Hairian, 1986) and symbiotic efficiency (Graw, 1979) is evident. Further, the optimal pH requirement may differ between endophyte to endophyte and between host and endophytes. Studies on legumes have indicated that soil acidity severely affects efficiency of tripartite symbiosis (Skipper and Smith, 1979; Newbould and Rangeley, 1984; Mays de Manzi and Cartwright, 1984). Like in leguminous system, evidences of stimulation of growth of non-leguminous hosts by dual inoculation under nutrient -deficient conditions are well documented (Rose and Youngberg, 1981; Gardner *et al.*, 1984; Russo, 1989; Jha *et al.*, 1993). However, information on the

effect of soil pH in the survival, infection and their effectivity in tripartite association involving alder (nonlegume) - *Frankia* - VAM microsymbionts is lacking and has practical importance as the soil system supporting alder forests is continuously becoming acidic due to release of litter¹ bound acidic substances and rapid replenishment of exchangeable with increase in soil N (Crocker and Major, 1955; Debell and Redwan, 1983; Van Miegroet and Cole, 1984).

Keeping above facts under consideration, the present work was conducted to assess the interaction between *Frankia* and VAM fungi and their effect on seedling growth of *A. nepalensis* at various soil pH regime under controlled environmental conditions.

Materials and Methods :

Collection and sterilization of soil :

Sandy loam soil collected from the *Alnus nepalensis* forest stand possessing following physico-chemical properties i.e. CEC - 22 Meq/100 g soil, sand 54.1%, silt 25.9% and clay 20%, pH - 5.21, organic matter - 4.11%, Total N - 0.229%, available P - 0.021 %, exchangeable K - 0.090 %, exchangeable Ca- 0.061% and extractable Mn - 0.0011% was mixed with sand in the ratio of 2:1 (soil: sand). After

moistening to field capacity, the soil was autoclaved at 15 lb/inch² for 1 h. The sterilization was repeated again after a gap of 24 h and was allowed to stand for 15 days.

Adjustment of soil pH :

The soil used for the experiment was acidic (pH 5.21). Four pH levels were maintained with above soil i.e. pH 4, 5, 6 and 7. The pH of the soil was raised with application of fine CaCO_3 powder following pH-base saturation method as suggested by Thompson and Troeh (1985). For the determination of cation exchange capacity (CEC), 10 g dry soil sample was leached with 250 ml ammonium acetate (1 M) to occupy all the exchangeable cation sites. Excess ammonia was removed by leaching with ethyl alcohol (90 %). The amount of ammonia held in the sample was replaced with 1 M NaCl and distilled into another container and was titrated with N/140 HCl. The CEC was expressed in terms of milliequivalent per 100 g soil (Meq/100 g). Further soil pH was determined as described in chapter I and pH-base saturation curve prepared using which CaCO_3 requirement of the soil was evaluated. Conversion of soil into acidic condition was achieved by application of 3% H_2SO_4 (Ryan *et al.*, 1975) and the amount of H_2SO_4 (3 %) required to lower pH was calculated (Thompson and Troeh, 1985).

The pH of the soil was adjusted after the steam-sterilization of the soil.

Collection of seeds and raising of seedlings :

The mature seeds of *A. nepalensis* were collected in the month of March 1992 from Himalayan alder stand at Upper Shillong. The husked, healthy seeds were surface sterilized with 5% w/v bleaching powder (CaOCl_2) and were thoroughly washed with sterile water. After 24 h of soaking, the imbibed seeds were then aseptically transferred to sterile solid water agar (1%) in Petridishes and grown under controlled growth conditions maintained at 10 Klx light intensity with 14 h light and 10 h dark cycle at 25°C day and 18°C night temperatures. Relative humidity of 60-80 % was maintained throughout the experimental period.

Preparation of *Frankia* inoculum :

Frankia strain AnF₁ (abbreviated as *Frankia*) isolated from root nodules of *A. nepalensis* was selected for this experiment. Ten week old culture growing in DPM liquid medium was harvested aseptically by centrifuging at 2000 g for 15-20 min and washed 3 to 4 times with sterile distilled water. The inoculum was homogenized by passing 15-20

times through 0.1 mm diameter syringe and diluted to known concentration i.e. 10^4 cells per ml with sterile distilled water by following the method of Burggraaf and Shipton, (1983). After mild sonication, inoculum density of *Frankia* was estimated with the help of Petroff Houser chamber (height 0.01 mm) using phase contrast microscope.

Preparation of VA Mycorrhizal inoculum :

Glomus mosseae (Nicol. & Gerd.) Gerdemann & Trappe, a dominant VAM fungus in Himalayan alder stand (chapter 1) was maintained on maize and onion host plants in the glass house. Eighty five *endomycorrhizal spores* were suspended in 50 ml of sterile distilled water along with 200 mg of infected maize/onion roots (infection level > 60%).

Inoculation of root microsymbionts and plant growth conditions :

One month old, uniform sized and healthy Himalayan alder seedlings growing in water agar were harvested, washed 4-5 times with sterile distilled water and transplanted at the rate of 5 plants in 1L conical flasks containing soils of various pH. Each seedling was inoculated with 1ml *Frankia* inoculum in the rhizosphere with the help of sterile syringe. VAM fungal inoculum was placed 2 cm below soil surface before

transplanting of alder seedlings in mycorrhizal and *Frankia* + mycorrhizal treatments. They were watered with sterile automated pipettes at the rate not exceeding 20% soil moisture level. The conical flasks were capped and transferred to controlled growth conditions and maintained as described earlier in this chapter.

Harvests :

Harvests of seedlings were made after 30, 60, 90 and 120 days after inoculation. 200 seedlings from each treatment were harvested along with intact root system from randomly sampled conical flasks. The soil adhering to the root system was collected and stored at 4°C and the roots of the seedlings were washed thoroughly under a jet of tap water.

Evaluation of seedling growth :

On each occasion, plant growth performance was evaluated by recording root, shoot and plant height with the help of simple scale and was expressed in mm seedling. Leaf area was measured using planimeter and expressed in mm seedling. Stem diameter was measured with slide caliper and expressed in mm seedling. The plants were oven¹dried at 60°C till constant with weight and shoot weight and R/S ratio

was calculated.

Nodulation, nodule biomass and nitrogen fixation :

After each harvest, the number of nodules formed per plant was recorded. After thorough washing, the clean root system of the seedlings was excised, transferred to 10 ml capacity serum bottle and capped with suba seal and sealed with a layer or two of parafilm to avoid loss of gases from tube. Thereafter 1 ml of air was withdrawn and 1 ml of C_2H_2 was injected into and let at room temperature for 1 h. The details of the estimation of nitrogenase activity are described in Chapter 1. However, the activity was expressed as n mole of C_2H_4 h⁻¹ seedlings⁻¹. Thereafter the nodules were oven dried at 60°C till constant weight ~~was achieved~~ and dry weight was recorded with the help of electronic balance.

Assessment of rhizospheric *Frankia* population :

Frankia population of rhizospheric^e soils was estimated following sucrose gradient density technique of Baker and O'Keefe (1984) with some modification as described in Chapter I.

Determination of VAM infection, arbuscules and vesicles :

On each sampling, the root system of seedlings was washed thoroughly under tap water and cut into 1 cm segments. Hundred randomly sampled root segments were cleared, ~~and~~ stained following the method of Koske and Gemma (1988) and VAM infection was determined following the root slide technique of Read *et al.* (1976) as described in Chapter I. The population of fungal arbuscules and vesicles was determined using above stained root segments.

Estimation of endogonaceous spore population :

Wet sieving and decanting technique of Gerdemann and Nicolson (1963) was used to isolate and enumerate endogonaceous spores from rhizosphere soils as described in Chapter I and was expressed in g dry soil.

Determination of phosphatase activity :

The rhizosphere activity of soil phosphatase was determined following the method of Tabatabai and Bremner (1969). To 1 g sieved soil, 4 ml of modified universal buffer (pH 6.5), 0.25 ml of toluene and 1 ml of 0.115 mM p-nitrophenyl phosphate solution were added. The conical flasks were

swirled, plugged and incubated at 37°C for 1 h. The details of the technique followed are described earlier in Chapter I.

Analysis of nutrients in seedlings :

Oven-dried root and shoot plant materials were separately ground and sieved through 0.2mm sieve and finer material (< 0.2mm) was employed for the analysis of N, P, K and Mn.

Plant N was determined following semi-microkjeldahl method (Allen, 1974). Ground plant material^{0.2g} was digested in microkjeldahl flasks along with 2 g of K₂SO₄-HgO mixture and 3 ml of conc. H₂SO₄. After digestion, the volume of aliquot was made up to 20 ml with distilled water. The technique used is described earlier in Chapter I.

For the estimation of P, molybdenum blue method (Allen, 1974) was adopted. Two hundred mg ground material was digested in triacid mixture (Conc. HNO₃, H₂SO₄ and 60% HClO₄ in a ratio of 10:1:4). The digested material was made up to 50 ml with distilled water and filtered through Whatman filter paper (no. 1). Two ml of filtrate was taken in a test tube to which 5 ml distilled water and 1 ml of ammonium molybdate were added and shaken vigorously. Thereafter,

1 ml of stannous chloride was added and stirred thoroughly. After making up the volume to 50 ml, the absorbance was read after 30 min at 660 nm and converted into known units through standard curve. The following formula was used to calculate P in plant tissues.

$$P (\%) = \frac{P(\text{mg}) \times \text{extraction solution used (ml)}}{10 \times \text{aliquot (ml)} \times \text{weight of sample (g)}}$$

C = Concentration of P obtained from standard curve.

Plant K and Mn were determined by atomic spectrophotometric method (Allen, 1974) ^{the} by using same aliquot employed for estimating P. However, the absorbance was recorded at 766.5 and 279.5 nm for K and Mn respectively and their contents were determined using formula as stated for K (Chapter I).

Statistical analysis :

The effect of growth regimes, treatments (symbionts) and harvests on various characteristics of microendophytes and plant growth performance was evaluated using 3 way analysis of variance (ANOVA). The coefficient of correlation was calculated using Karl Pearson's correlation coefficient (Zar, 1974).

RESULTS

Plant growth :

The growth response of *A. nepalensis* seedlings inoculated singly or dually with *Frankia* or / and VAM fungi and noninoculated ones was markedly influenced by soil pH. Better seedling growth was achieved by seedlings of all treatments at pH 6.0 below which growth was *reduced*.

Shoot height, root length, plant height and stem collar diameter increased as the soil pH increased and highest response was recorded at soil pH 6.0. The most effective increase was achieved by dually inoculated plants. A variation in above plant growth characteristics in most pH levels and harvests amongst singly, dually or noninoculated plants was evident (Fig. 30, 31, 32 & 33). However, mycorrhizal and dually inoculated plants showed better growth than *Frankia* inoculated and uninoculated ones at low pH soils.

Biomass of shoot, root and whole plant increased in time and with increase in soil pH. Mycorrhizal plants performed better than *Frankia* nodulated and noninoculated plants especially at low pH but was less efficient in terms of biomass accumulation than dually inoculated ones at corresponding pH (Tables 13, 14 and 15a). Maximum biomass

production by seedlings in all the treatments was, however, achieved at pH 6.0. Besides, clearcut differences in above plant characteristics of singly, dually inoculated or noninoculated plants at any pH level was evident. The R/S ratio increased with the decrease in soil pH. However, dually inoculated plants had low ratio than other singly inoculated and noninoculated counterparts at all soil pH and harvests (Table 15b). Besides, mycorrhizal seedlings exhibited lower ratio as compared to *Frankia* inoculated or noninoculated seedlings.

Variations in leaf number and leaf area between singly, dually or noninoculated plants emerged after 30 days of growth. High values of leaf number and leaf area were obtained on pH 6.0 and declined with decrease in soil pH. However, both plant characteristics increased with increase in time and this increase varied with the pH of the soil and the type/(s) of endophytes involved. (Fig. 34 & 35).

3 way ANOVA showed that most of above plant growth characteristics were significantly (ANOVA $P < 0.01$) affected by soil pH, harvest and endophyte/(s) (Table 16).

Nodulation, nodule biomass and nitrogen fixation :

The formation of root nodules increased in time and with increase in soil pH and decreased with decrease in pH value above or below pH 6.0. Dually inoculated seedlings developed more nodules by these only with *Frankia* inoculated at most pH levels (Fig. 36).

The biomass of root nodule was markedly affected by soil pH and the endophyte/(s) (Fig. 37). Better growth of root nodules was noted at higher soil pH. At low pH level, the growth of root nodules in *Frankia* inoculated seedlings was poor as compared to that in dually inoculated plants.

The rate of nitrogenase activity of root nodule was most favoured at soil pH 6.0. However, considerable difference was recorded in nitrogenase activity between *Frankia* inoculated and *Frankia* + *G. mosseae* inoculated seedlings at most pH levels (Fig. 38).

Frankia population :

Frankia population of rhizosphere soil generally increased in rhizosphere of *Frankia* and *Frankia* + *G. mosseae* inoculated plants and was maximum pH 6 and decreased with increasing soil acidity. Besides soil *Frankia* abundance varied markedly in singly (*Frankia*) and dually (*Frankia* + *G. mosseae*)

inoculated plants at all soil pH levels except at pH 4.0 (Fig. 39).

Both nodule characteristics and *Frankia* population were significantly (ANOVA $P < 0.01$) affected by soil pH, harvest and symbionts (Table 17).

Mycorrhizal infection and dynamics of arbuscules and vesicles:

The intensity of VA mycorrhizal infection varied greatly between *Frankia* + *G. mosseae* and only *G. mosseae* inoculated plants at all pH levels. However, infection level of dually inoculated seedlings was significantly higher than those of mycorrhizal inoculated ones at pH range 5-6 (Fig. 40).

The arbuscules were more abundant than the vesicles at higher soil pH. However, with the increase in time their ratio declined. More arbuscules were produced at pH range from 5.0 - 6.0 than at any other pH level. Generally, dually inoculated plants produced more arbuscules than singly inoculated ones at more pH and harvests (Fig. 41).

In vivo production of vesicles by *G. mosseae* was highest at pH range of 5-6 for both singly and dually inoculated plants but latter maintained their lead throughout

the experimental period (Fig.42).

Endogonaceous spore population :

The abundance of endogonaceous spores increased with time and increase in soil pH. The highest number of endogone spores was encountered at soil pH 6.0 which decreased with increase or decrease in soil pH. A marked variation in the production of endogonaceous spores was evident between *G. mosseae* and *Frankia* + *G. mosseae* inoculated seedlings especially at higher pH levels (Fig. 43).

Soil phosphatase activity :

Total phosphatase activity in the rhizosphere soil decreased with the increase in soil pH. However, the activity was detected only from the rhizosphere soils of *Frankia* + *G. mosseae* and *G. mosseae* inoculated plants. Generally higher phosphatase activity was recorded from rhizosphere soil of dually inoculated plants as compared to *G. mosseae* inoculated ones at pH range 5-7 (Fig. 44).

Generally VA mycorrhizal infection, arbuscule and vesicle populations and spore counts were significantly (ANOVA

P < 0.01) controlled by soil pH levels, harvest periods and symbionts (Table 18). Similarly, rhizosphere phosphatase activity was affected by soil pH, harvests and symbionts (Table 18).

N, P, K and Mn contents of seedlings :

Generally, N, P, K and Mn content in the shoot were more in plants growing in slightly acidic soil pH than at low pH levels. However, differences in the nutrients content of singly, dually and noninoculated plants at most pH levels and harvests was recorded (Fig. 45 a, 46 a, 47 a & 48 a).

In root too, nutrient content varied among the plants of various groups. However, nodulated + mycorrhizal plants contained increasingly more element than singly or noninoculated counterparts (Fig. 45 b, 46 b, 47 b & 48 b).

The nutrient (N, P & K) contents of both shoot and root tissues were significantly (ANOVA P < 0.01) affected by soil pH, harvests and endophytes (Table 19). Mn was affected by above factors only in shoots (Table 19).

Significant positive correlation was established between mycorrhizal infection and nitrogenase activity, number of nodules, nodule dry weight and *Frankia* population at all soil pH levels except at pH 4.0 where insignificant relationships between mycorrhizal infection and nitrogenase activity was observed (Table 20). Similarly, correlation between mycorrhizal infection and *Frankia* population was negatively established at pH 4 (Table 20).

Correlation between nodule nitrogenase activity and mycorrhizal characteristics showed that former was positively but insignificantly related to mycorrhizal infection except at pH 5 whereas relationship between former and endogone spore number was positively established only at pH 5 and 6 (Table 21). Correlation between nitrogenase activity and arbuscule and vesicle populations was insignificant (Table 21).

Discussion :

The variation in the growth performance of *A. nepalensis* seedlings in various soil pH may be attributed mainly to the availability of nutrients for their growth and development.

VA mycorrhizal fungi exert profound influence on

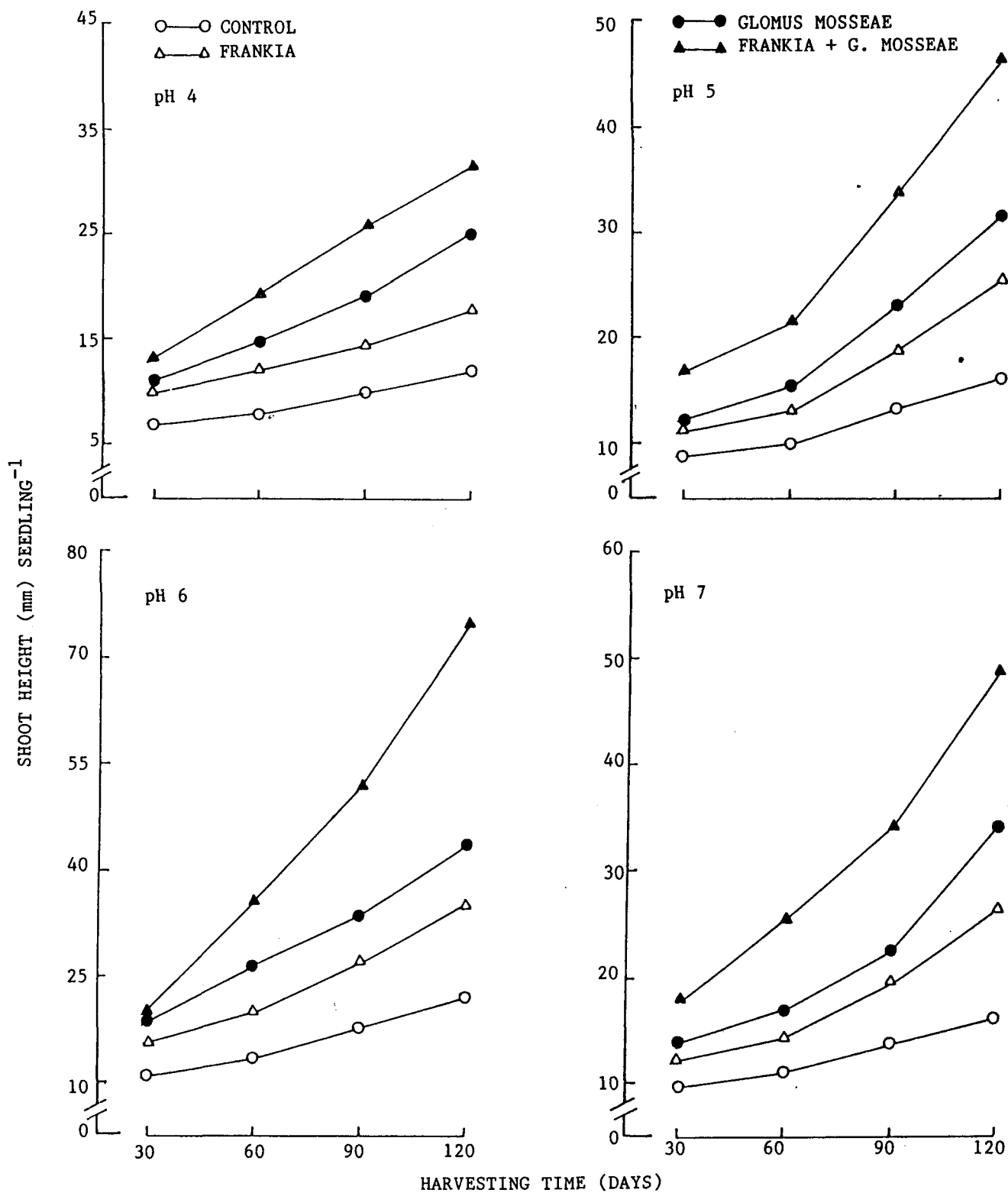


Fig.30. Effect of Frankia, G. mosseae and Frankia + G. mosseae on shoot height of A. nepalensis seedlings grown on soils with various pH.

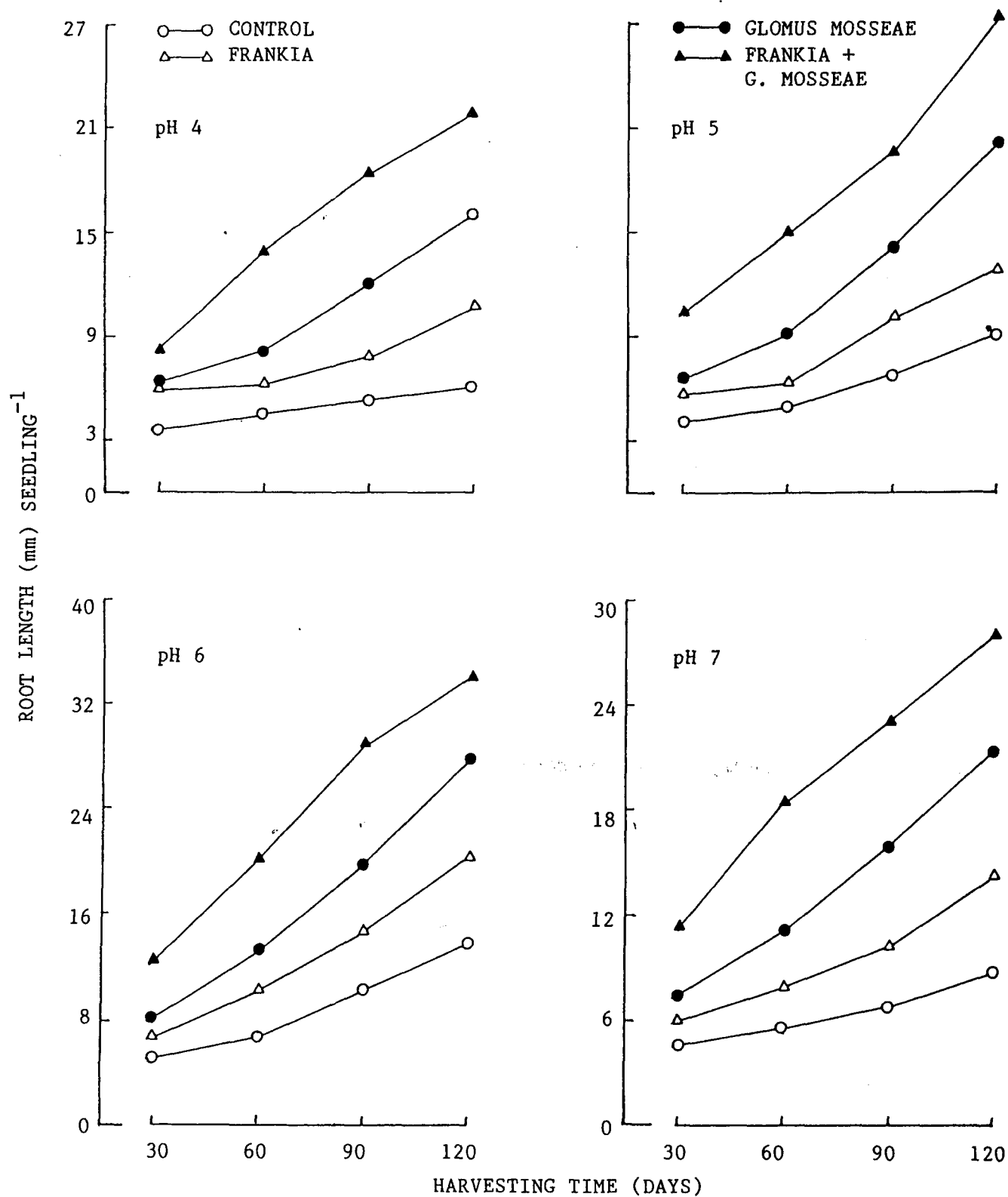


Fig.31. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root length of *A. nepalensis* seedlings grown at different soil pH.

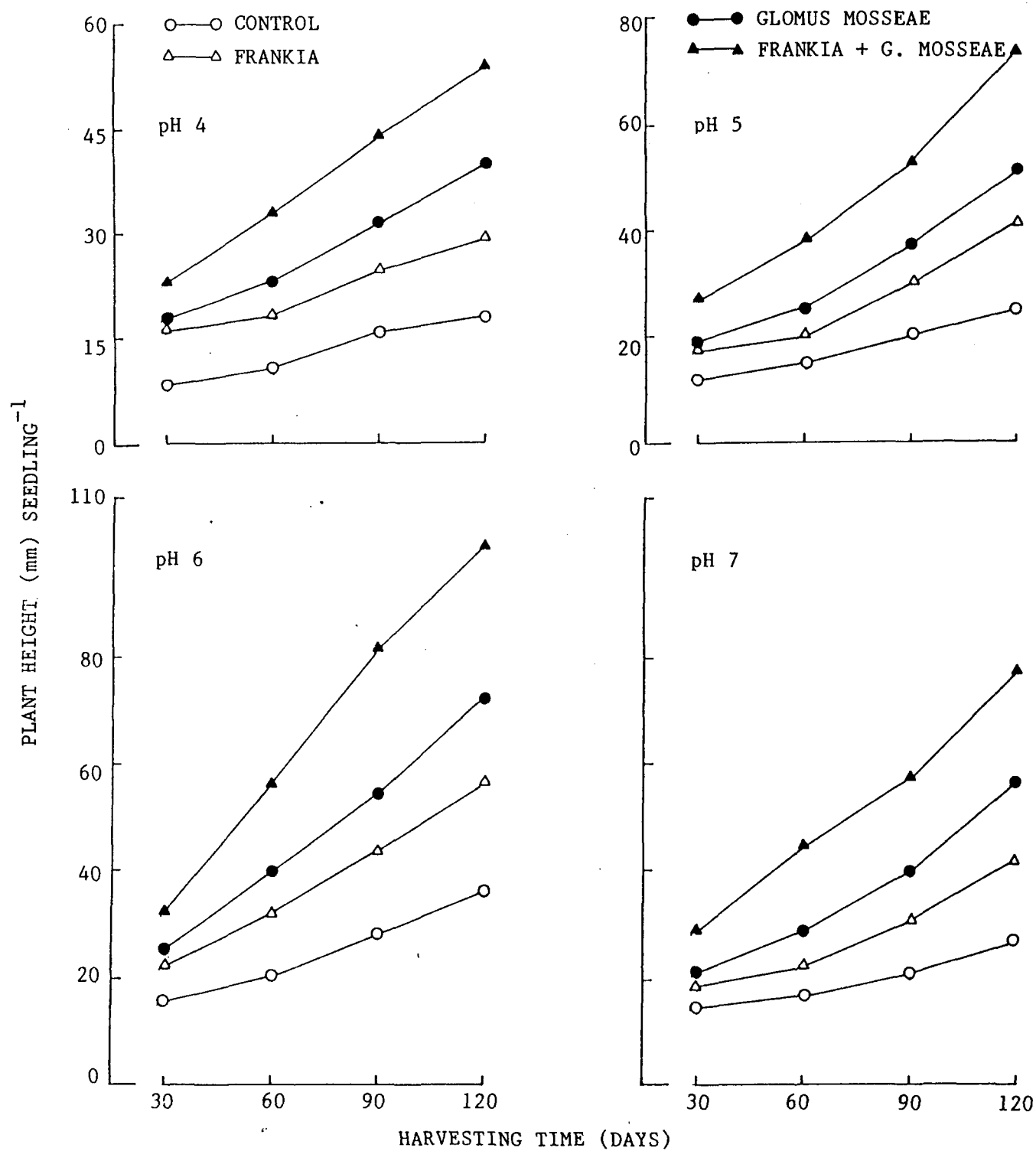


Fig. 32. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on the height of *A. nepalensis* seedlings grown at different soil pH.

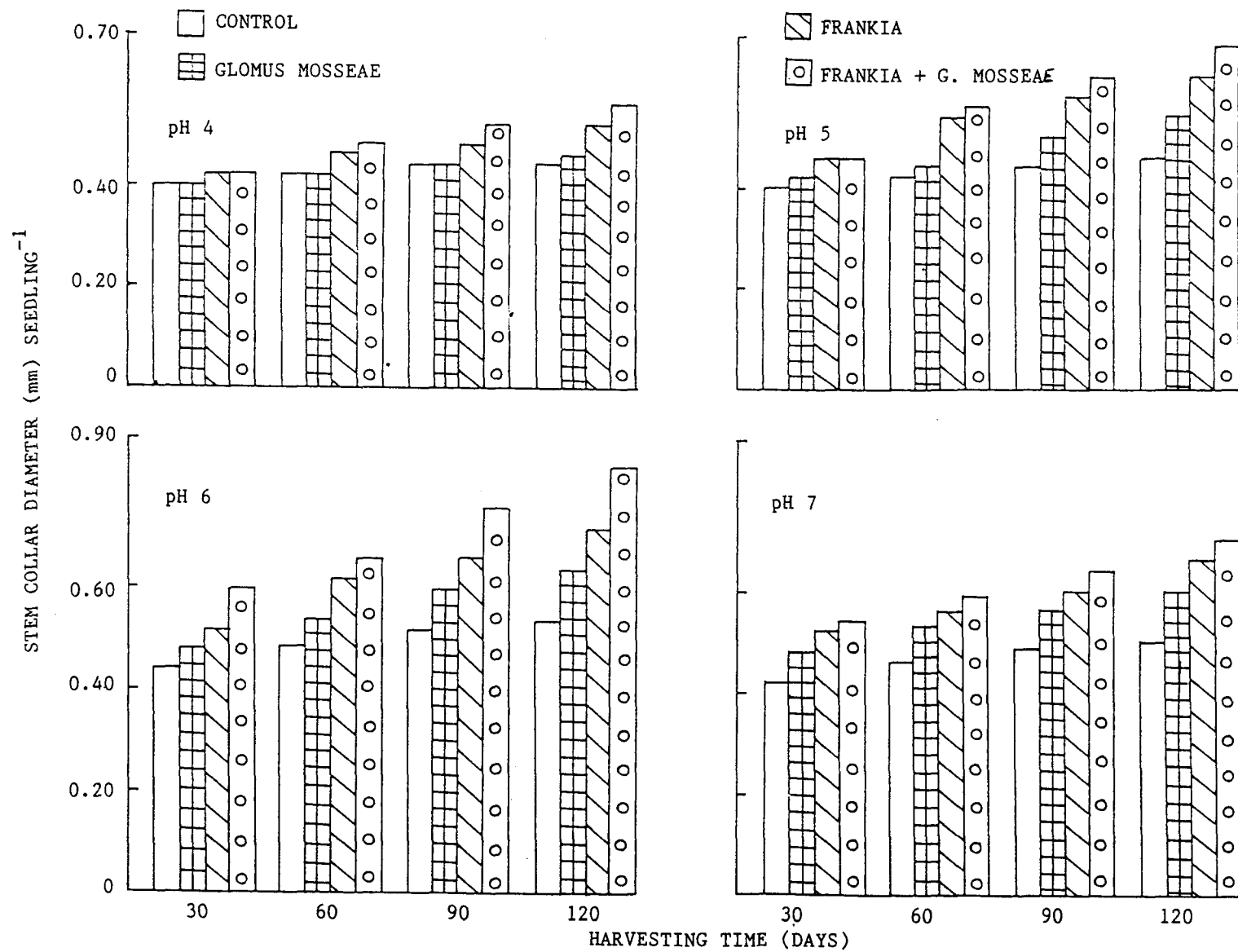


Fig.33. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on stem collar diameter of *A. nepalensis* seedlings grown on soils with different pH.

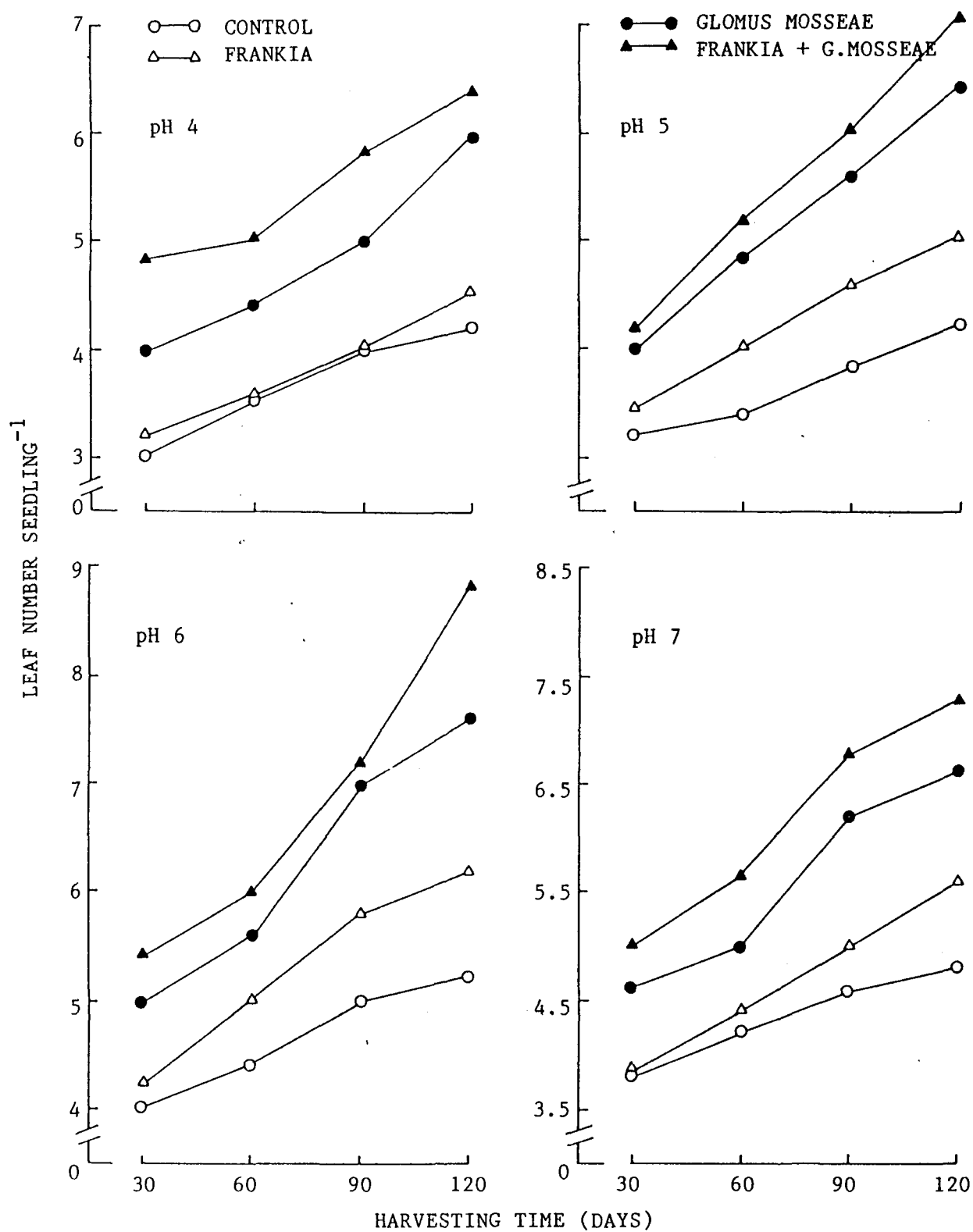


Fig.34. Effect of *Frankia*, *G. mosseae* and *Frankia + G. mosseae* on leaf number of *A. nepalensis* seedlings grown at different soil pH.

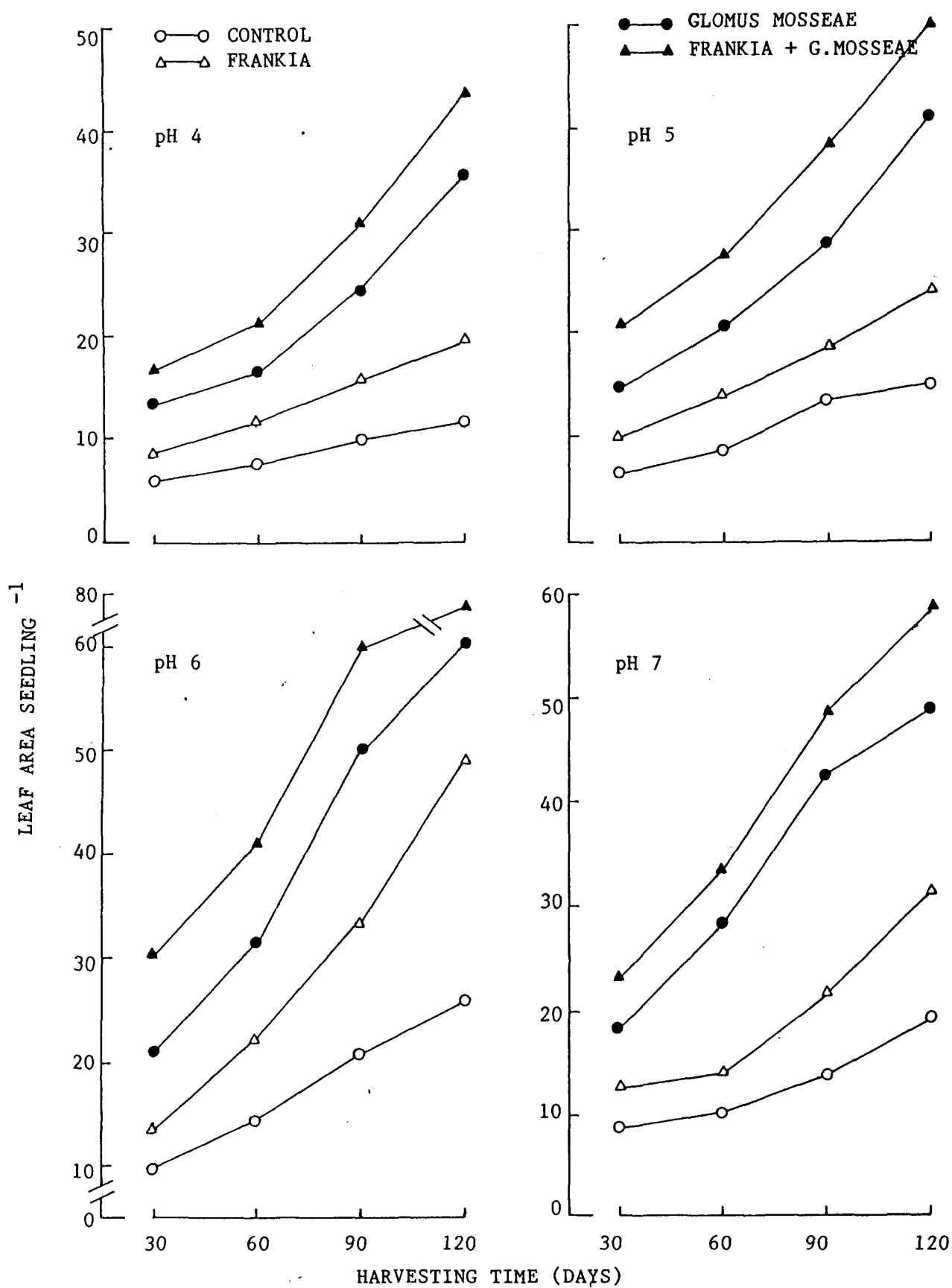


Fig.35. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on leaf area of *A. nepalensis* seedlings at different levels of soil pH.

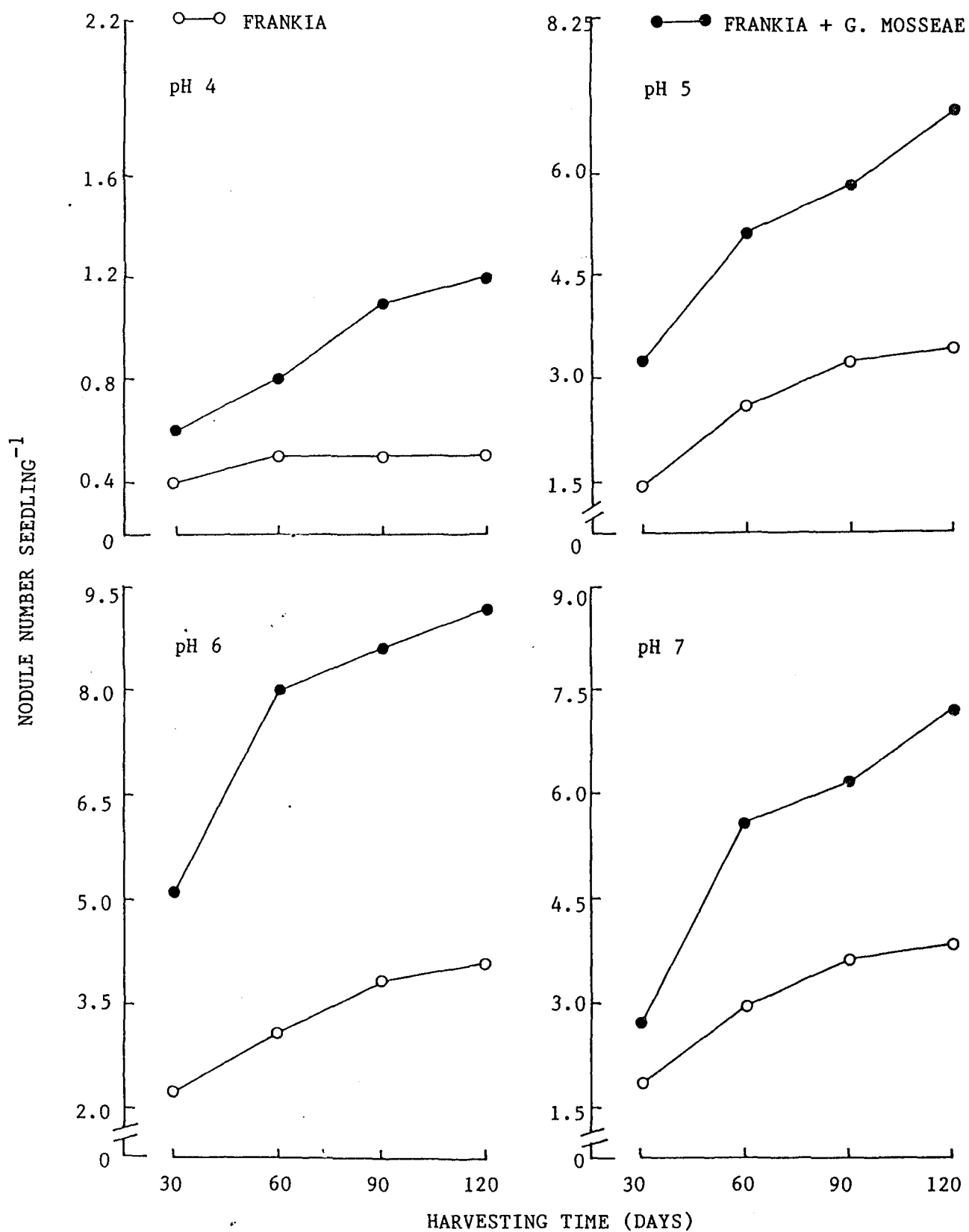


Fig.36. Effect of *Frankia* and *Frankia* + *G. mosseae* on nodule number of *A. nepalensis* grown at various levels of soil pH.

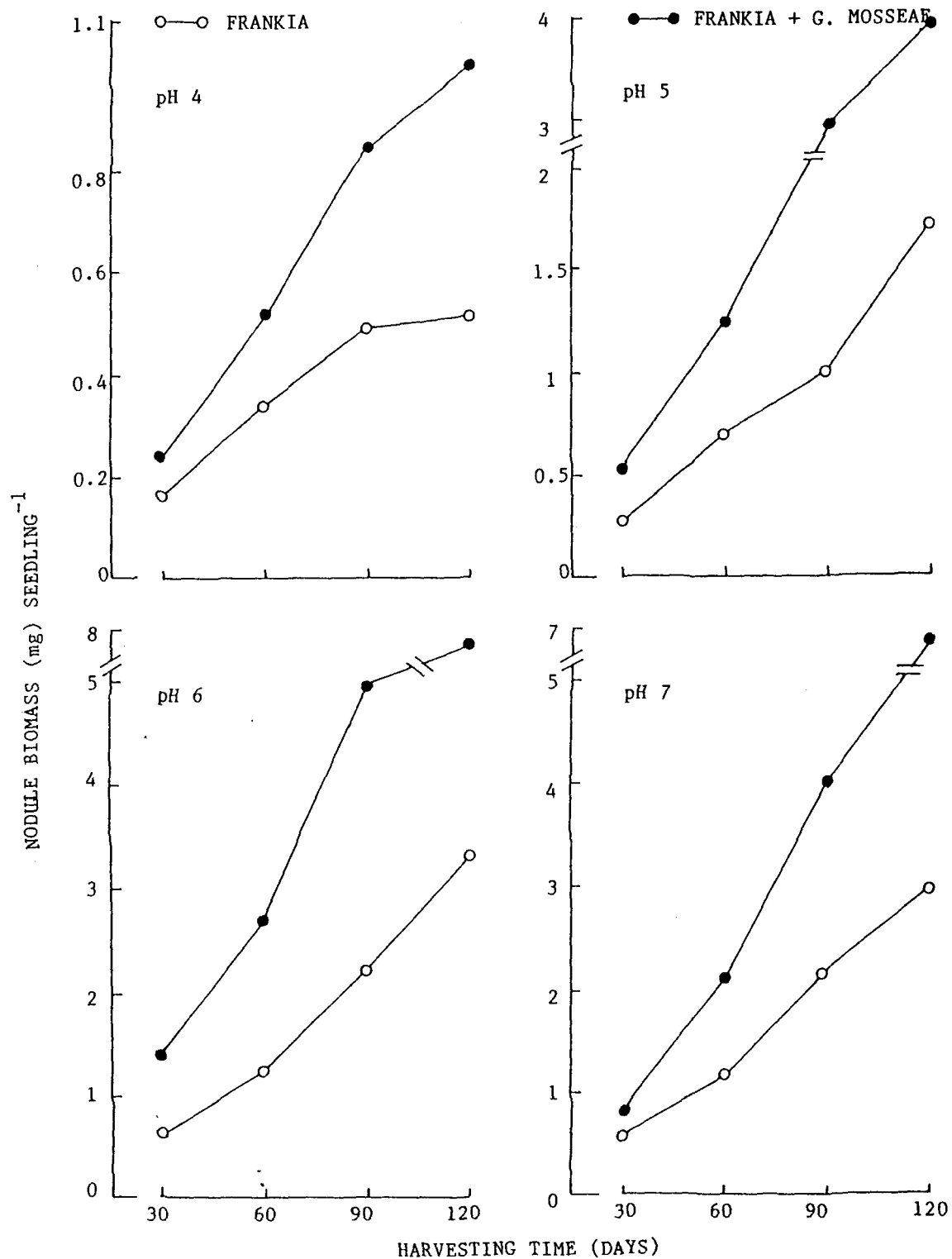


Fig. 37. Effect of Frankia and Frankia + G. mosseae on nodule biomass in A. nepalensis seedlings grown at different soil pH.

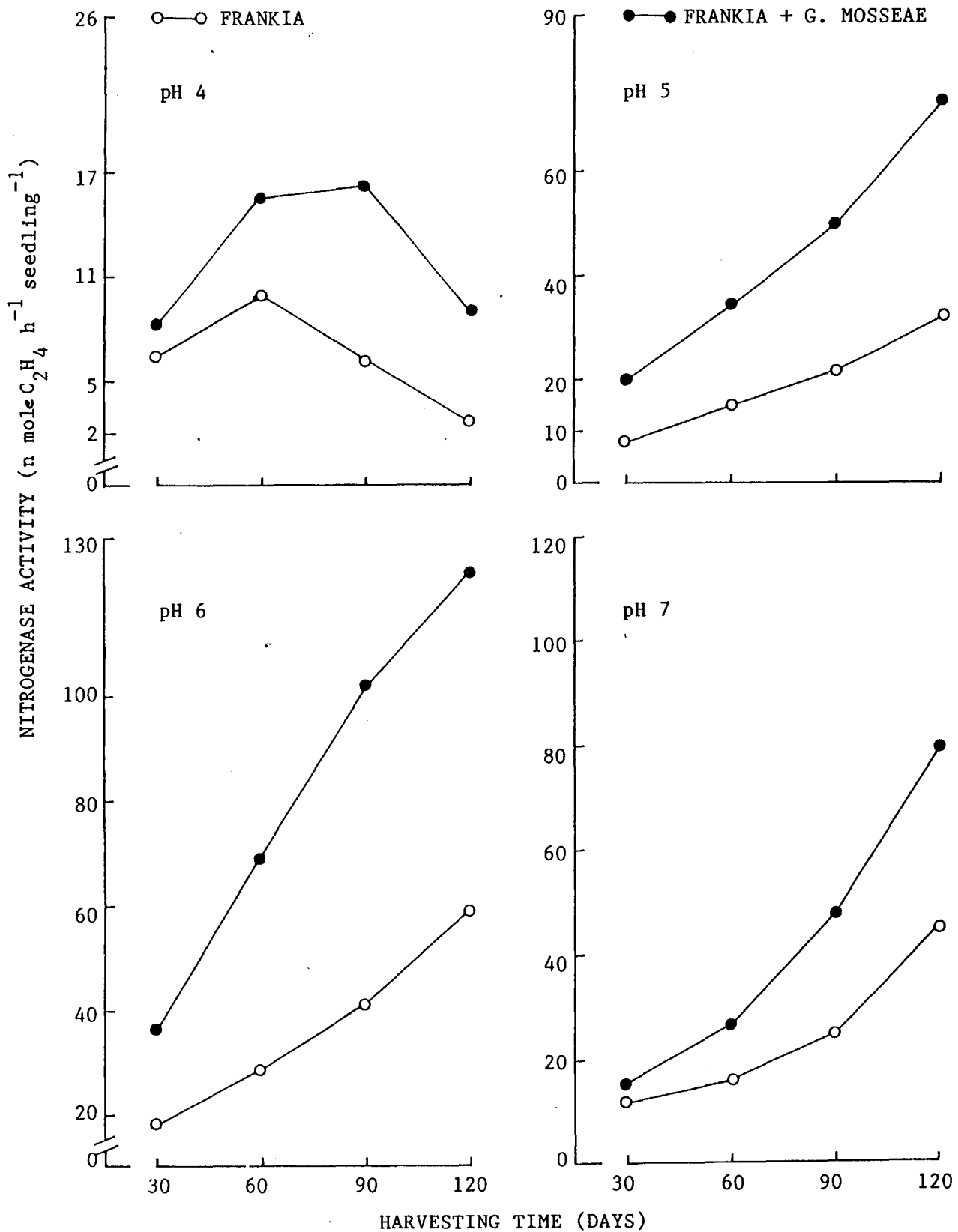


Fig. 38. Effect of *Frankia* and *Frankia* + *G. mosseae* on nodule nitrogenase activity of *A. nepalensis* seedlings grown at different soil pH.

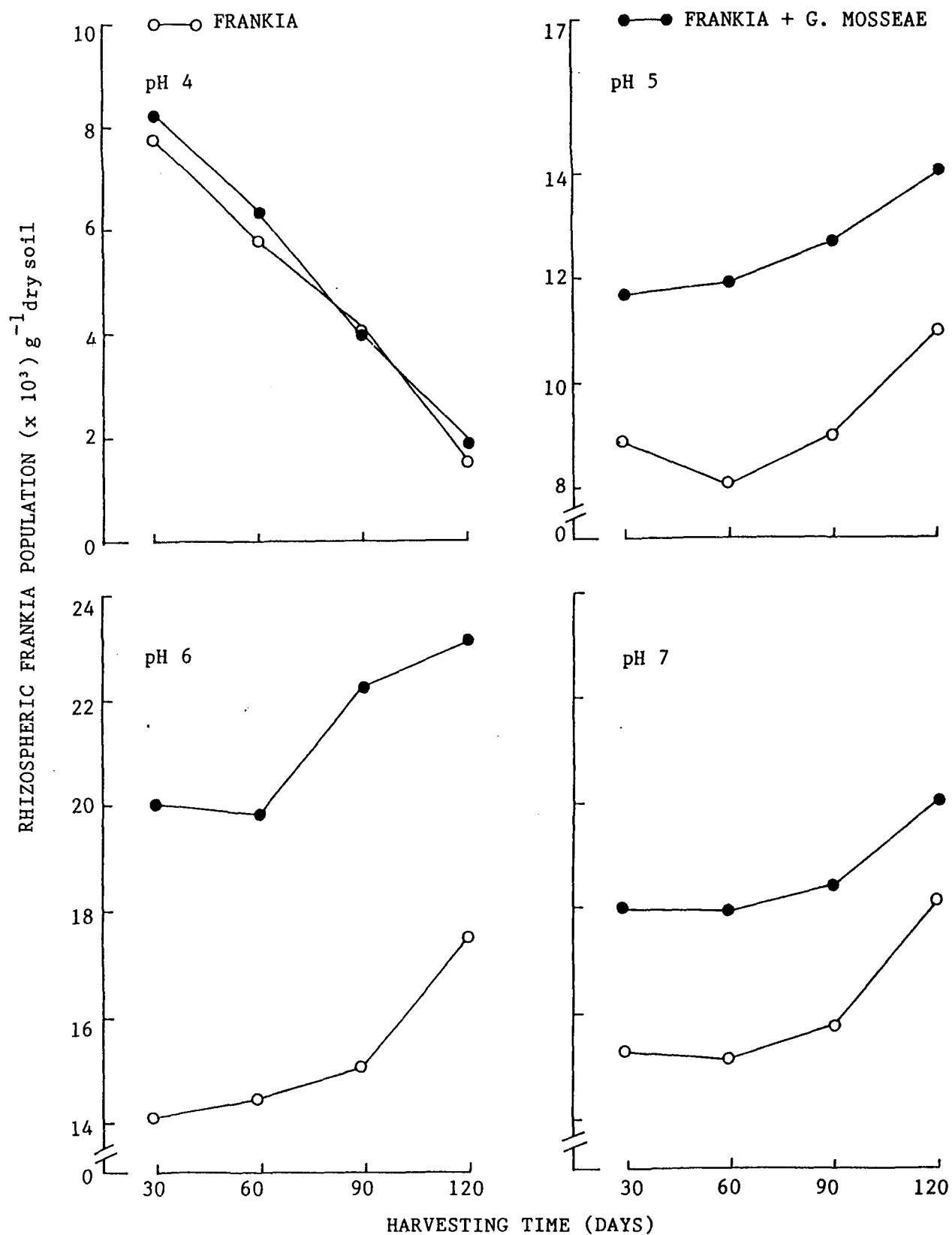


Fig. 39. Abundance of *Frankia* in the rhizosphere soil of *A. nepalensis* with *Frankia* and *Frankia* + *G. mosseae* at different levels of soil pH.

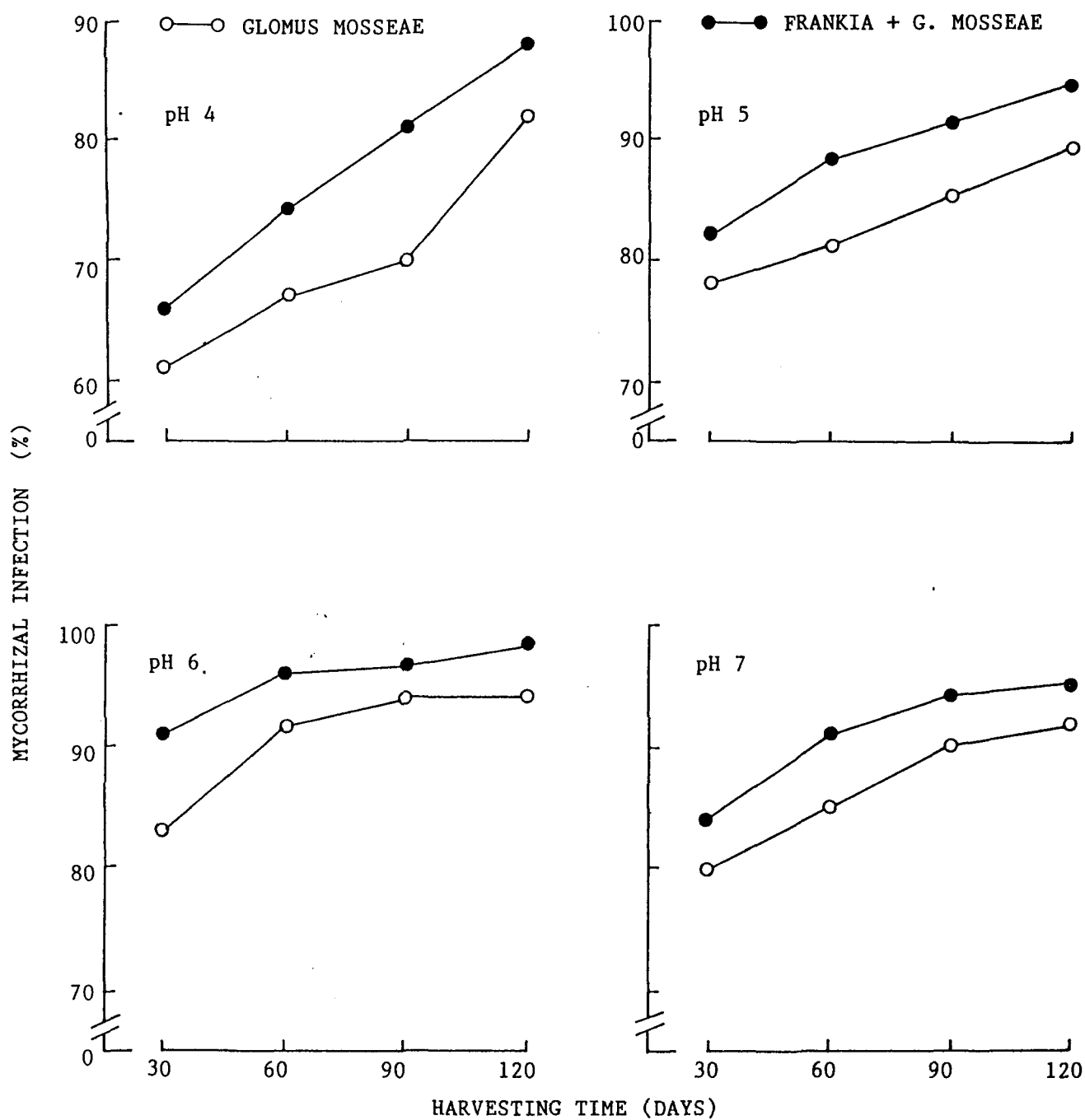


Fig.40. Effect of *G. mosseae* and *Frankia* + *G. mosseae* on mycorrhizal infection of *A. nepalensis* grown at different levels of soil pH.

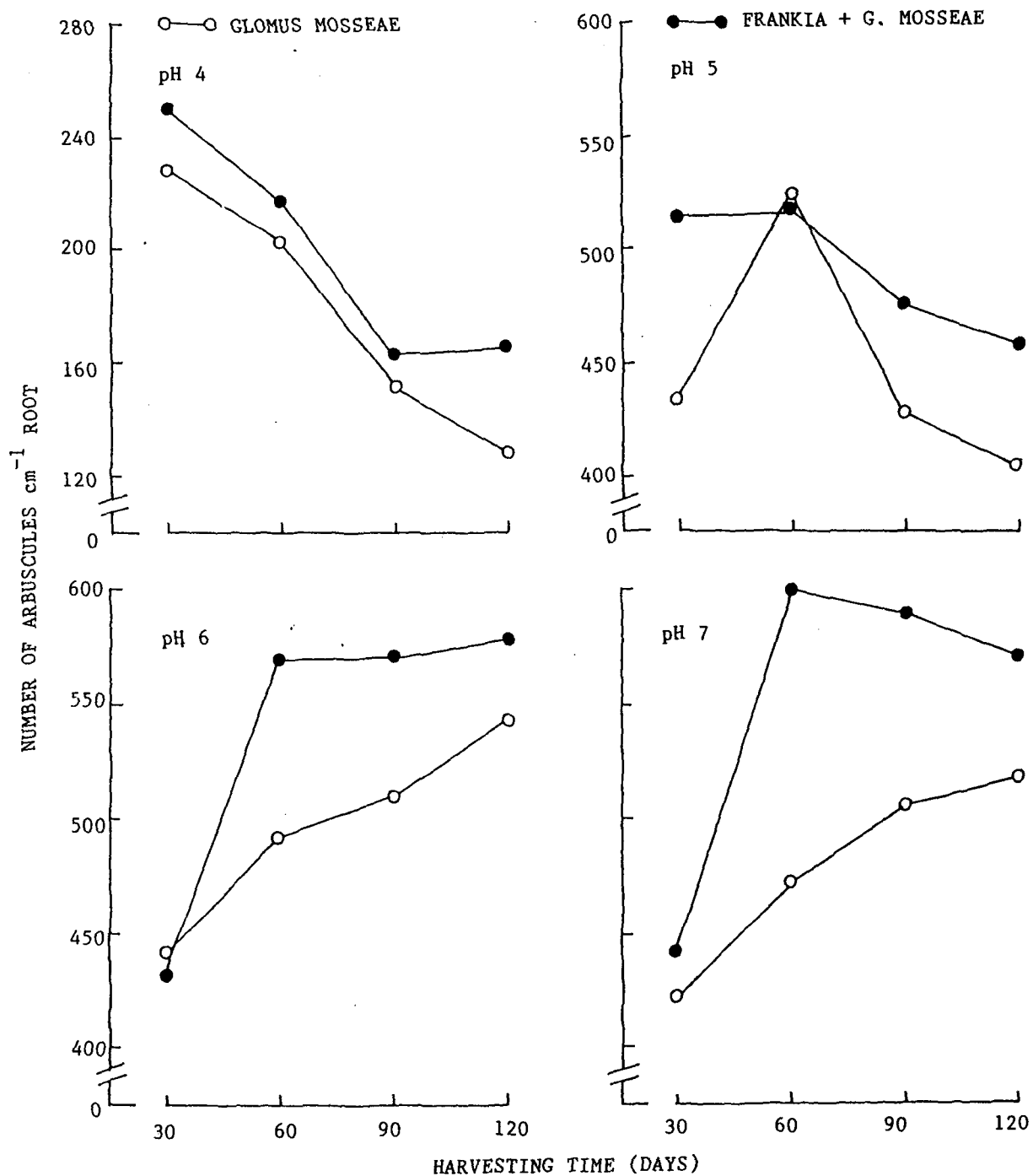


Fig. 41. Number of arbuscules in mycorrhizal roots of *A. nepalensis* inoculated with *G. mosseae* and *Frankia + G. mosseae* at different levels of soil pH.

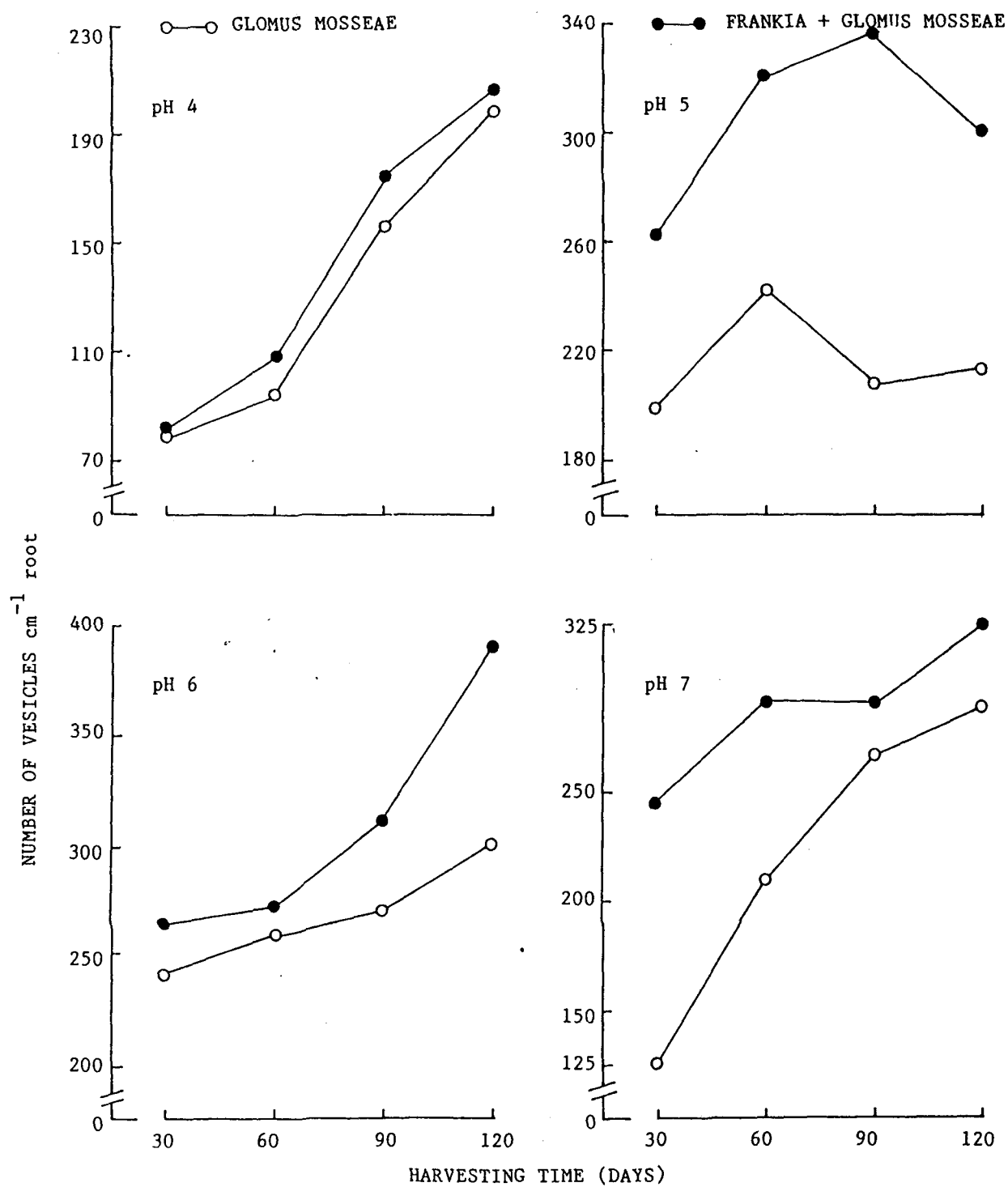


Fig.42. Number of vesicles in mycorrhizal roots of *A. nepalensis* inoculated with *G. mosseae* and *Frankia + G. mosseae* at various levels of soil pH.

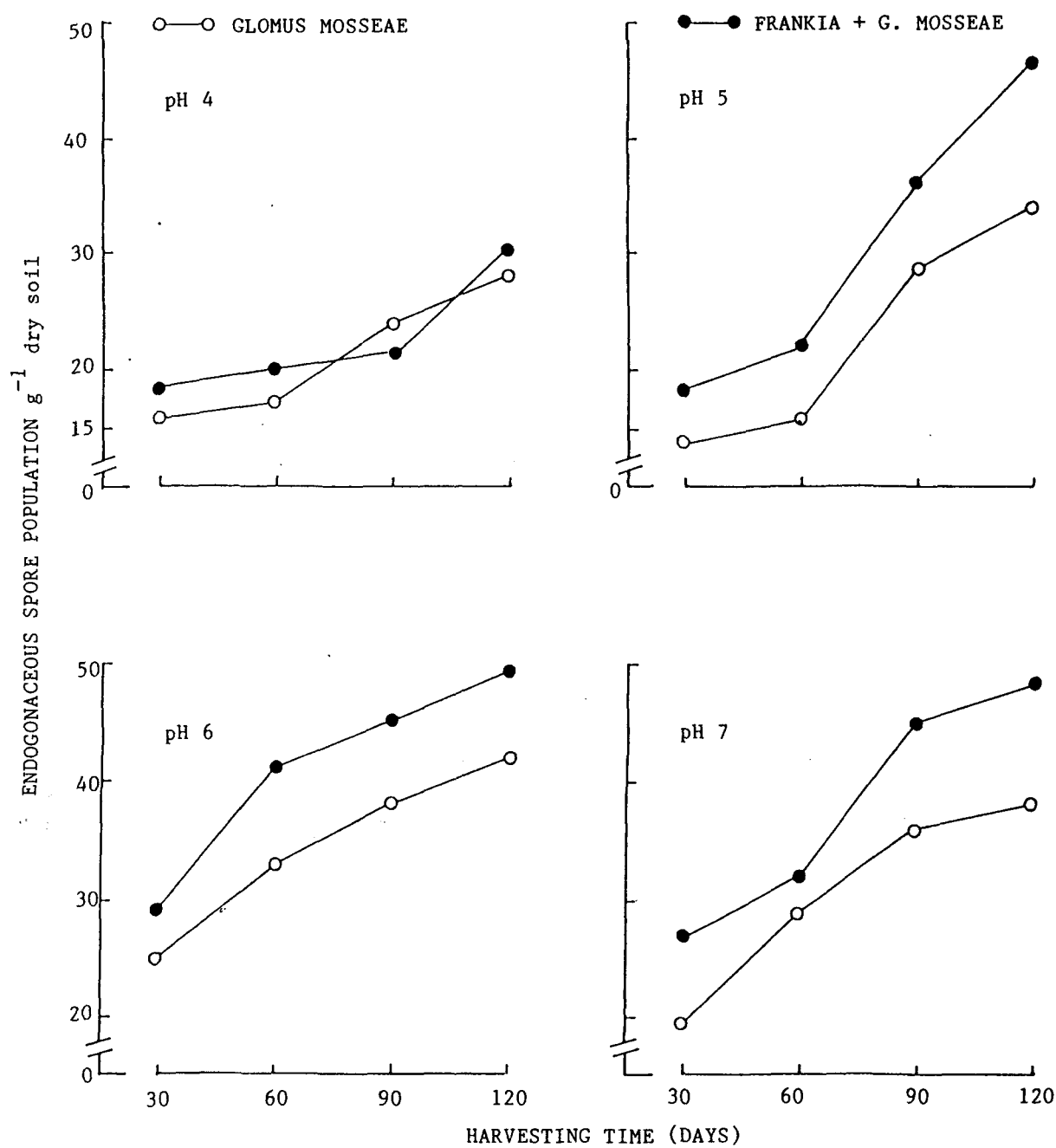


Fig.43. Endogonaceous spore population in the rhizosphere soil of *A. nepalensis* inoculated with *G. mosseae* and *Frankia* + *G. mosseae* at various levels of soil pH .

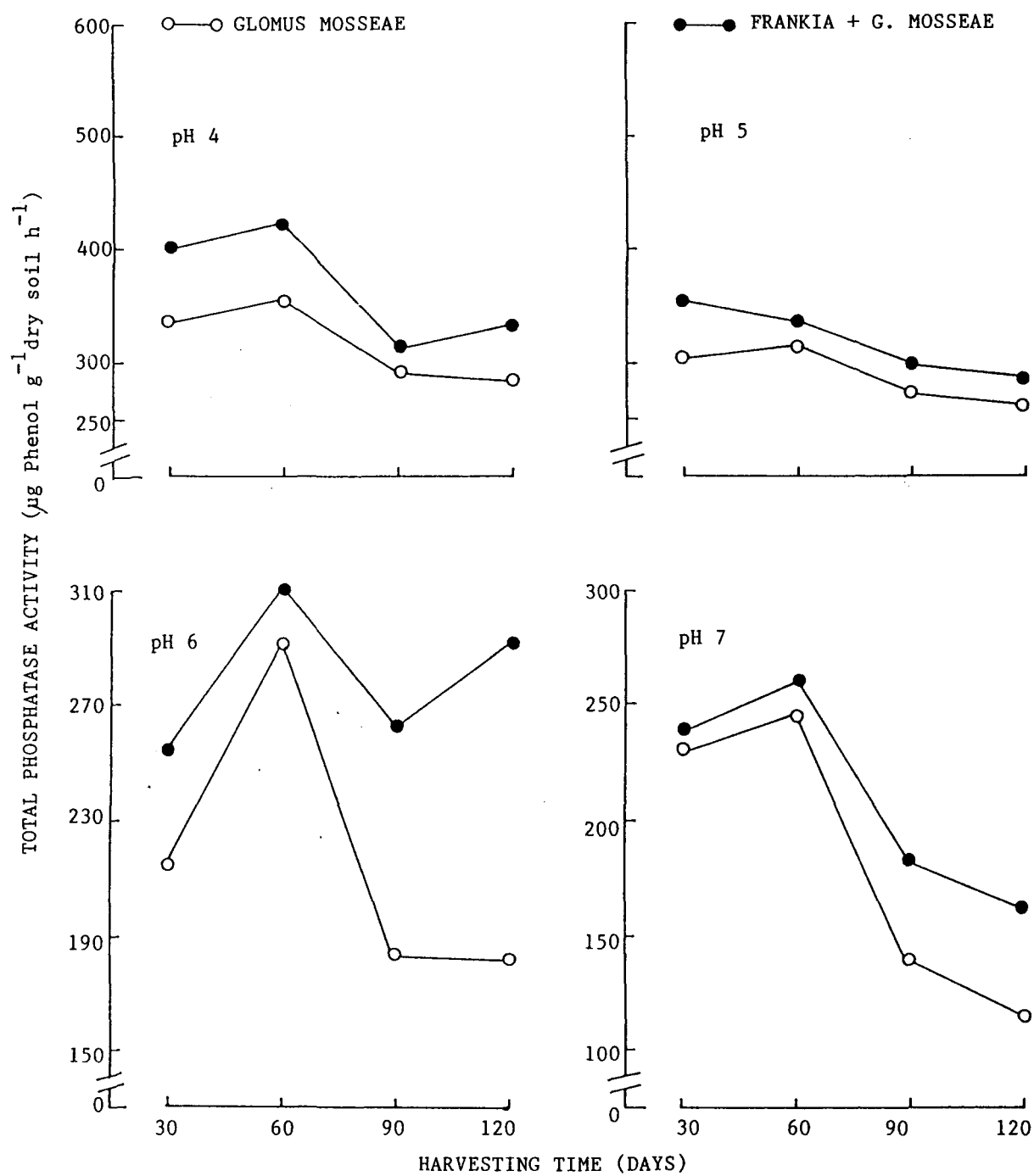


Fig.44. Total phosphatase activity of rhizosphere soil of *A. nepalensis* seedlings inoculated with *G. mosseae* and *Frankia + G. mosseae* at different levels of soil pH.

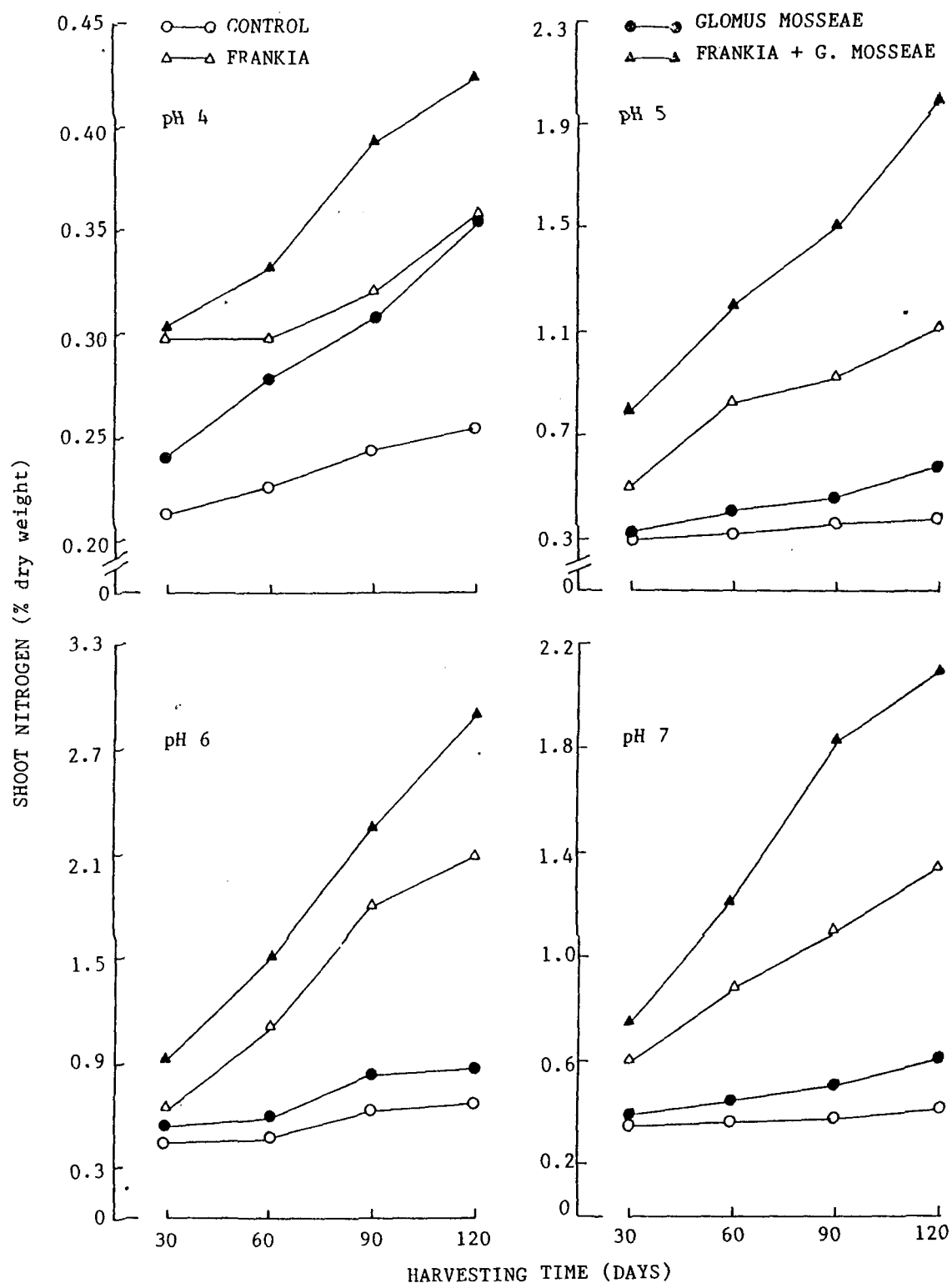


Fig.45a, Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on shoot nitrogen content of *A. nepalensis* at various levels of soil pH.

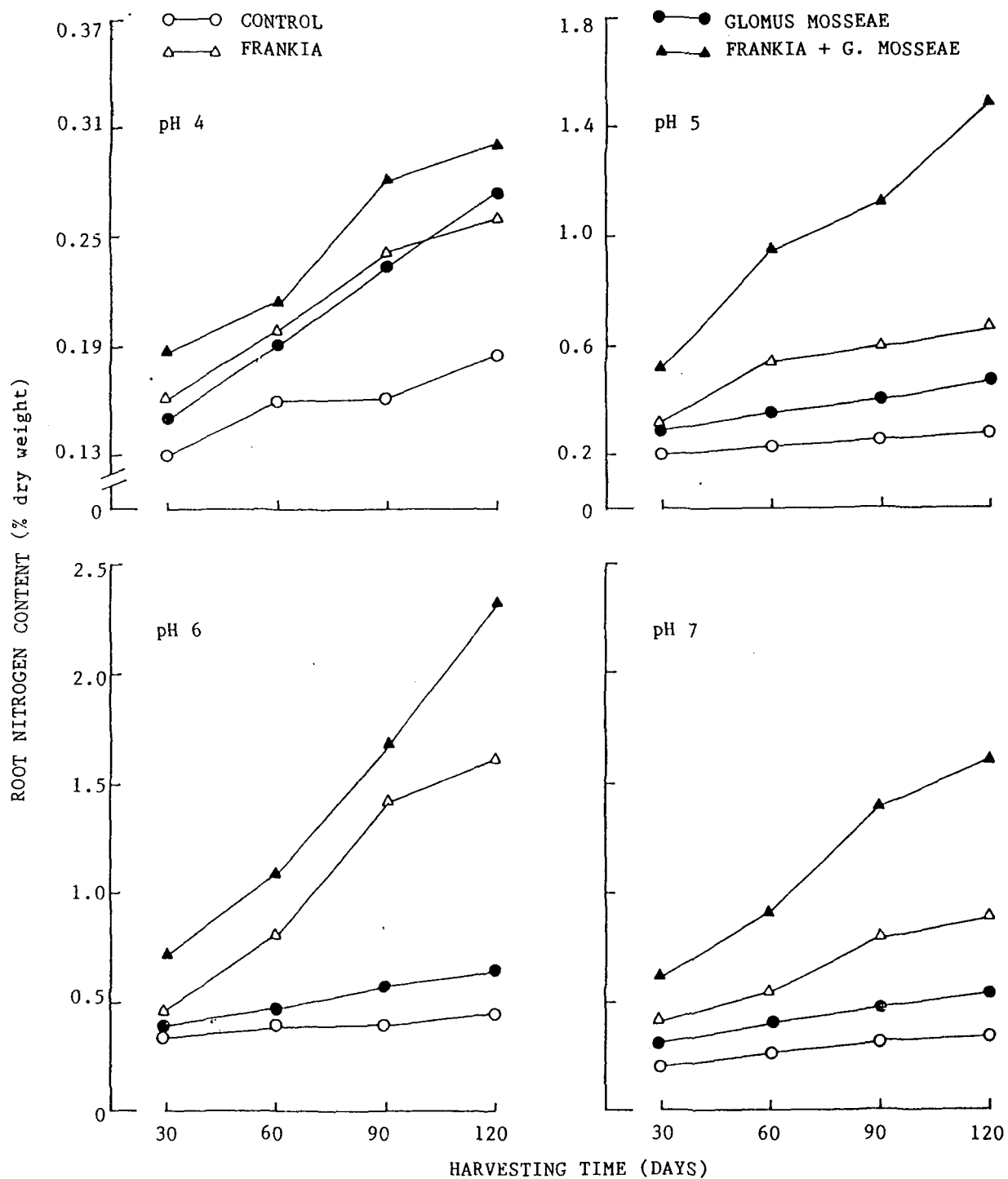


Fig. 45b. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root N content of *A. nepalensis* seedlings at various soil pH.

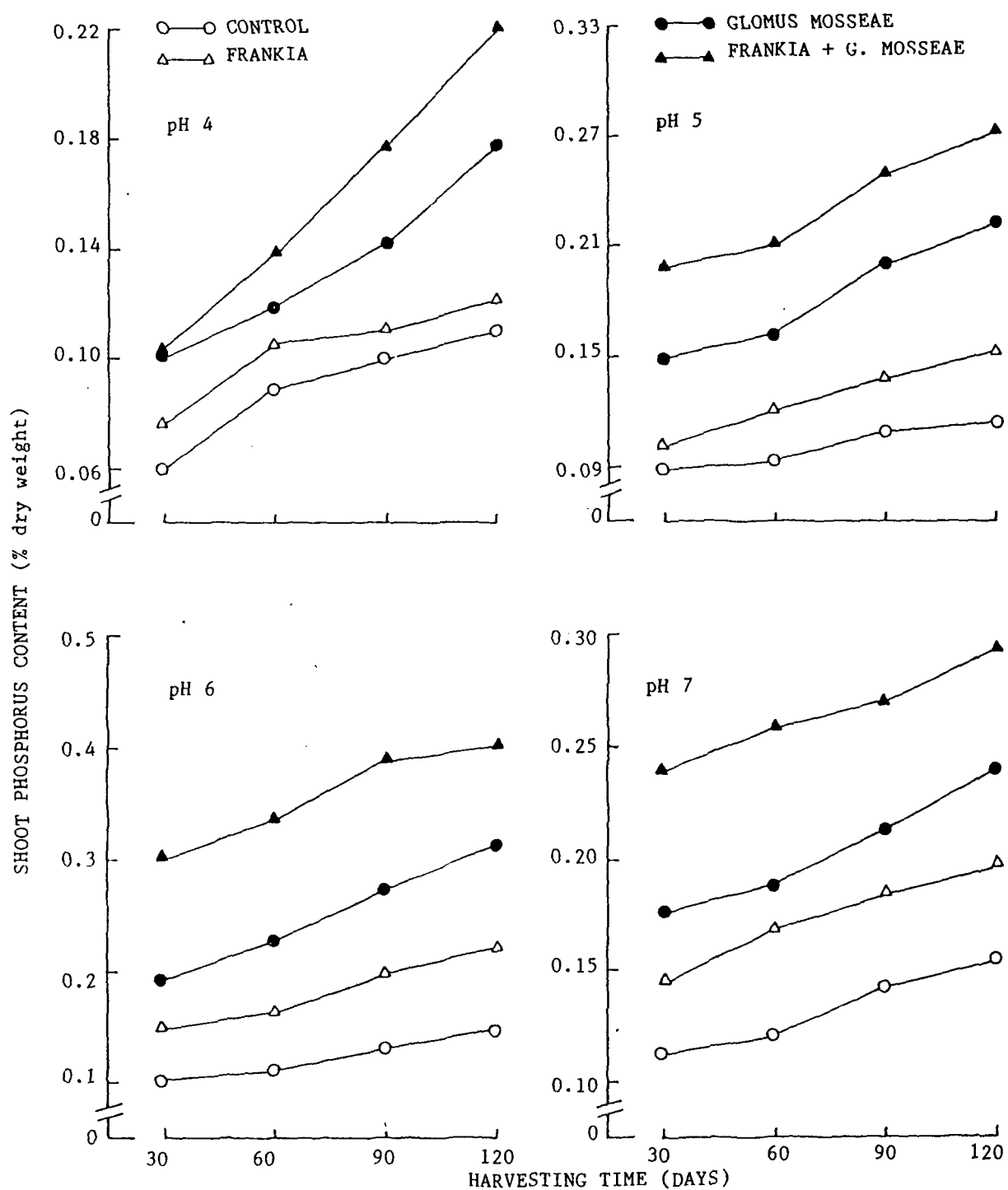


Fig. 46. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on shoot phosphorus content (%) of *A. nepalensis* seedlings grown at different soil pH.

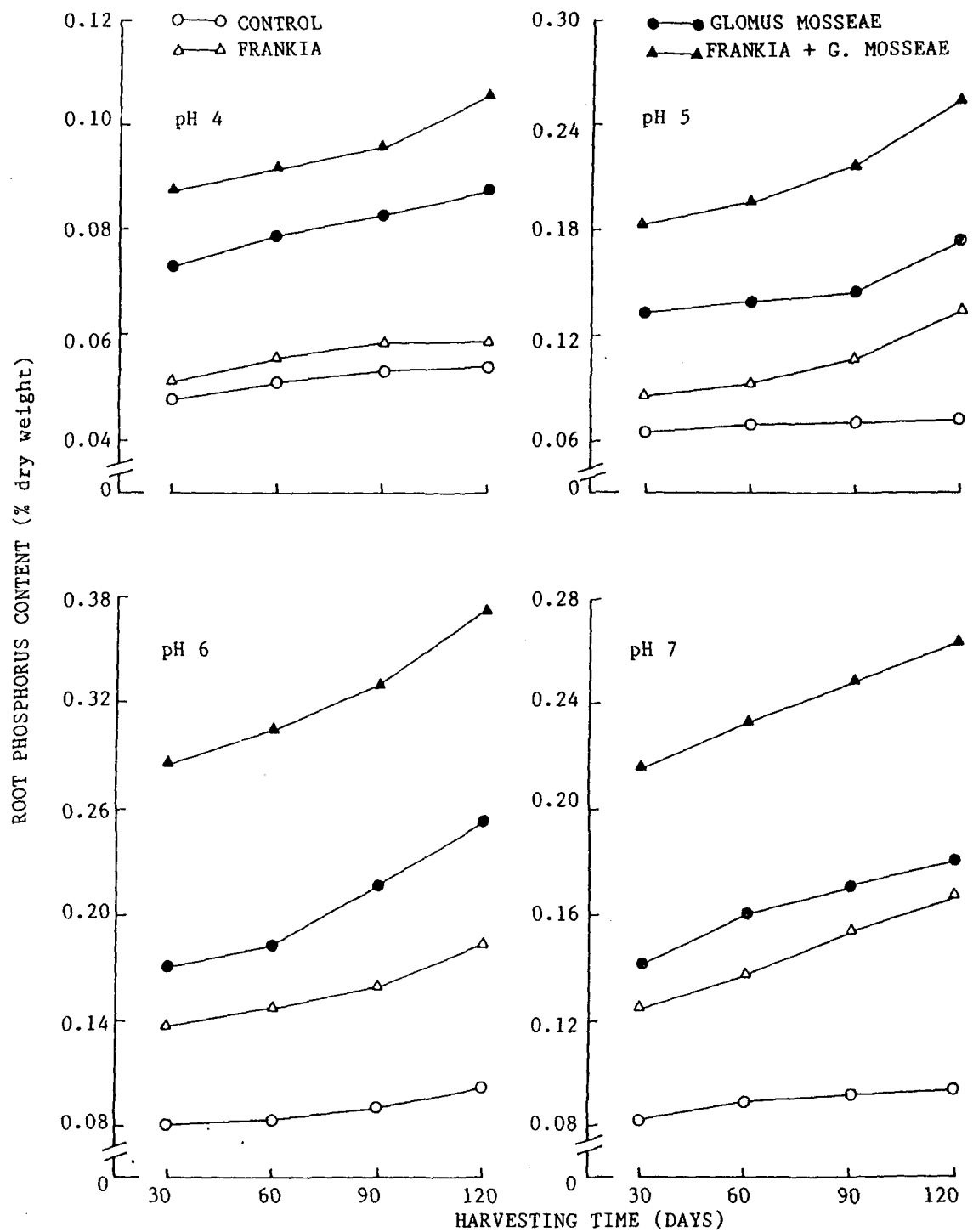


Fig.46b. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root phosphorus content (%) of *A. nepalensis* grown at different soil pH.

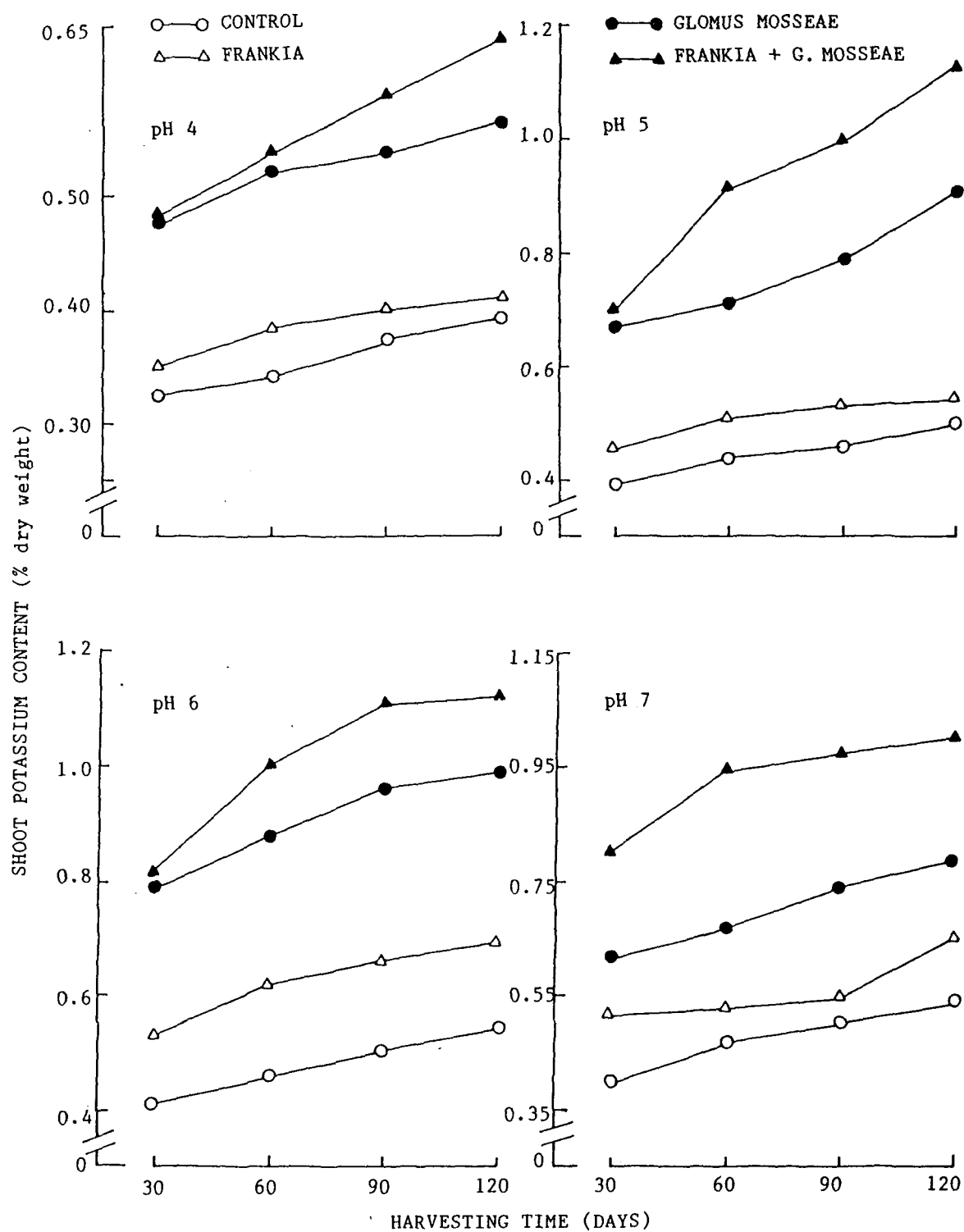


Fig.47a Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on shoot potassium content of *A. nepalensis* grown at various levels of soil pH.

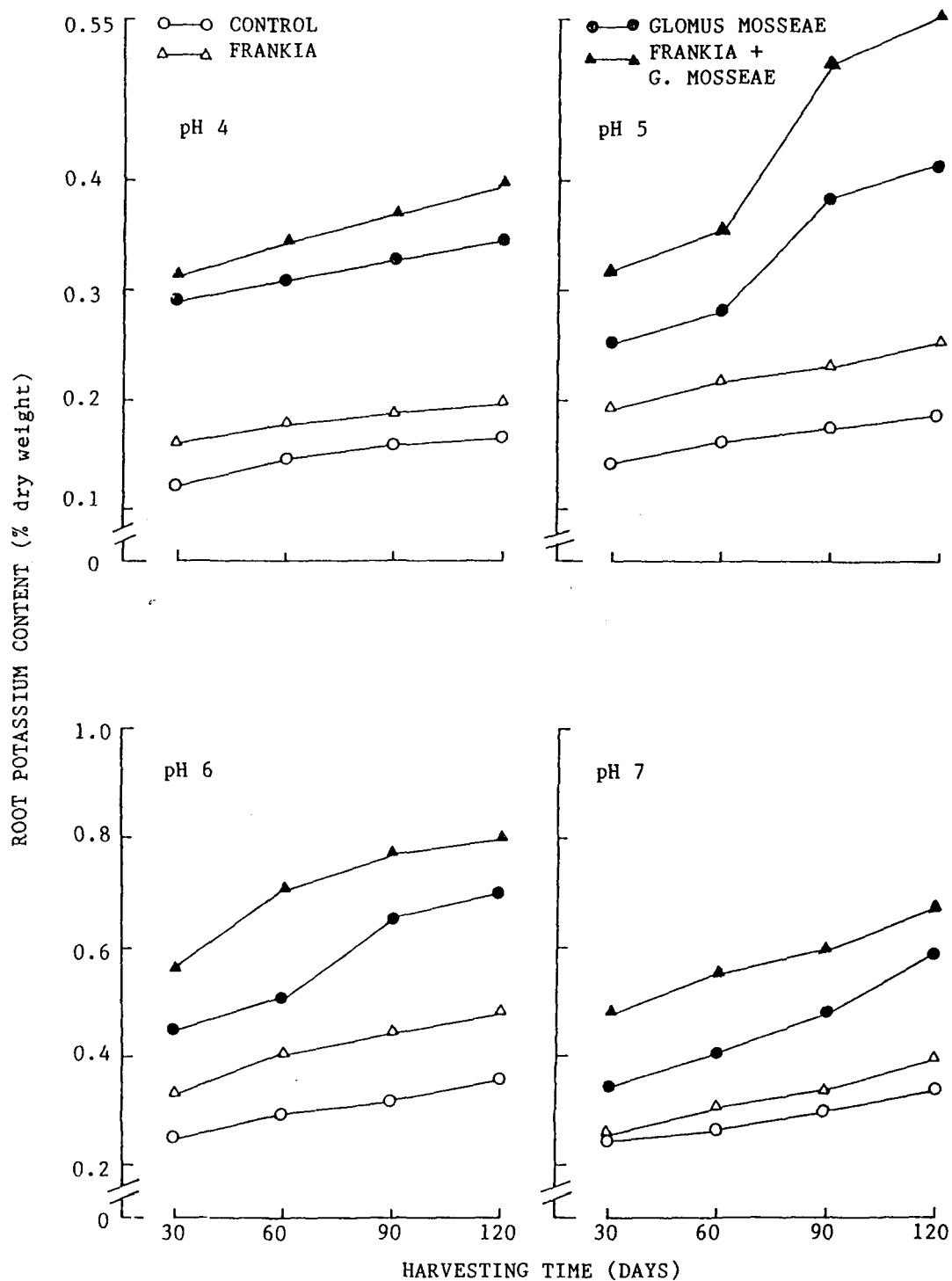


Fig.47b. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root potassium content (%) of *A. nepalensis* grown at different soil pH.

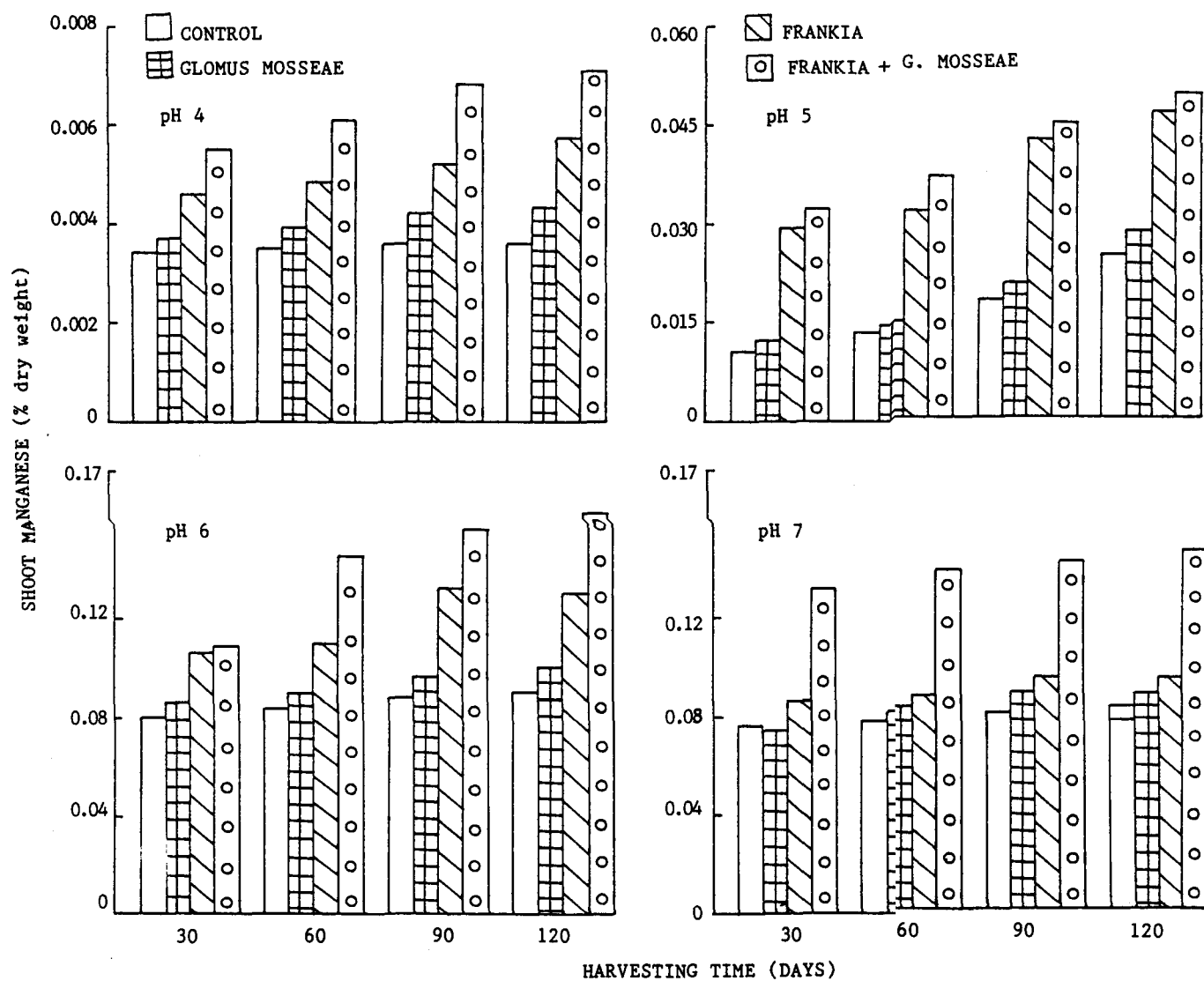


Fig.48a. Effect of *Frankia*, *G. mosseae* and *Frankia + G. mosseae* on shoot manganese content of *A. nepalensis* grown at different levels of soil pH.

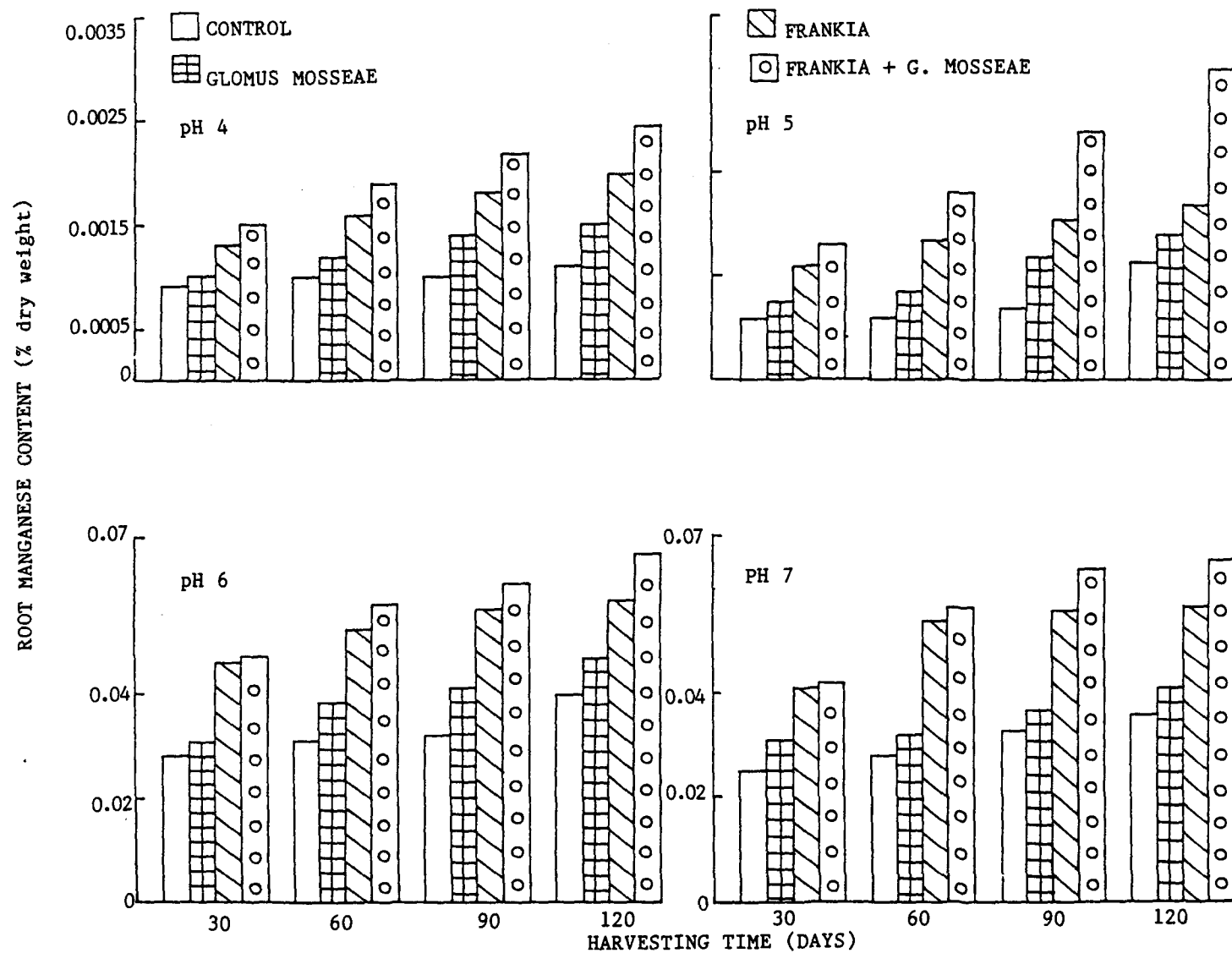


Fig.48b. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root manganese content of *A. nepalensis* seedlings grown at different levels of soil pH.

Table 13. Effect of *Frankia*, *Glomus mosseae* and *Frankia* + *G. mosseae* on shoot dry weight (mg seedling⁻¹) of *A. nepalensis* seedlings grown at various soil pH.

Soil pH	Harvesting period (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
4	2.4	2.8	3.7	3.9	2.7	3.2	4.2	5.6	2.9	3.7	5.1	8.5	3.0	4.0	6.3	10.0
5	3.1	4.2	5.0	7.1	5.4	7.2	8.3	13.6	6.9	11.1	13.4	21.3	8.8	16.2	18.1	30.1
6	3.6	5.6	6.3	9.2	6.1	9.2	11.0	17.3	8.8	15.9	18.1	28.5	11.0	27.9	29.1	45.9
7	3.4	5.2	5.7	8.1	6.1	8.4	10.3	15.6	7.9	15.4	16.9	27.8	9.2	25.1	26.4	41.4

1 = Control 2 = *Frankia* 3 = *G. mosseae* & 4 = *Frankia* + *G. mosseae*

Table 14. Effect of *Frankia*, *B. mosseae* and *Frankia* + *B. mosseae* on dry weight of root (mg seedling⁻¹) of *A. nepalensis* seedlings grown at various pH levels.

Soil pH	Harvesting period (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
4	0.8	0.9	1.1	1.2	0.9	1.1	1.3	1.7	1.1	1.4	1.7	2.6	1.1	1.6	2.0	3.1
5	0.9	1.1	1.2	1.5	1.6	2.2	2.2	3.1	2.1	3.4	3.3	4.5	3.1	5.1	4.4	6.2
6	1.0	1.4	1.5	2.1	1.8	2.5	2.4	3.8	2.5	4.5	3.9	6.1	3.1	7.5	6.2	9.8
7	1.0	1.5	1.4	2.0	1.9	2.2	2.3	3.5	2.4	4.1	3.7	6.0	2.8	7.3	6.0	9.5

1 = Control 2 = *Frankia* 3 = *B. mosseae* 4 = *Frankia* + *B. mosseae*

Table 15a. Effect of *Frankia*, *Glomus mosseae* and *Frankia* + *G. mosseae* on plant dry weight(mg/seedling-1) of *A. nepalensis* grown at various soil pH levels.

Soil pH	Harvesting period (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
4	3.2	3.7	4.9	5.2	3.7	4.3	5.6	7.3	4.0	5.2	6.8	11.1	4.2	5.6	8.3	18.5
5	4.1	5.4	6.3	8.6	7.0	9.5	10.6	16.7	9.0	14.6	16.7	25.8	12.0	21.3	22.5	36.3
6	4.7	7.1	7.8	11.4	7.9	10.7	13.4	21.1	11.3	20.5	22.1	34.6	14.2	35.4	35.3	55.7
7	4.4	6.8	7.1	10.1	8.0	10.6	12.7	19.1	10.4	19.5	20.7	33.8	12.0	32.5	32.4	50.9

1 = Control 2 = *Frankia* 3 = *G. mosseae* 4 = *Frankia* + *G. mosseae*

Table 15b. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on R/S ratio of *A. nepalensis* seedlings grown at various soil pH levels.

Soil pH	Harvesting period (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
4	0.33	0.32	0.31	0.32	0.35	0.34	0.32	0.31	0.37	0.37	0.33	0.31	0.38	0.39	0.31	0.29
5	0.31	0.27	0.25	0.22	0.30	0.31	0.25	0.22	0.30	0.30	0.25	0.21	0.35	0.31	0.24	0.20
6	0.297	0.258	0.244	0.23	0.29	0.27	0.22	0.22	0.28	0.28	0.21	0.21	0.28	0.26	0.21	0.21
7	0.308	0.283	0.245	0.24	0.31	0.27	0.22	0.22	0.30	0.29	0.22	0.21	0.30	0.29	0.23	0.22

1 = Control 2 = *Frankia* 3 = *G. mosseae* & 4 = *Frankia* + *G. mosseae*

Table 16. F values of three way ANOVA for shoot height, root length, plant height, shoot weight, root weight, plant dry weight, R/S ratio, stem collar diameter, leaf number and leaf area as affected by soil pH, symbionts and harvests.

Sources of variation	Shoot height	Root length	Plant height	Shoot weight	Root weight	Total plant wt.	R/S ratio	Stem collar diameter	Leaf number	Leaf area
pH	77.17 ^{††}	293.46 ^{††}	144.88 ^{††}	5.61 ^{††}	84.27 ^{††}	100.25 ^{††}	205.43 ^{††}	95.84 ^{††}	156.27 ^{††}	261.41 ^{††}
Harvests	158.70 ^{††}	1139.77 ^{††}	379.00 ^{††}	7.90 ^{††}	191.78 ^{††}	169.56 ^{††}	0.79	3.74 [†]	312.80 ^{††}	545.01 ^{††}
Symbionts	164.44 ^{††}	1380.16 ^{††}	409.73 ^{††}	9.08 ^{††}	75.54 ^{††}	104.33 ^{††}	174.33 ^{††}	53.92 ^{††}	357.50 ^{††}	675.25 ^{††}
pH x H	5.91 ^{††}	25.86 ^{††}	11.45 ^{††}	1.61	14.13 ^{††}	14.81 ^{††}	2.58 ^{††}	1.08	2.91 ^{††}	15.13 ^{††}
pH x S	5.24 ^{††}	8.52 ^{††}	7.63 ^{††}	1.23	4.00 ^{††}	5.79 ^{††}	4.12 ^{††}	7.16 ^{††}	1.64	10.11 ^{††}
H x S	13.76 ^{††}	62.70 ^{††}	26.21 ^{††}	1.83	9.79 ^{††}	12.42 ^{††}	4.53 ^{††}	0.92	9.22 ^{††}	28.15 ^{††}

†† Significant at P < 0.01 level.

Without asterisk statistically insignificant at above level.

Table 17. F values of three way ANOVA for nodule number, nodule weight, nitrogenase activity and rhizospheric *Frankia* abundance as affected by soil pH, symbionts and harvests.

Sources of variation	Nodule number	Nodule weight	Nitrogenase activity	<i>Frankia</i> population
pH	251.45* *	43.13* *	102.62* *	2023.19* *
Harvests	63.42* *	56.16* *	62.58* *	9.97* *
Symbionts	365.49* *	66.10* *	136.99* *	495.44* *
pH x H	5.36*	5.53*	9.83* *	49.54* *
pH x S	39.05* *	6.40*	17.28* *	76.49* *
H x S	8.59* *	9.39* *	9.13* *	0.81

*, * * Significant at $P < 0.05$ and 0.01 level respectively.

Without asterisk statistically insignificant at above levels.

Table 18. F values of three way ANOVA for VAM infection, arbuscule and vesicle populations *in vivo* and rhizospheric spore number and phosphatase activity as affected by soil pH, symbionts and harvests.

Sources of variation	VAM infection	Arbuscule number	Vesicle number	Endogonaceous spore population	Total phosphatase activity
pH	226.54* *	251.13* *	53.35* *	109.11* *	84.63* *
Harvests	104.58* *	4.99*	17.56* *	143.86* *	30.40* *
Symbionts	94.02* *	19.51* *	29.53* *	64.99* *	36.95* *
pH x H	5.54* *	6.51* *	2.01	5.54* *	2.34
pH x S	1.51	1.04	3.55	4.95*	1.31
H x S	0.36	0.34	0.02	1.15	0.50

*, * * Significant at $P < 0.05$ and 0.01 respectively.

Without asterisk statistically insignificant at above levels.

Table 19. F values of three way ANOVA for nutrient contents of *A. nepalensis* in shoot and root tissues as affected by soil pH, root symbionts and harvests.

Sources of variation	Shoot N	Root N	Shoot P	Root P	Shoot K	Root K	Shoot Mn	Root Mn
pH	10.17 ^{††}	57.81 ^{††}	106.73 ^{††}	914.70 ^{††}	254.63 ^{††}	360.70 ^{††}	342.35 ^{††}	6.23 ^{††}
Harvests	7.97 ^{††}	28.71 ^{††}	33.06 ^{††}	84.51 ^{††}	76.93 ^{††}	93.65 ^{††}	8.20 ^{††}	1.47
Symbionts	9.55 ^{††}	77.80 ^{††}	213.73 ^{††}	1185.18 ^{††}	586.66 ^{††}	498.53 ^{††}	19.26 ^{††}	0.84
pH x H	1.39	3.37 ^{††}	1.31	7.72 ^{††}	2.67 [†]	5.24 ^{††}	3.62 ^{††}	1.11
pH x S	1.98	8.72 ^{††}	11.18 ^{††}	93.00 ^{††}	21.72 ^{††}	9.87 ^{††}	13.03 ^{††}	0.97
H x S	0.97	5.81 ^{††}	3.42 ^{††}	5.92 ^{††}	5.60 ^{††}	5.52 ^{††}	0.85	0.99

†† Significant at $P < 0.01$ level.

Without asterisk statistically insignificant at above level.

Table 20. Correlation coefficients (r) between mycorrhizal infection and nodule number, nitrogenase activity, nodule dry weight and *Frankia* population at different levels of soil pH.

Sources of variation	Soil pH	Nodule number	Nitrogenase activity	Nodule dry weight	<i>Frankia</i> population
	4	0.985*	- 0.181	0.998**	- 0.996**
Mycorrhizal infection (%)	5	0.992**	0.954*	0.953*	0.732
	6	0.986*	0.918	0.847	0.554
	7	0.987*	0.850	0.866	0.550

*, ** Significant at P<0.05 and 0.01 levels respectively.

Without asterisk statistically insignificant at above levels.

Table 21. Correlation coefficients (r) between nitrogenase activity and mycorrhizal infection, arbuscule, vesicle and rhizospheric spore numbers at various levels of soil pH.

Sources of variation	Soil pH	Mycorrhizal infection	Arbuscule number	Vesicle number	Spore number
	4	0.128	- 0.375	0.110	- 0.293
Nitrogenase activity	5	0.954*	- 0.937	0.393	0.981*
	6	0.918	0.833	0.897	0.970*
	7	0.847	0.506	0.908	0.943

* Significant at $P < 0.05$ level.

Without asterisk statistically insignificant at above level.

growth of seedlings, which was attributed to better uptake of nutrients by them in soils of low fertility (Gerdemann, 1975). Increased growth of mycorrhizal plants has often been attributed to improved uptake of phosphorus. Harley (1969) demonstrated that mycorrhizal roots take up several time more P than non-mycorrhizal ones. Improved growth of nodulated + mycorrhizal plants at slightly acidic pH may be associated with higher availability of nutrients which are explored and translocated to host root by extra-radical mycorrhizal hyphae. The efficiency of mycorrhizal plants may also depend on pH of the medium. Kruckelmann (1975), Seikh *et al.* (1975) and Hayman and Tavares (1985) also revealed that soil pH considerably influenced VAM fungal activity. This may be due to effect of soil pH conditions on the extent of formation of external hyphae by VAM fungi (Zhao, 1988). Decreased growth of mycorrhizal plant at low pH may be related to decrease in efficiency of VA mycorrhizal endophyte (Mosse, 1972; Graw, 1979; Skipper and Graw, 1979). Adaptation of mycorrhizal fungi to a particular soil conditions (Lambert *et al.*, 1980) may also be responsible for decreased activity of *G. mosseae* at pH 4-5 range as latter was isolated from *A. nepalensis* forest soil with average pH of 5.3-5.8. It has also been demonstrated that *Glomus* spp. perform better mostly at slightly acidic to neutral pH conditions (Green *et al.*, 1976). Further, depression in root and overall growth of seedlings at highly acidic soil may

be attributed to aluminium and manganese toxicity as noted in other plant species (Mugwira, 1980; Sheppard and Floate, 1984). Danielson and Vesser (1989) and Arines and Vilarino (1990) revealed that the colonization of host root by VAM fungus under hostile acidic soil condition failed to improve establishment of host plant. Besides, detrimental effect on the survival of microendophytes caused by toxicity of metals in acidic pH may have considerably affected plant growth. Toxicity of metals on the survival of other micro-organisms has previously been demonstrated (Harding and Whitton, 1977; Rai *et al.*, 1981). A decrease in plant growth at pH 7 may be explained by lowered availability of micronutrients essential for growth.

Higher nodulation, nodule biomass and nitrogen fixation in nodulated + mycorrhizal seedlings at pH range 5-7 is in accordance with the findings of Wheeler *et al.* (1981). This improvement in dually inoculated plants may have been accomplished by P mediated effects of VAM fungi (Gardner *et al.*, 1984; Russo, 1989; Jha *et al.*, 1993) as observed in legumes (Asai, 1944; Carling *et al.*, 1978). Besides, these improvements in leguminous system have been demonstrated to be mediated by qualitative and quantitative changes in the free amino acids (Young *et al.*, 1972; Krishna and Bagyaraj, 1983) or

hormones (Munne and Mosse, 1980; Manjunath and Bagyaraj, 1984). A similar situation may be conceived in non-leguminous (*Alnus*) - *Frankia-G. mosseae* symbioses. However, Fraga-Baddier and Tacon (1990) revealed that stimulation of *Frankia* by VAM fungus may be mediated by other unknown mechanism. An ameliorated nutritional status due to improved uptake of N (Smith, 1982; Ames *et al.*, 1983; Smith *et al.*, 1985; Johnsen *et al.*, 1992), K (Smith, 1982; Nielsen and Jensen, 1983), Ca (Ross, 1984; Tang *et al.*, 1989) and Mn (Kothari *et al.*, 1989) by VA mycorrhizal endophyte may have additive effect on above. A decrease in nodulation and nitrogenase activity at low pH may have resulted due to toxicity of aluminium on nodulation (Paulino *et al.*, 1987; Mays de Manzi and Curtwright, 1984) which directly interferes with uptake and translocation of P and other vital nutrients essential for nodulation (Hutchinson *et al.*, 1971; Mugwira, 1980).

A marked stimulation of soil *Frankia* by VAM fungi may be brought about by selective proliferation of *Frankia* by root exudates of mycorrhizal plants. Qualitative and quantitative changes in the root exudates of mycorrhizal legumes have been previously shown to selectively attract and proliferate rhizobia thereby increasing nodulation (Gitte *et al.*, 1978).

Mycorrhizal infection was markedly influenced by *Frankia* in dually inoculated plants. Our observation is supported by the findings of Berlinger and Torrey (1989). The stimulation of mycorrhizal infection may have brought about by changes in hormonal quality and levels (Wheeler *et al.*, 1979; Berry *et al.*, 1989). The possibility of *Frankia* affecting directly on fungal metabolism by secretion of fungal specific polysaccharide or other mechanism cannot be excluded as observed in legume-rhizobium-VAM fungus association (Azcon *et al.*, 1980; Paccini *et al.*, 1988). However, the root infection at low level of soil pH was not improved even in *Frankia* + *G. mosseae* combination which may be related to hampered survival and affectivity of *Frankia* (Rodriguez-Burrueco, 1968; Smolander *et al.*, 1988) and VA endophyte (Green *et al.*, 1976; Graw, 1979; Zhao, 1988).

Increase in arbuscules and vesicle population in the presence of *Frankia* may be a synergistic effect related to exchange of more nutrients and its storage which is directly related to symbiotic nitrogen fixation and improved plant growth.

An ameliorative effect of *Frankia* on endogonaceous spore in dually inoculated plants may be important which finally lead to more root infection and improved uptake of

nutrients from the soil. This synergistic influence of nodule endophyte on *G. mosseae* may be mediated by qualitative and quantitative changes in hormones (Wheeler *et al.*, 1979).

The confinement of phosphatase activity in nodulated + mycorrhizal and mycorrhizal seedlings possibly indicates the origin of enzyme system from VA mycorrhizal (*G. mosseae*) fungus (Gianinazzi-Pearson and Gianinazzi, 1978). The increased activity of phosphatase enzyme in soils of low pH may signify P stress. A decrease in its activity at pH range 6-7 may thus be attributed to availability of P in above pH range. A similar observation was made by Harrison (1983). An increased activity of the enzyme in *Frankia* + *G. mosseae* infected plants may be synergistic effect of former on latter which may lead to increased acquisition of P, a critical factor involved in nodulation and nitrogen fixation.

Improved N, P, K and Mn content of dually inoculated plants may be associated with greater uptake of these nutrients by mycorrhizal fungi. This is in agreement with the findings of Rose and Youngberg (1981). Ameliorated N status in dually inoculated plants may be attributed to two-fold influence of VAM fungus i.e. the stimulation of nitrogen

fixation of *Frankia* (Rose and Youngberg, 1981; Gardner *et al.*, 1984; Russo, 1989; Jha *et al.*, 1993) and absorption of N directly from soil (Smith, 1982; Ames, 1983; Smith *et al.*, 1985; Barea *et al.*, 1988; Azcon-Aguilar and Barea, 1992; Johnsen *et al.*, 1992). These complimentary effects of microendophytes on each other and overall plant growth under nutrient depleted (acidic conditions) habitats may be promising for the establishment and productivity of actinorhizal tree species, which may be exploited for the reclamation of degraded wastelands.

Chapter	:	Interaction between <i>Frankia</i> and <i>Glomus mosseae</i> on
V	:	P uptake, nitrogen fixation and growth performance
	:	of <i>Alnus nepalensis</i> seedlings under various soil
	:	organic matter levels.

Introduction :

A wide variety of actinorhizal trees and shrubs harbour nitrogen-fixing endophyte i.e. *Frankia* in their root nodules (Bond, 1976). They fix atmospheric nitrogen at rates comparable to legumes and consequently play an important role in replenishing N in forest soils (Tarrant and Miller, 1963; Berg and Doerksen, 1975; Miller and Murray, 1978). Constant fixation and accretion of nitrogen and deposition of organic matter through annual litterfall (Tarrant and Miller, 1963; Bormann and Debell, 1981) may predispose soils harmful for the growth, survival, infectivity and effectivity of VA mycorrhizal (Davidson and Visser, 1989) and *Frankia* endophytes. Besides, inhibitory role of peat (Sheppard *et al.*, 1989) substrate N (Bond and MacKintosh, 1975; Ingestad, 1980; Huss-Danell *et al.*, 1982; MacKay *et al.*, 1987) and nitrate (Kohls and Baker, 1989) in nodulation and nitrogen fixation by *Frankia* is evident. In

addition plant phenolic compounds released from organic matter may affect growth and survival of *Frankia* (Perradin *et al.*, 1983; Vogel and Dawson, 1985). VAM fungi which form symbiotic association with legumes and nonlegumes and induce efficient uptake of soil P unavailable to nonmycorrhizal ones. This leads to ameliorative nodulation and nitrogen fixation by these hosts under P deficient conditions (Rose and Youngberg, 1981; Gardner *et al.*, 1984; Russo, 1989; Jha *et al.*, 1993).

Changes in soil fertility due to amendments with mineral fertilizers or organic matter may affect efficiency and survival of mycorrhizal fungus. Organic matter/manures may often favour mycorrhizal development compared to mineral fertilizers (Sharma *et al.*, 1986; Jha, 1990). Despite the ecological significance of tripartite association involving nonlegume-*Frankia*-VAM fungi in improving soil fertility, short term energy plantations, reclamation of denuded and hostile habitats and other economical importance, the information on the influence of organic matter amendment on the infectivity and effectivity of microendophytes i.e. *Frankia* and VAM fungi, their interactions during the growth of host are not available, which could add to the present limited knowledge on tripartite association. This is practically important from the view point of reclamation of nutrient-deficient wastelands.

The present investigation aims to evaluate the interaction between *Frankia* and VAM microendophytes, *in vitro* survival, infectivity and effectivity on P uptake, nitrogen fixation and seedling growth of *A. nepalensis* under various soil organic matter levels.

Materials and methods

Collection and sterilization of soil :

Sandy-loam laterite soil with CEC - 25.2 Meq/100 g, sand - 66 %, silt - 24 %, clay - 10 %, pH - 5.34, organic matter - 3.40 %, total N - 0.343 %, available P - 0.022 %, exchangeable K - 0.121 %, Ca - 0.094 %, Mg - 0.072 % and Mn 0.0013 % was collected from Botanical garden of North-Eastern Hill University, Shillong (Meghalaya). It was mixed thoroughly with builder's sand in a ratio of 2:1 (Soil: Sand), moistened to field capacity and was steam sterilized at 15 lb/inch² for 1 h. The sterilization was repeated twice at a gap of 24 h and was allowed to stand for 15 days.

Amendment of soil with organic matter :

Litter was collected after 1 month of litterfall (February, 1992) from Himalayan alder stand, upper Shillong.

It was oven-dried at 60°C till constant weight was achieved and was milled in a mechanical grinder. On the basis of rate of annual litterfall in *Alnus nepalensis* stand and organic matter contents in soil supporting various forests (Ugolini, 1979; Thompson and Troeh, 1985), four levels of soil organic matter i.e. 0 N (unamended soil), 1 N (1 lac kg organic matter/ ha soil), 2 N (2 lacs kg organic matter / ha) and 4 N (4 lacs kg organic matter / ha) were maintained by adding and mixing thoroughly the milled litter to the sterilized soil and filled in sterile 1 L conical flasks and capped with sterile cotton plugs.

Organic bound phenolic compound in the litter was determined following the method of Chattopadhyay and Samaddar (1986). One g of ground fine litter in a centrifuged tube was treated with 5 ml of sodium lauryl sulphate (3% w/v) and centrifuged at 2000 g for 5 min. After discarding supernatant, the residue was washed successively, once with 5 ml sodium lauryl sulphate, twice with 5 ml of water, twice with 5 ml of ethanol and twice with 10 ml of diethyl ether. After each washing they were centrifuged and supernatant discarded. The residue was allowed to dry and was resuspended in 3 ml of 0.5 M NaOH in centrifuge tube. After standing overnight it was centrifuged and supernatant was saved. Then 1 ml of the

extract was diluted to 8 ml mixed with 1 ml 0.5 M NaOH and Folin-Ciocalteu reagent and absorbance read at 290 nm in a spectrophotometer. The O.D. was converted into known units using standard curve of phenol and was expressed in ug/g litter. The organic-bound phenol estimated was 84 ug/g dry litter.

Collection of seeds and raising of seedlings :

Seeds of *A. nepalensis* were collected in the month of March, 1992 from Himalayan alder stand located at Upper Shillong. They were husked from female catkins and were transferred to conical flask containing 5% (w/v) CaOCl_2 and allowed to stand for 20 min at room temperature. Further process of seedling germination and growth was carried out as described in Chapter IV.

Preparation of *Frankia* inoculum :

Inoculum of *Frankia* isolate *AnF₁* (abbreviated as *Frankia*) was prepared and diluted to known concentration as described earlier in chapter IV.

Preparation of VA Mycorrhizal inoculum :

VA mycorrhizal fungus *Glomus mosseae* was selected,

cultured and maintained as described in chapter IV. Eighty five endomycorrhizal spores suspended in 50 ml sterile water and 200 mg infected root (infection level >60%) were prepared as mentioned in chapter IV.

Inoculation of root microsymbionts and plant growth conditions:

One month old healthy seedlings of *A. nepalensis* growing on water agar were harvested and washed several times in sterile distilled water. Five seedlings were transplanted in each conical flask containing different level of organic matter. Transplanted seedlings in each conical flask were inoculated either with *Frankia*, *G. mosseae* or both (*Frankia* + *G. mosseae*). The controls were maintained by addition of steamed sterilized inoculum of *Frankia* and *G. mosseae*. The seedlings were watered to about 20 % w/v soil moisture content and plugged with sterile cotton. They were transferred to growth chamber and maintained under similar conditions as mentioned in chapter IV. They were watered whenever necessary in order to maintain 20 % w/v moisture content.

Harvests :

Harvesting of seedlings was conducted after 30, 60, 90 and 120 days of inoculation as mentioned in Chapter IV.

Evaluation of seedling growths :

At each harvest, growth characteristics like root length, shoot height, total plant height, root, shoot and plant dry weight, R/S ratio, stem collar diameter, leaf number and leaf area were determined similarly as described in chapter IV.

Nodulation, nodule biomass and nitrogen fixation :

At each harvest, the root system of the seedlings was thoroughly washed, excised and transferred to 10 ml serum bottle. After stoppering with suba seal, 1 ml of air was withdrawn and replaced with 1 ml C_2H_2 with the help of a syringe. Further process of estimation of nitrogenase activity was done similarly as described in Chapter I. However, the activity was expressed as $nmole\ C_2H_4\ h^{-1}\ seedling^{-1}$. Thereafter number of nodules formed per seedling was counted with the help of stereomicroscope. The dry weight of the nodules was determined after drying them in a hot air oven at $60^{\circ}C$ till constant weight and was expressed in $mg\ seedling^{-1}$.

Assessment of rhizosphere *Frankia* population :

Population of *Frankia* in the rhizosphere soil was estimated following the method of Baker and O'Keefe (1984) as

described in Chapter I.

Assessment of VAM infection, arbuscules and vesicles :

On each sampling, the roots of seedlings were washed and cut into 1 cm segments. One hundred segments were randomly selected, cleared and stained employing the method of Koske and Gemma (1988). The assessment of VAM infection was carried out following the method of Read *et al.* (1976). The detail of the above techniques is mentioned in Chapter I. Number of arbuscules and vesicles were determined using stained root segments and expressed as cm^{-1} root length.

Determination of endogonaceous spore population :

Air dried rhizosphere soil was used to determine endogonaceous spore number after its isolation using wet sieving and decanting technique (Gerde mann and Nicolson, 1963) and expressed as g^{-1} dry soil.

Determination of total soil phosphatase activity :

Phosphatase activity of the rhizosphere soil was assayed following the method of Tabatabai and Bremner (1969)

as described in Chapter I.

Analysis of nutrient contents in seedlings :

Oven dried root and shoot samples were ground separately, sieved (0.2 mm) and used for elemental (N, P, K and Mn) analysis. The N content of the plant tissues was estimated following semi-microkjeldahl method (Allen, 1974) as elaborated in Chapter I. Phosphorus content in plant tissues was estimated following the molybdenum blue method (Allen, 1974), potassium and manganese were determined employing atomic absorption spectrophotometric method (Allen, 1974) as outlined in chapter IV.

Statistical Analysis :

The effect of organic matter, root symbionts and harvests on various characteristics of microendophytes, nutrients content, nitrogen fixation and growth performance of seedlings were evaluated using 3-way Analysis of Variance (ANOVA). Interactions between endophytes were evaluated using coefficient of correlation (Zar, 1974).

RESULTS

Plant growth performance :

Seedlings of *A. nepalensis* inoculated with *Frankia* + *G. mosseae* responded best up to 1 N level of organic matter amendments, whereas, those seedlings either inoculated with single inoculum or without any, performed better at higher organic matter levels (1 N-2 N). The growth of seedlings with or without symbiosis was however, adversely affected at 4 N organic matter dose. A marked difference was observed in overall growth pattern between dually, singly and noninoculated seedlings especially at low organic matter level.

Root length , shoot and seedling height and stem collar diameter increased with organic matter amendment upto 1 N level for all groups of plants. However, this increase was more pronounced in dually (*Frankia* + *G. mosseae*) inoculated seedlings. With further increase in organic matter content of soil, seedling growth was depressed exponentially irrespective of inoculations with single or both microsymbionts. Difference in above growth characteristics of seedlings with organism/s or without it was clearcut at low organic matter level but narrowed with increasing level of latter (Fig.49, 50, 51 & 52).

The accumulation of shoot, root, and total seedling biomass increased in time for all treatments upto 1 N organic matter level beyond which it declined sharply. Higher biomass was achieved by *Frankia* + *G. mosseae* inoculated seedlings over singly or uninoculated counterparts (Table 22, 23 & 24). However, the difference narrowed markedly at higher level of soil organic matter. In terms of R/S ratio, mycorrhizal plants exhibited lower values than *Frankia* inoculated or uninoculated control ones but lacked behind dually inoculated ones at most levels of organic matter (Fig.53).

In terms of leaf number and leaf area too, dually inoculated plants generally maintained fair lead throughout the growth period over *G. mosseae*, *Frankia* inoculated and noninoculated ones at most levels of soil organic matter. However, this difference was more evident at low organic matter levels (Fig. 54 & 55). Seedlings inoculated with *G. mosseae* performed better than *Frankia* inoculated and uninoculated controls especially at lower organic matter level. Both leaf number and leaf area increased with time.

All above plant growth characteristics were significantly (ANOVA $P < 0.05$ & 0.01) influenced by soil organic matter levels, harvests and symbionts (Table 25).

Nodulation, nodule biomass and nitrogen fixation :

Number of nodules per seedling increased exponentially in time and with increasing soil organic matter dose upto 1 N level beyond which they showed an exponential decrease and was totally absent at 4 N level both for *Frankia* and *Frankia + G. mosseae* colonized seedlings. Difference in the number of nodules between *Frankia* inoculated and *Frankia + G. mosseae* inoculated seedlings was clear cut at lower level of soil organic matter (Fig. 56).

As for nodule number, nodule biomass per seedling also increased in time and upto 1 N level and then markedly decreased at 2 N level. It was totally depressed at 4 N level of organic matter (Fig. 57). Seedlings inoculated with *Frankia + G. mosseae* accumulated higher nodule biomass than *Frankia* inoculated counterpart especially at low organic matter doses (Fig. 58).

Nitrogenase activity of nodules, generally followed the similar trend as for nodule biomass (Fig. 58).

***Frankia* population :**

The population of rhizosphere soil *Frankia* was

markedly affected by level of soil organic matter and harvests. The number of *Frankia* increased with increase in soil organic content but was severely affected at higher doses. Profound difference in its abundance was noted in rhizosphere of *Frankia* and *Frankia* + *G. mosseae* inoculated seedlings (Fig. 59). Control and VA mycorrhizal fungus inoculated plants did not harbour *Frankia* in their rhizosphere.

Nodule number, nodule biomass, nitrogenase activity and *Frankia* population were significantly (ANOVA $P < 0.05$ & 0.01) influenced by levels of organic matter, harvests and root symbionts (Table 26).

Mycorrhizal infection, arbuscules and vesicles :

Root infection of Himalayan alder by *G. mosseae* increased in time and upto 1 N level of organic matter. Uninoculated and *Frankia* inoculated seedlings did not show root infection at any stage of growth. However, *G. mosseae* inoculated seedlings exhibited less mycorrhizal dependency than those of *Frankia* + *G. mosseae* inoculated ones between 0 N-1 N dose of organic matter (Fig. 60).

Number of arbuscules was higher than vesicles in the beginning but the ratio decreased as the time increased.

This decline was however, more pronounced at lower level of organic matter. The number of arbuscules and vesicles was generally more in dually inoculated plants as compared to mycorrhizal alone at a given level of organic matter and harvest (Fig. 61 & 62).

Endogonaceous spore population :

The number of rhizosphere endogonaceous spores increased with time at all soil organic matter levels. However, spore production by *G. mosseae* was more at lower levels than at higher doses of organic matter. Generally, marked difference in the spore population in singly and dually inoculated plants was observed at low organic matter levels (Fig. 63).

Total phosphatase activity :

Soil phosphatase activity increased with the increasing level of soil organic amendments at the beginning but declined substantially with increase in time. However, at low organic matter level the activity increased with increase in time. Generally at any given time and organic matter level, total phosphatase activity of rhizosphere soil differed between *G. mosseae* and *Frankia* + *G. mosseae* inoculated plants

but the difference narrowed with increase in soil organic matter level (Fig. 64). Uninoculated and *Frankia* inoculated plants did not exhibit phosphatase activity in its rhizospheric soil.

All above mycorrhizal characteristics were significantly (ANOVA $P < 0.05$ & 0.01) influenced by organic matter levels, harvests and symbionts (Table 27). Similarly, total phosphatase activity of the soil was influenced by organic matter levels, harvests and symbionts (Table 27).

N, P, K, and Mn contents in plant tissues :

Shoots of nodulated + mycorrhizal (*Frankia* + *G. mosseae*) seedlings contained more N, P, K and Mn than roots. Moreover, concentration of these nutrients was higher in dually inoculated seedlings compared to either *Frankia* nodulated, mycorrhizal and uninoculated controls. However, a marked variation in the nutrient contents in shoot of singly, dually and non-inoculated seedlings was observed at most harvests especially at low organic matter level (Fig. 65a, 66a, 67a & 68a).

Generally, the concentration of nutrients in root also followed the similar trend as above (Fig. 65b, 66b, 67b &

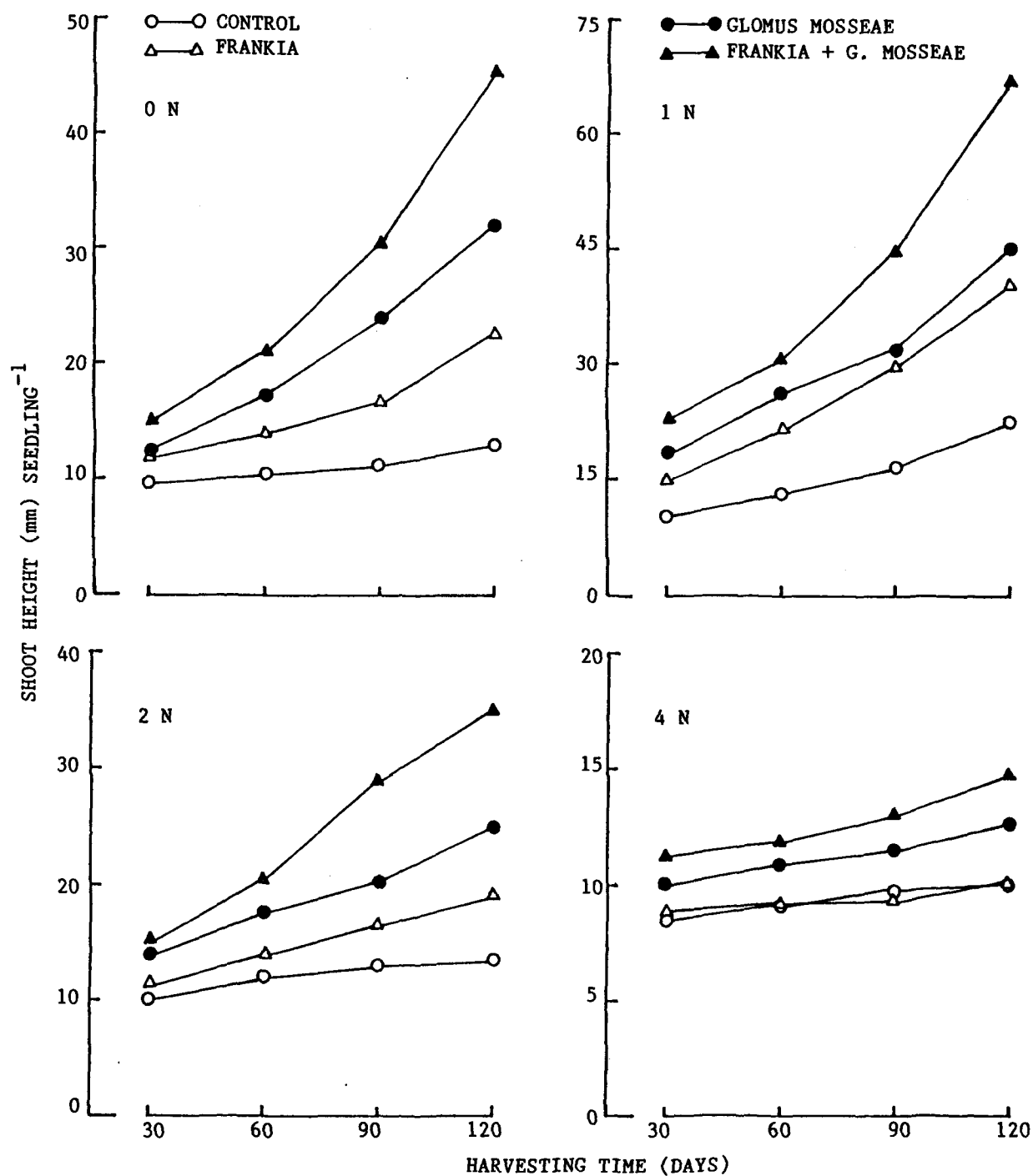


Fig.49. Effect of Frankia, G. mosseae and Frankia + G. mosseae on shoot height of A. nepalensis grown on soil amended with various levels of organic matter.

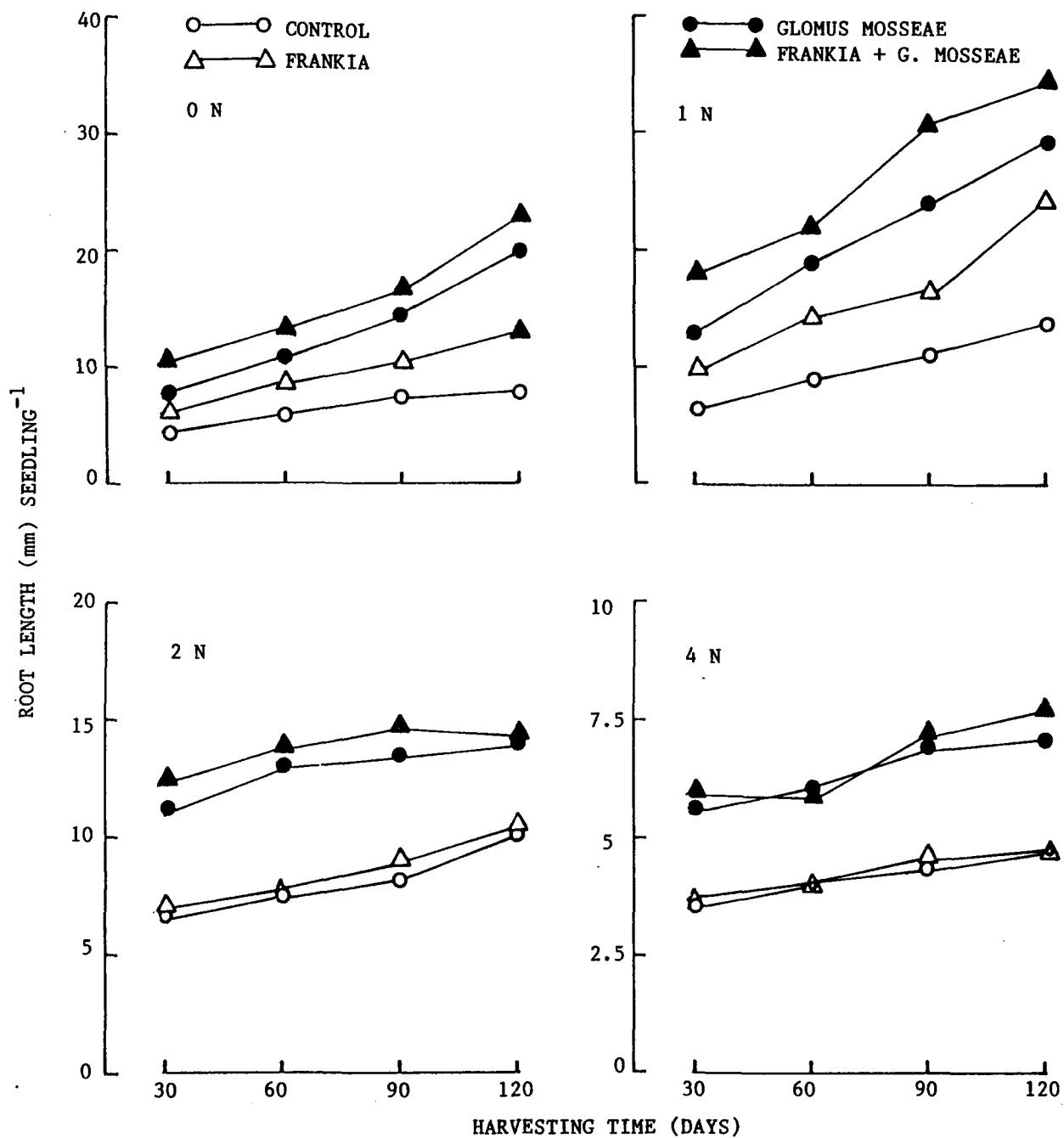


Fig.50. Effect of Frankia, G. mosseae and Frankia + G. mosseae on root length of A. nepalensis seedlings at different levels of organic matter.

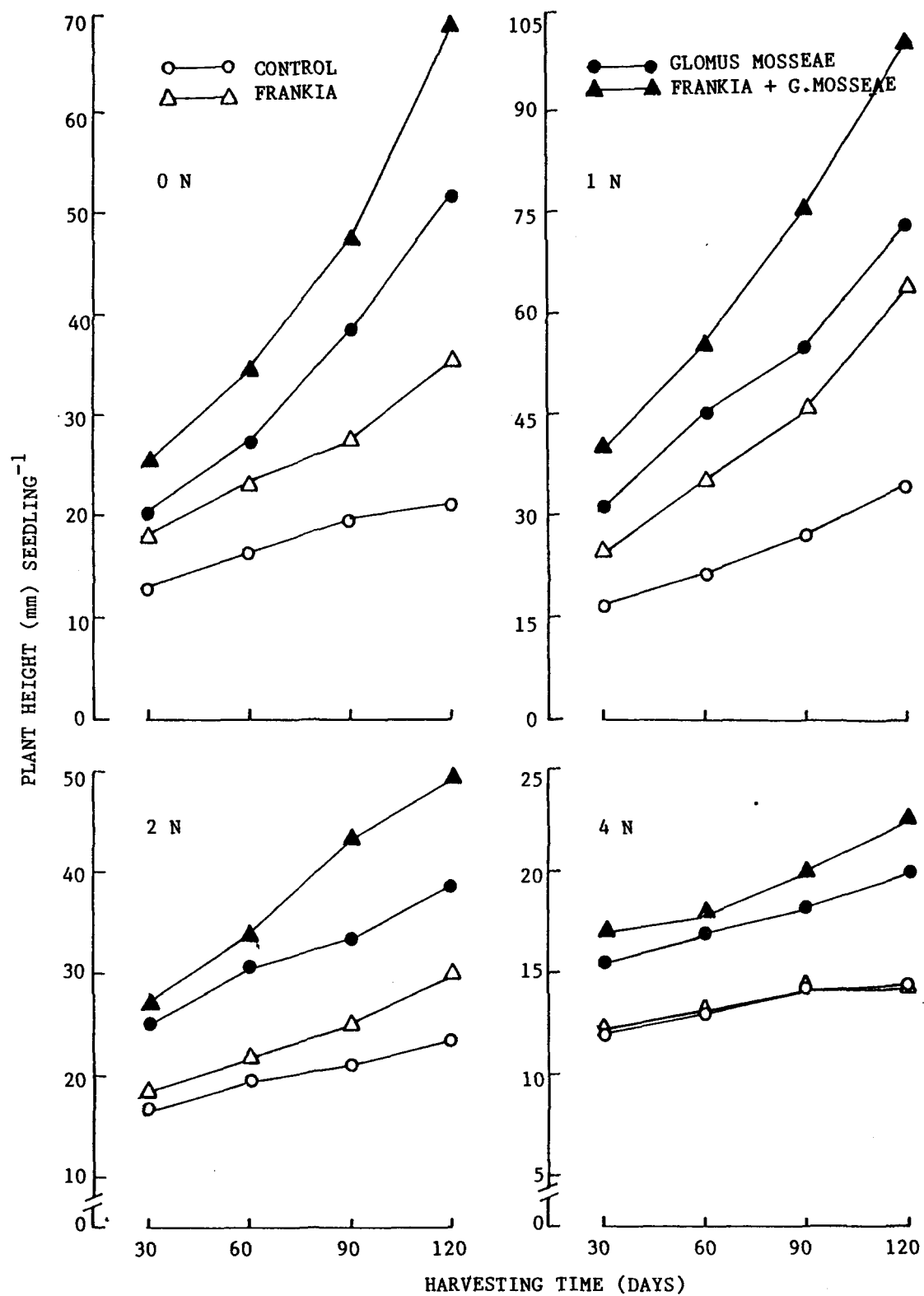


Fig.51. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on height of *Alnus nepalensis* seedlings at various levels of soil organic matter.

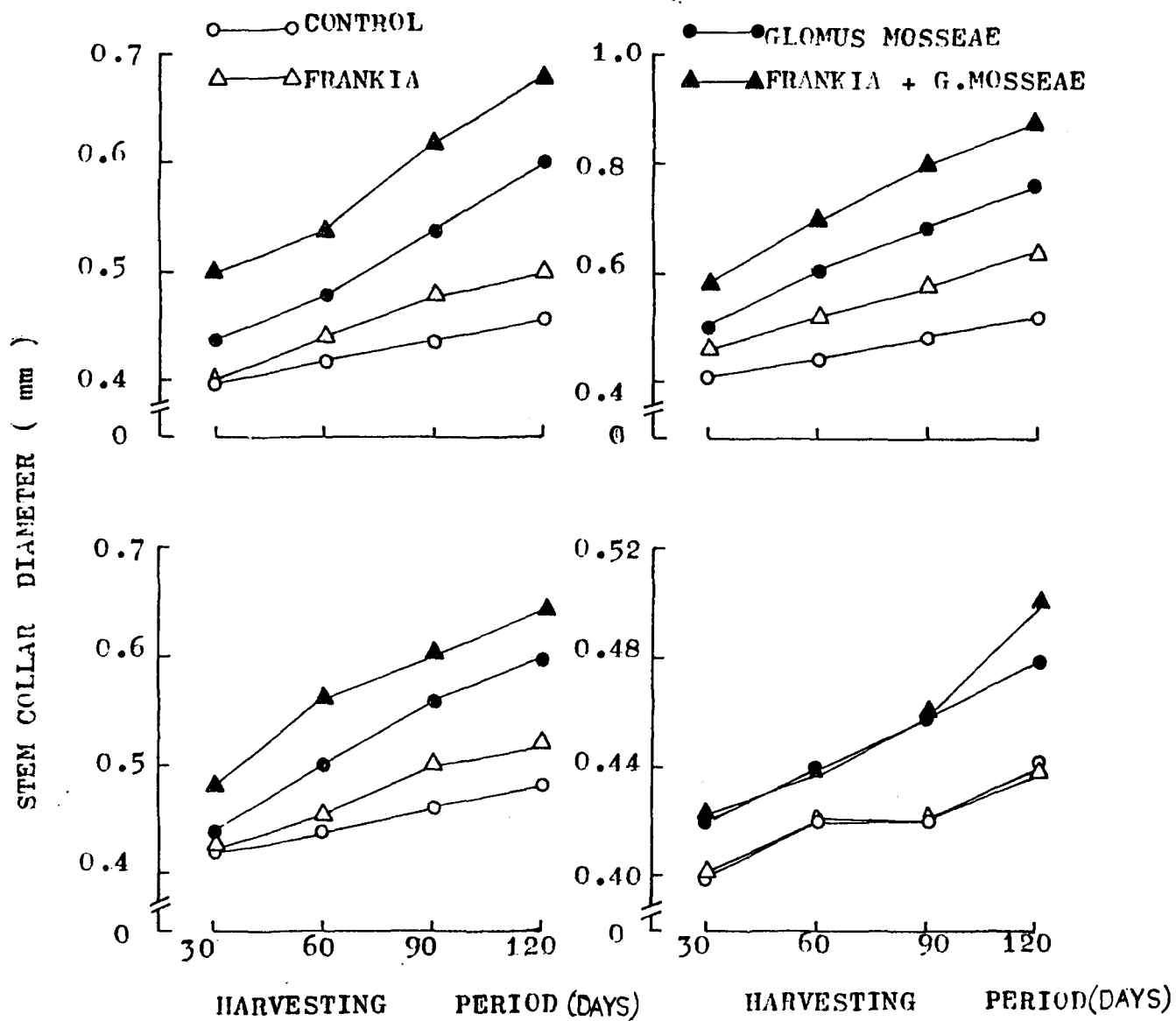


Fig. 52. Effect of Frankia, G. mosseae and Frankia + G. mosseae on stem collar diameter of A. nepalensis seedlings grown at various levels of organic matter.

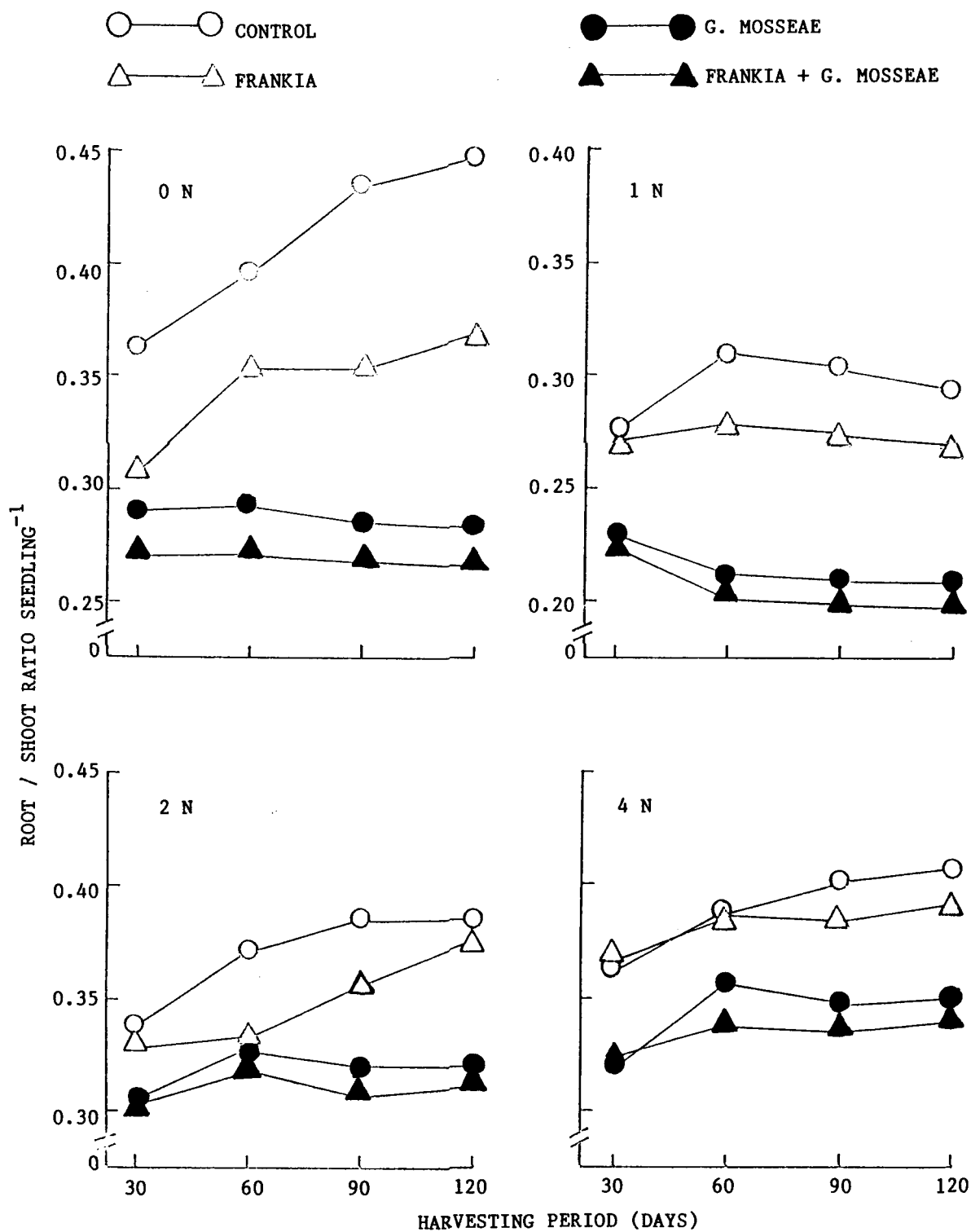


Fig.53. Effect of Frankia, G. mosseae and Frankia + G. mosseae on R/S ratio of A. nepalensis seedlings at various levels of organic matter.

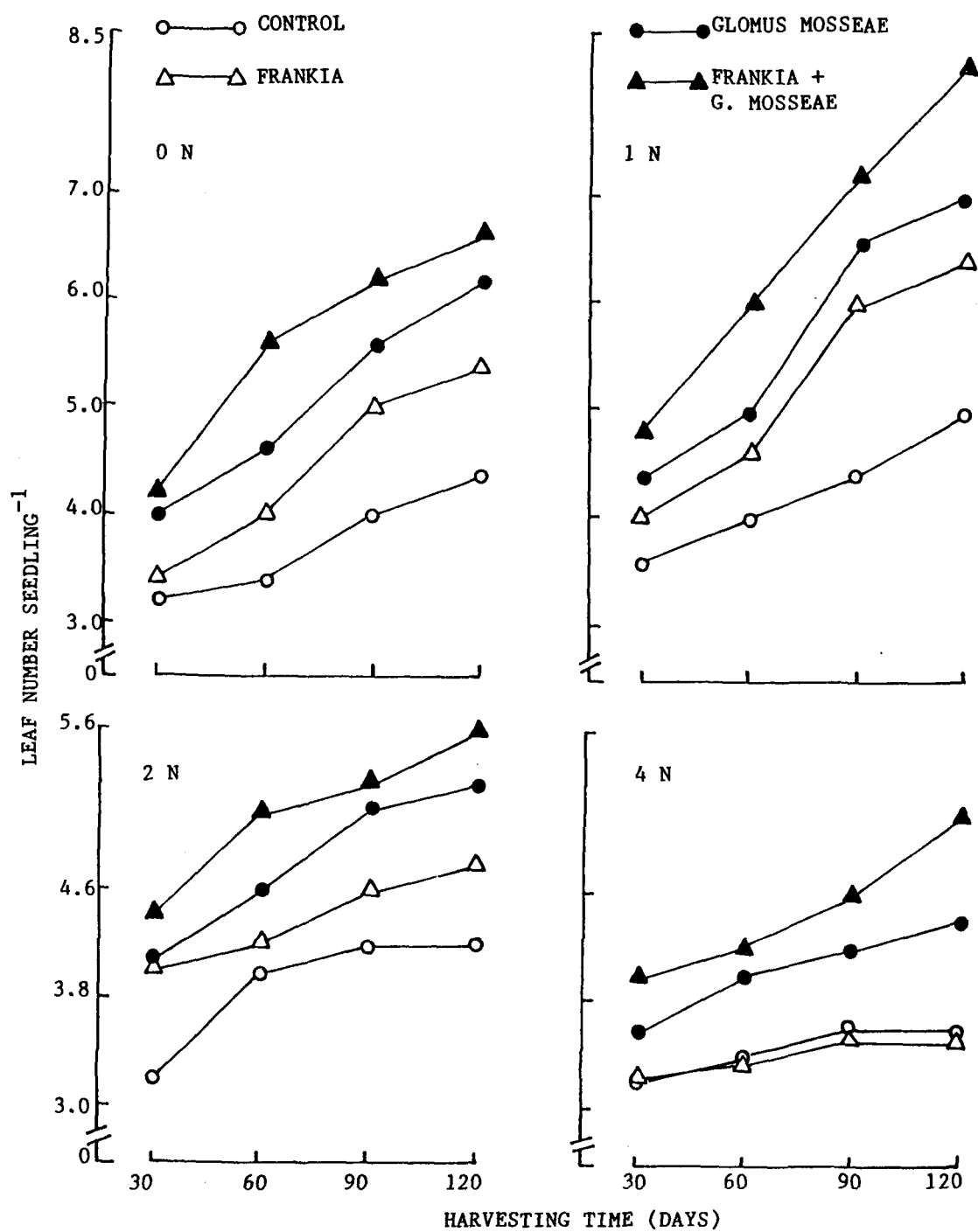


Fig.54. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on leaf number of *A. nepalensis* seedlings at various levels of soil organic matter.

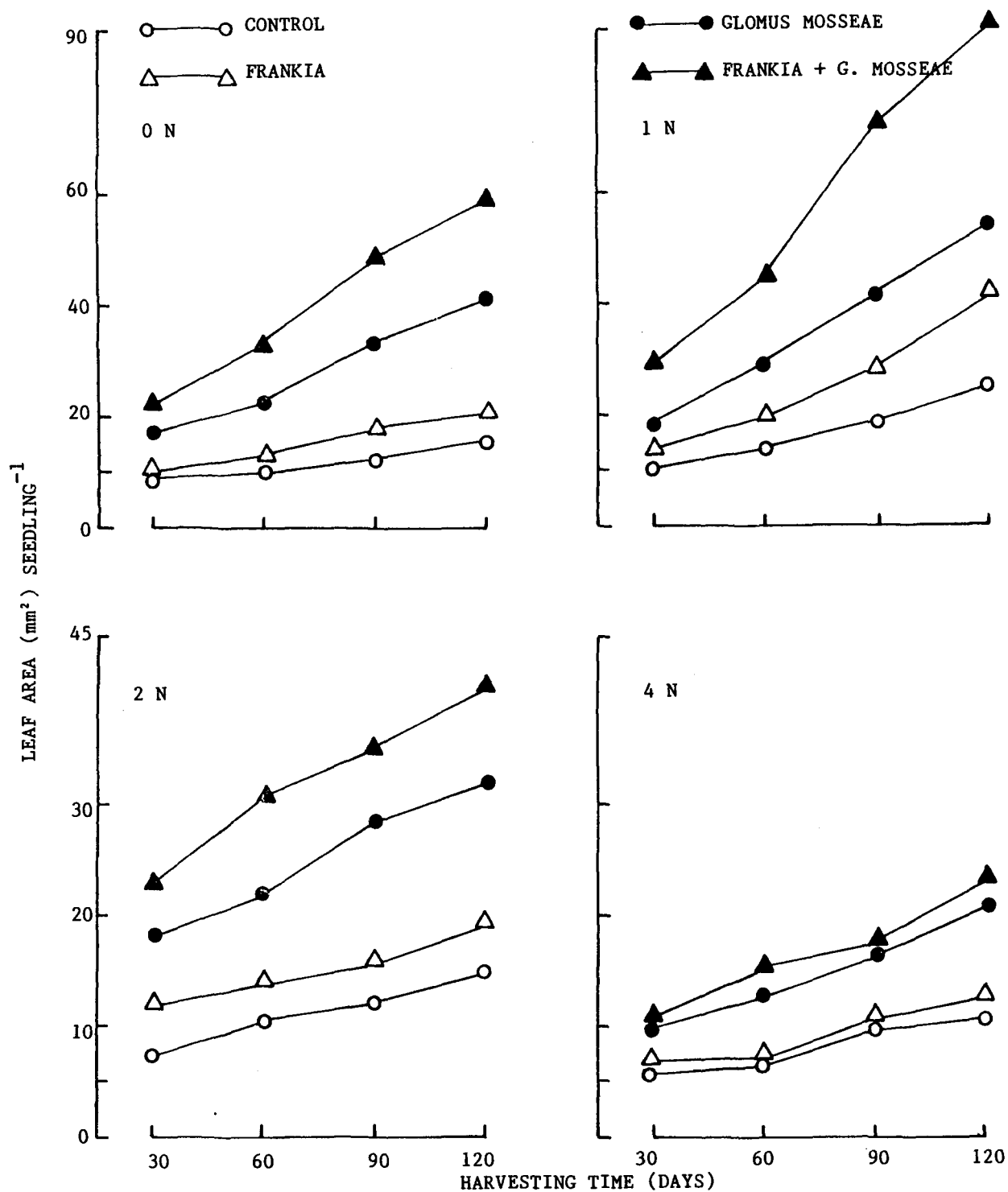


Fig.55. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on leaf area of *A. nepalensis* seedlings grown at various levels of organic matter.

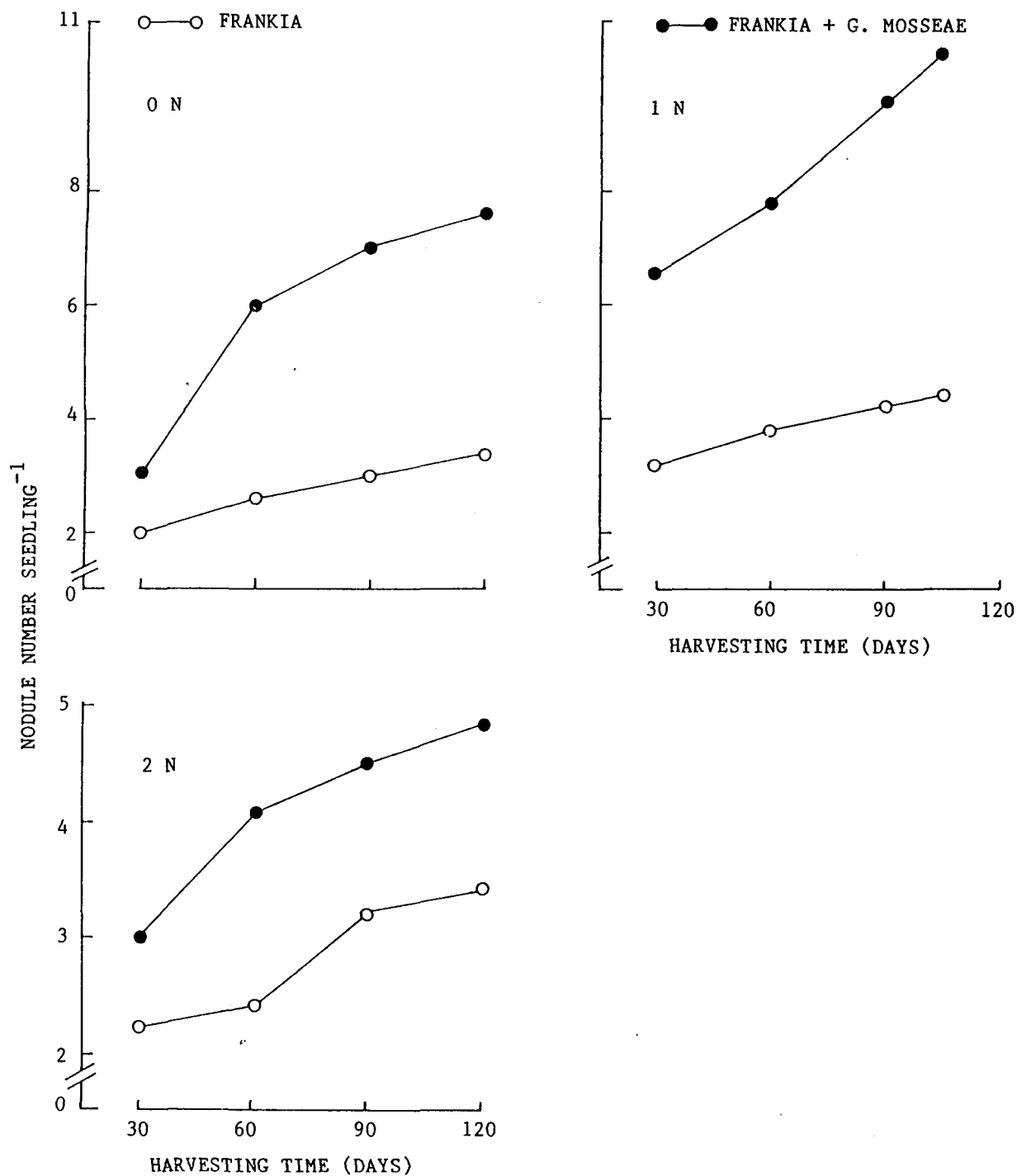


Fig. 56. Effect of inoculation of Frankia and Frankia + G. mosseae on nodule number of A. nepalensis seedlings at different levels of soil organic matter.

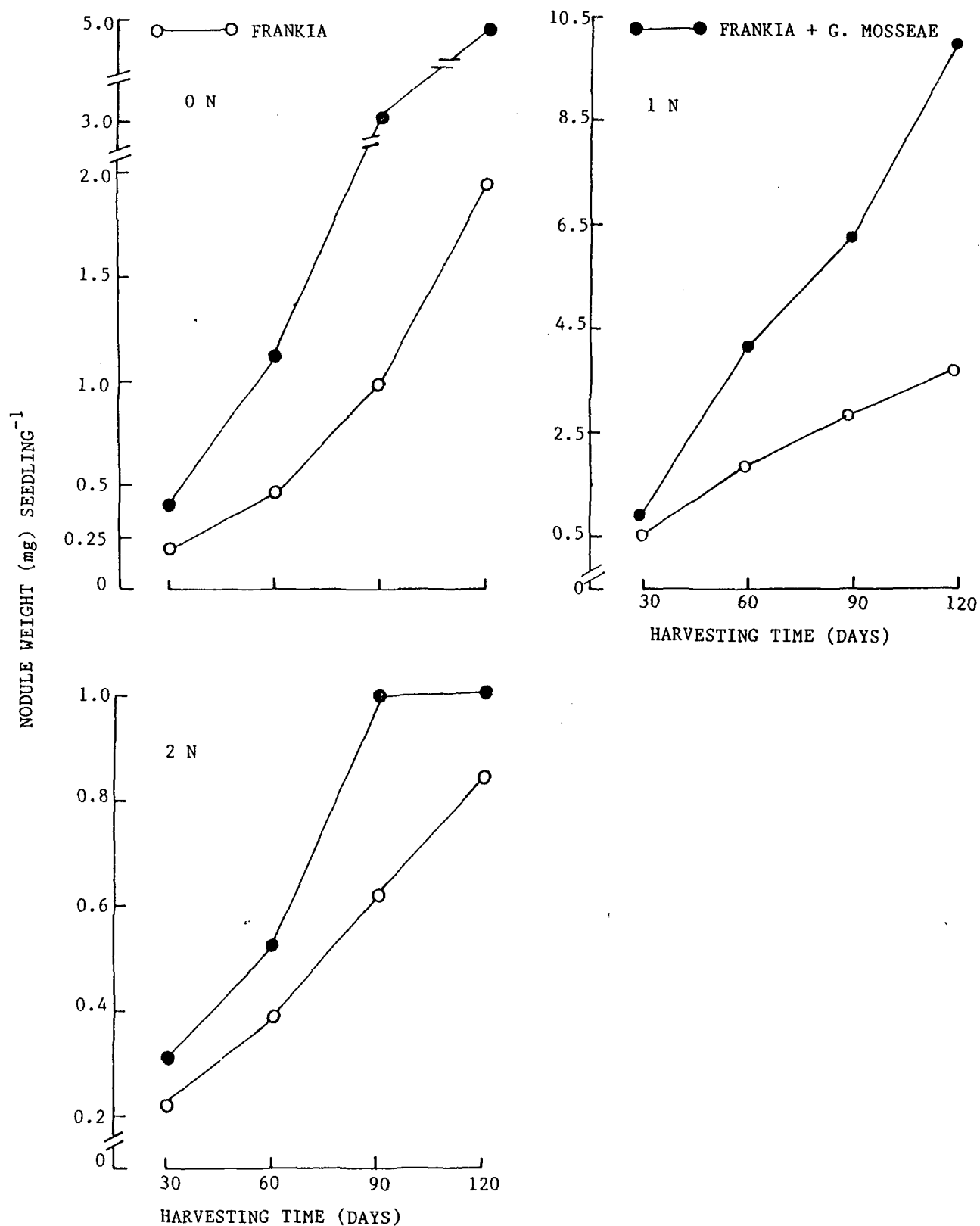


Fig. 57. Nodule dry weight of Frankia and Frankia + G. mosseae inoculated seedlings of A. nepalensis at various soil organic matter levels.

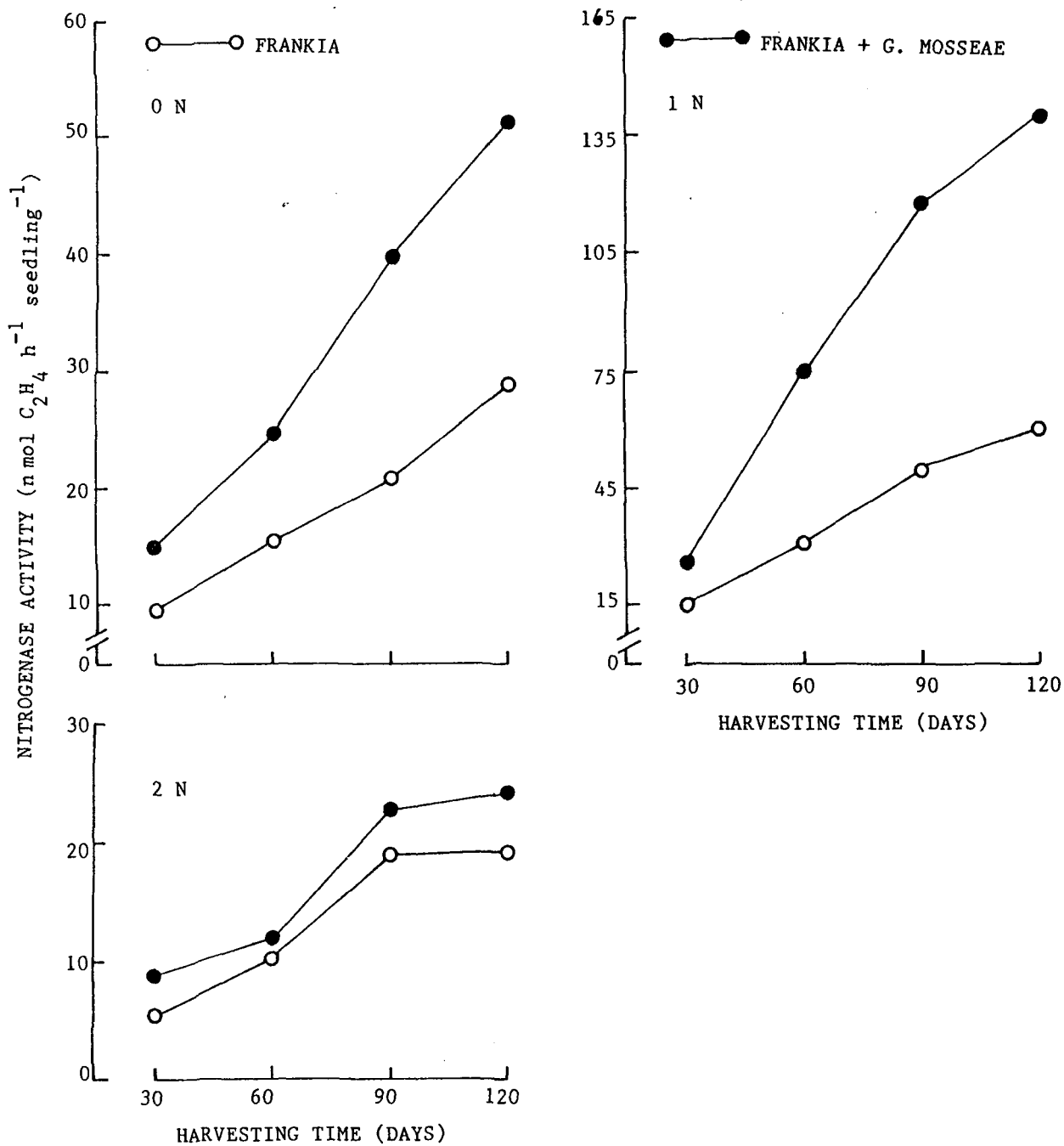


Fig.58. Effect of Frankia and Frankia + G. mosseae on nitrogenase activity of root nodules of A. nepalensis at various levels of soil organic matter.

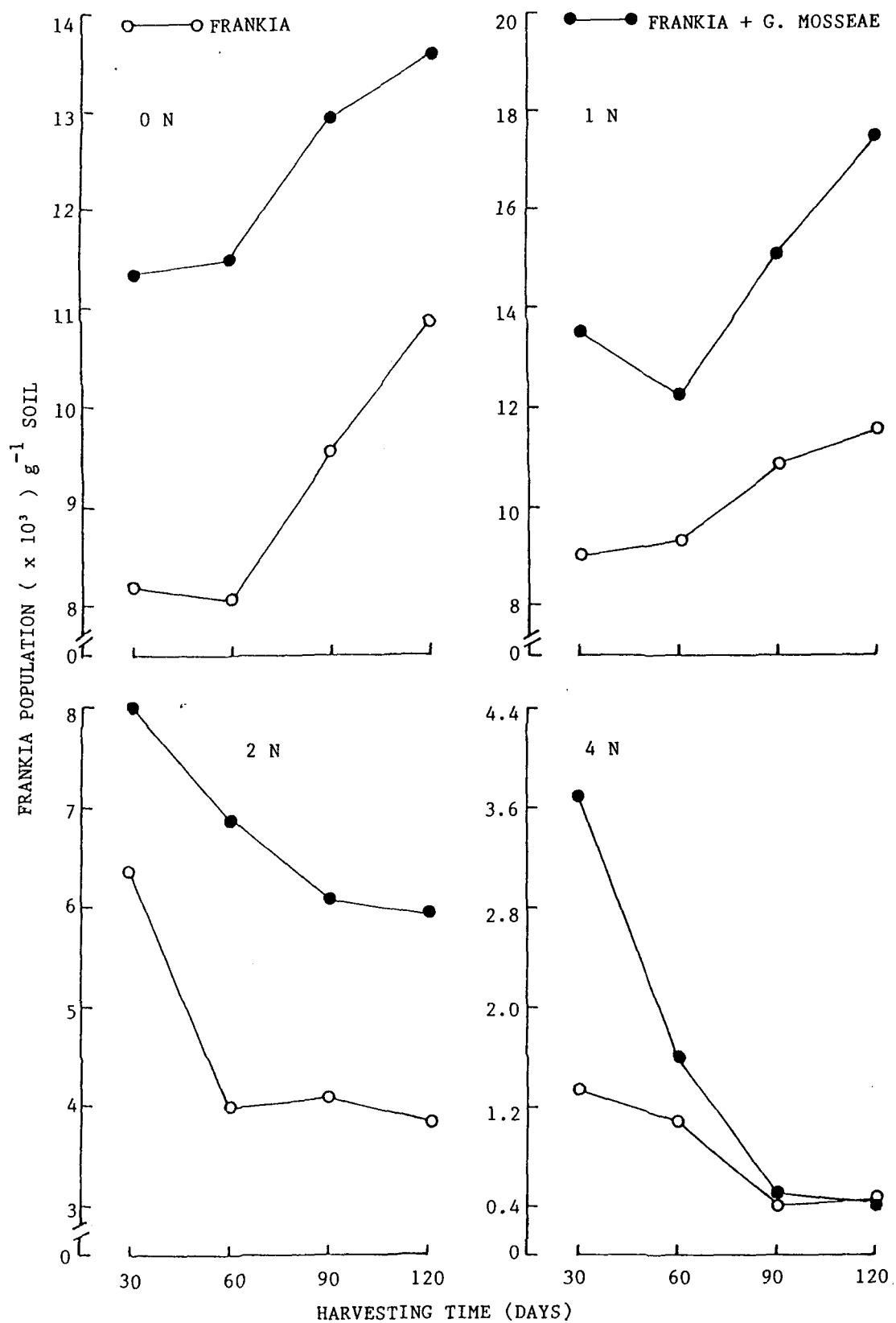


Fig.59. Population of *Frankia* in the rhizosphere soil of *A. nepalensis* inoculated with *Frankia* and *Frankia* + *G. mosseae* at various levels of soil organic matter.

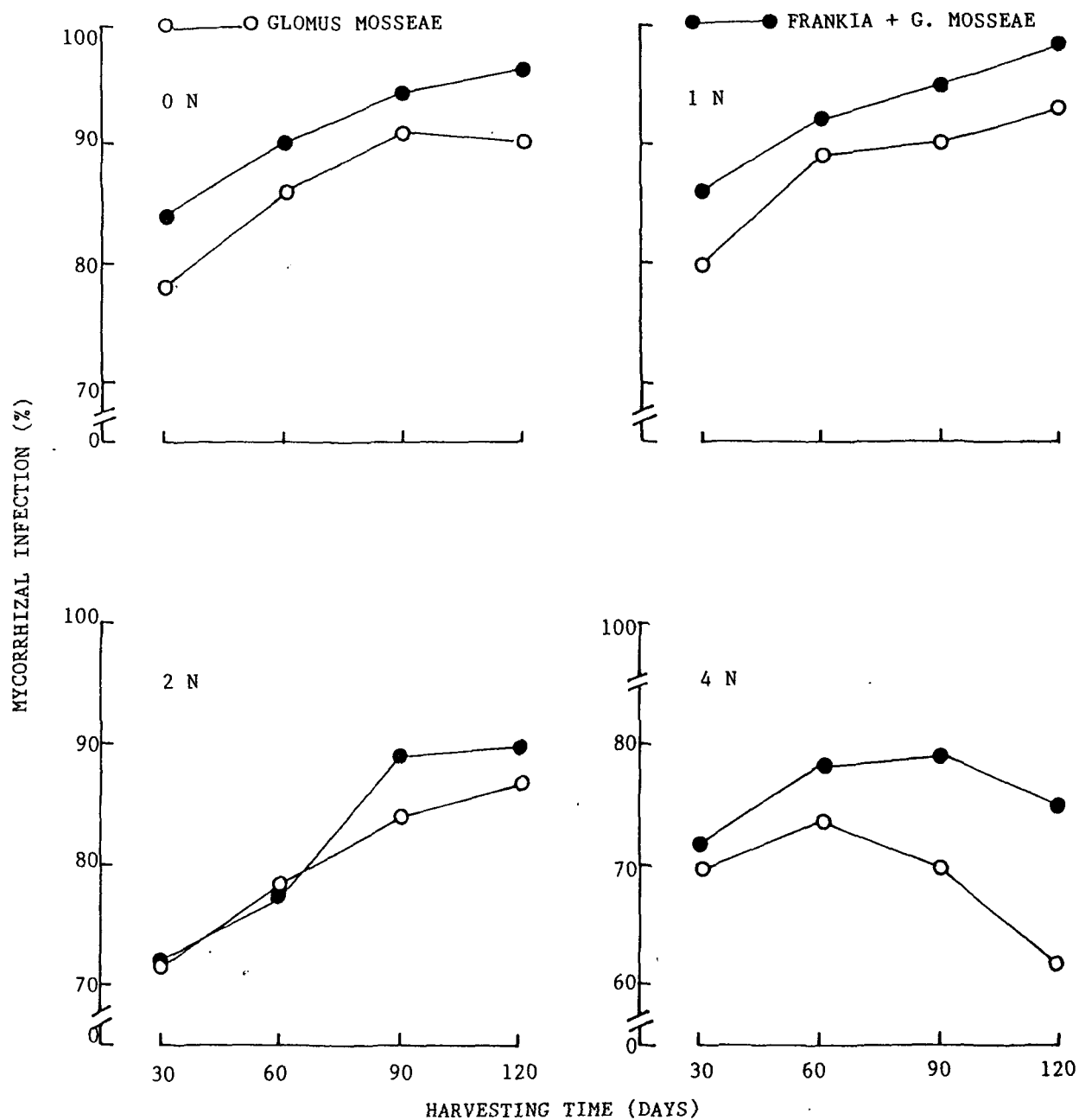


Fig. 60. Effect of *G. mosseae* and *Frankia + G. mosseae* on the mycorrhizal association (%) of *A. nepalensis* seedlings grown at different levels of soil organic matter

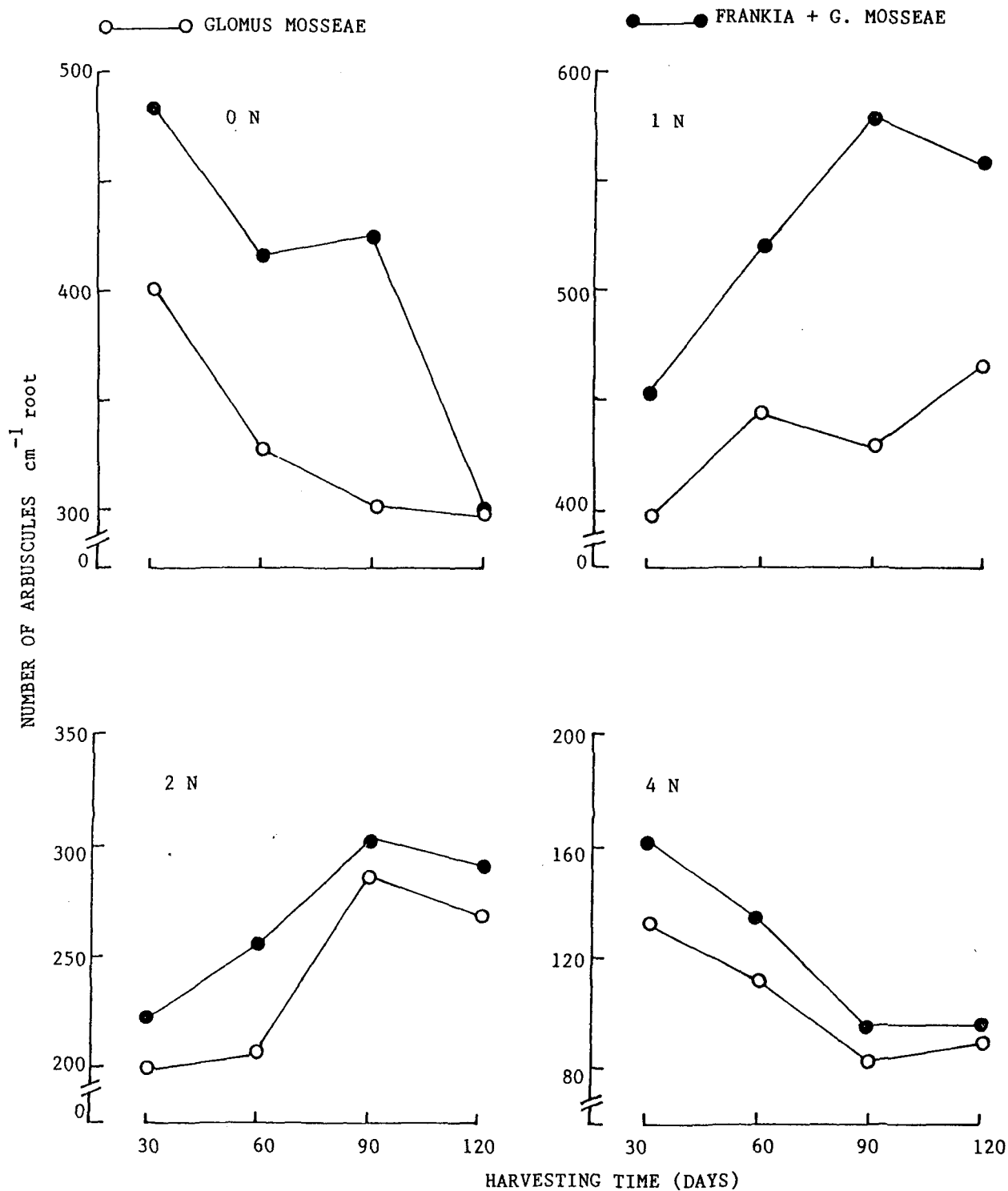


Fig.61. Number of arbuscules in mycorrhizal root of *A. nepalensis* seedlings grown at different levels of organic matter.

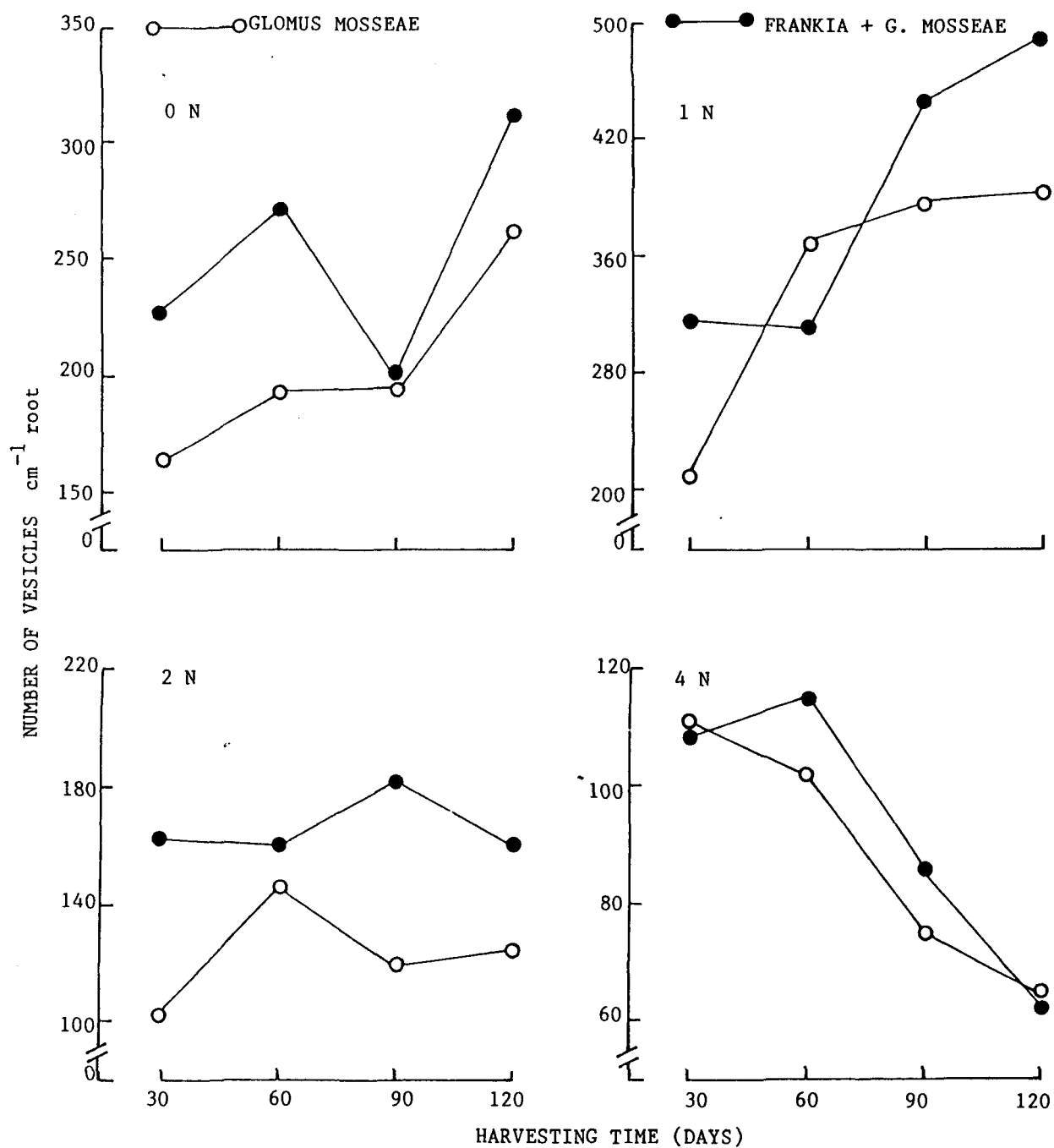


Fig.62. Number of vesicles in mycorrhizal root of *A. nepalensis* inoculated with *G. mosseae* and *Frankia + G. mosseae* at various levels of organic matter.

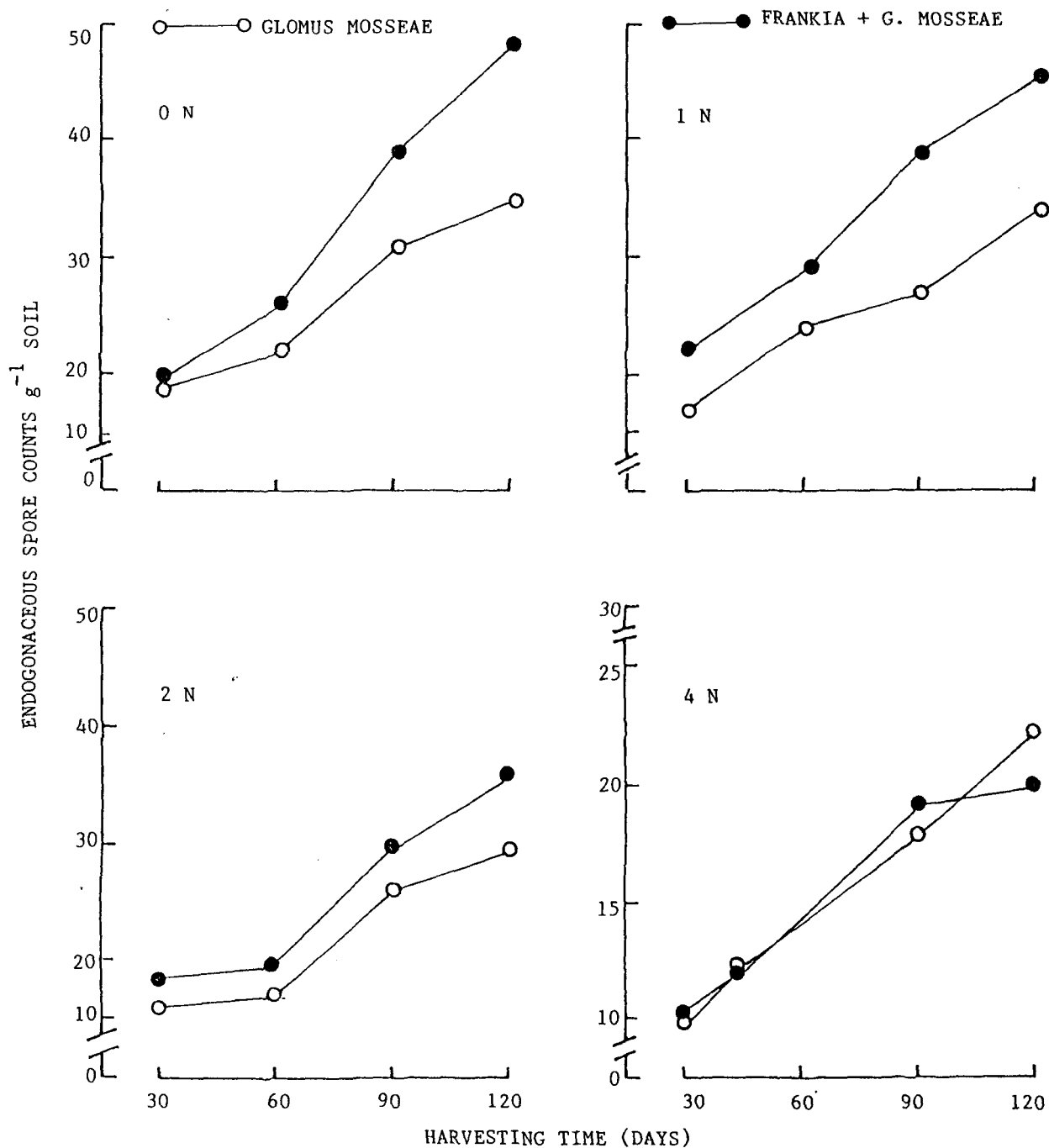


Fig.63. Endogonaceous spore population in the rhizosphere soil of *A. nepalensis* inoculated with *G. mosseae* and *Frankia* + *G. mosseae* at various levels of soil organic matter.

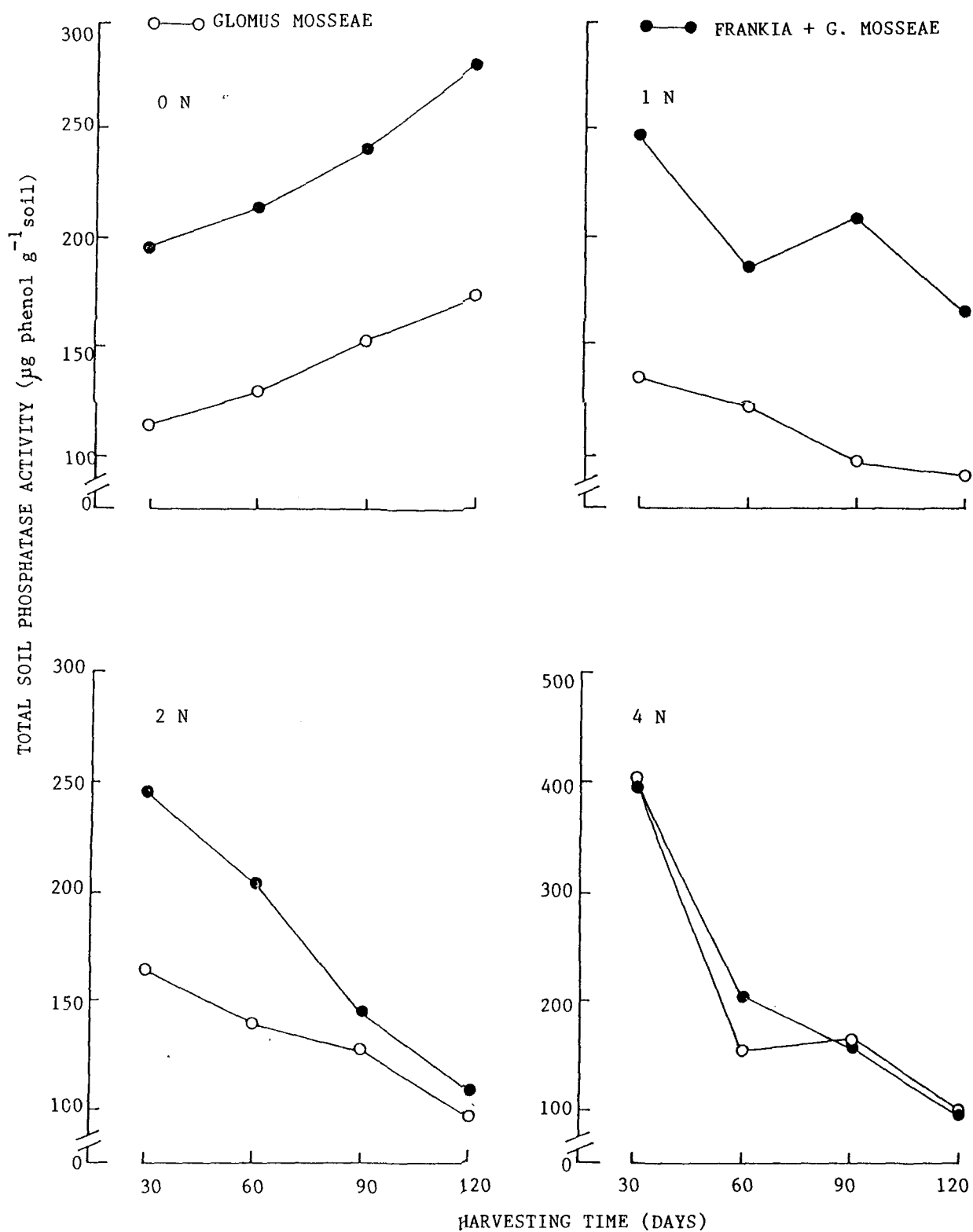


Fig.64. Variation in total phosphatase activity in rhizosphere soil of *A. nepalensis* seedlings inoculated with *G. mosseae* and *Frankia + G. mosseae* at various levels of soil organic matter.

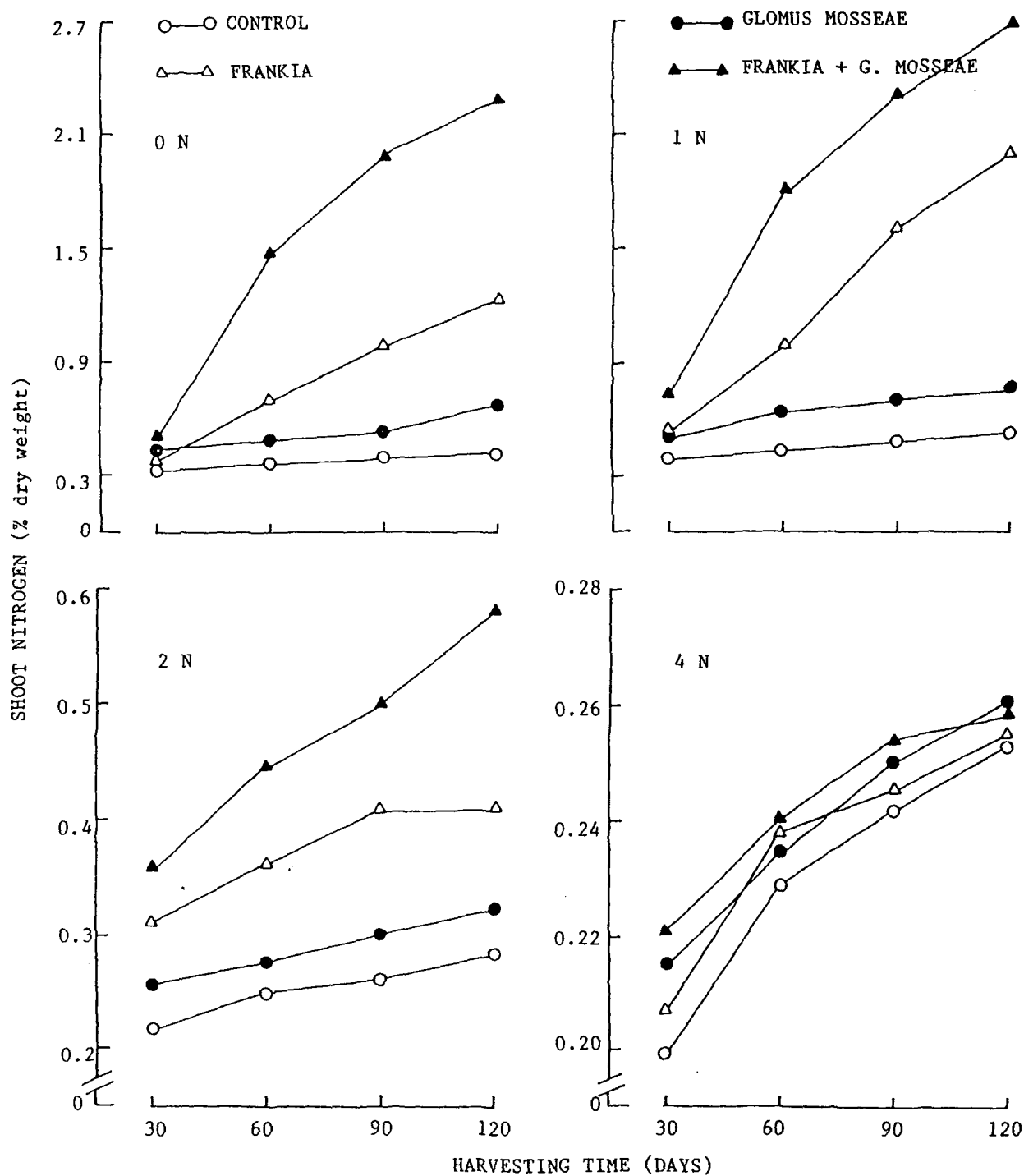


Fig.65aEffect of Frankia, G. mosseae and Frankia + G. mosseae on shoot nitrogen content of A. nepalensis seedlings at different levels of soil organic matter.

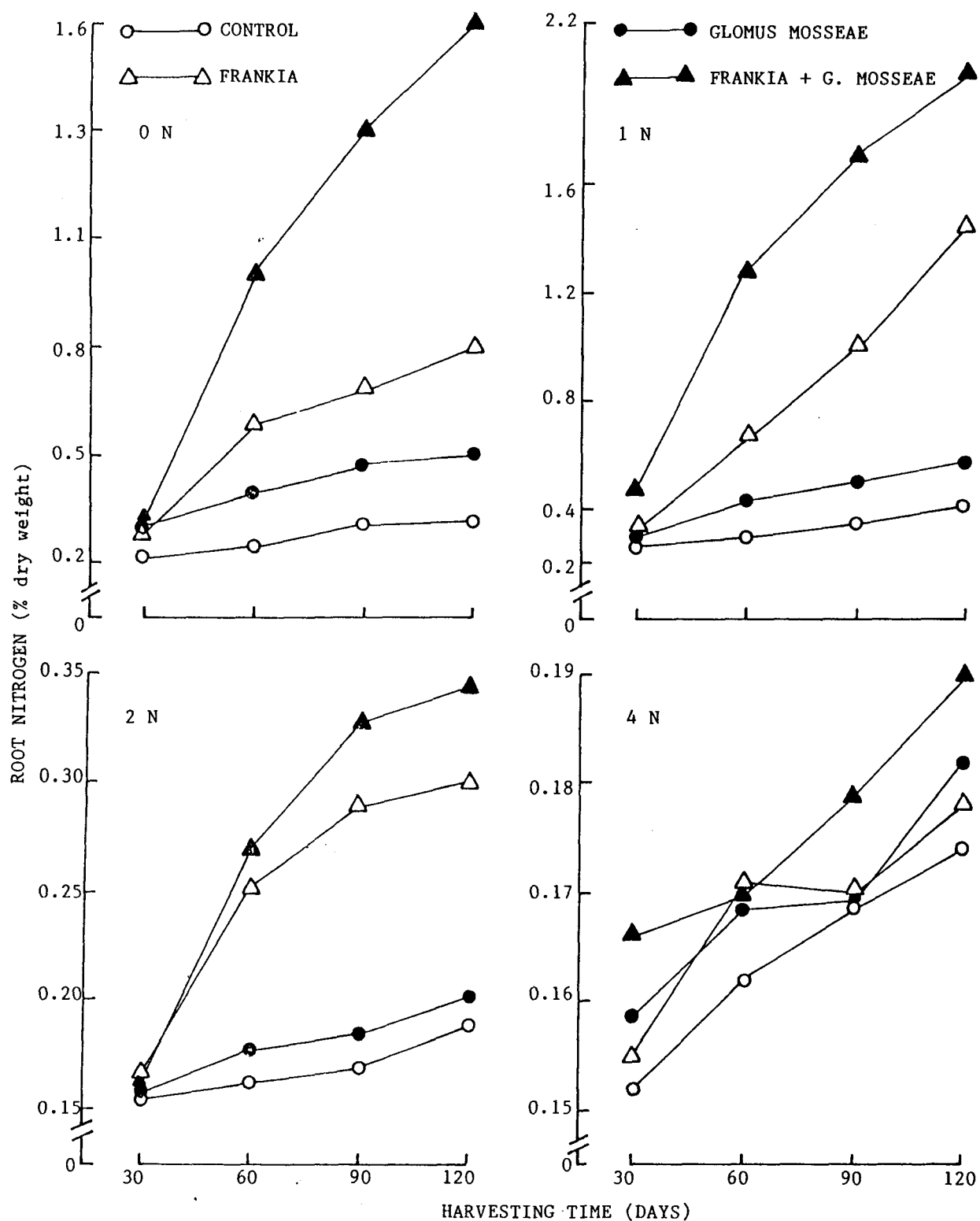


Fig.65b. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root nitrogen content (%) of *A. nepalensis* seedlings at different levels of soil organic matter.

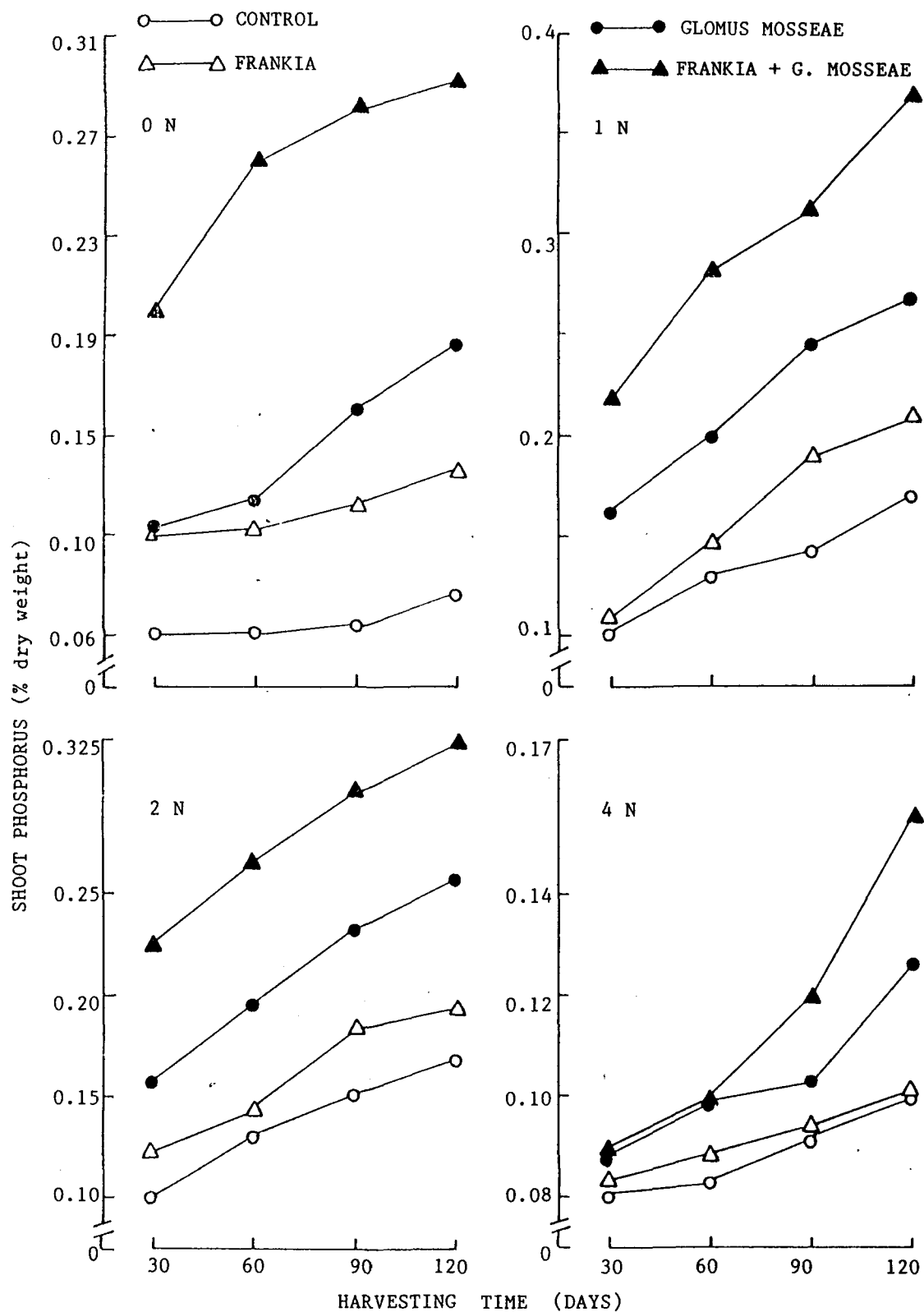


Fig.66a. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on shoot phosphorus (%) content of *A. nepalensis* seedlings grown at different levels of organic matter.

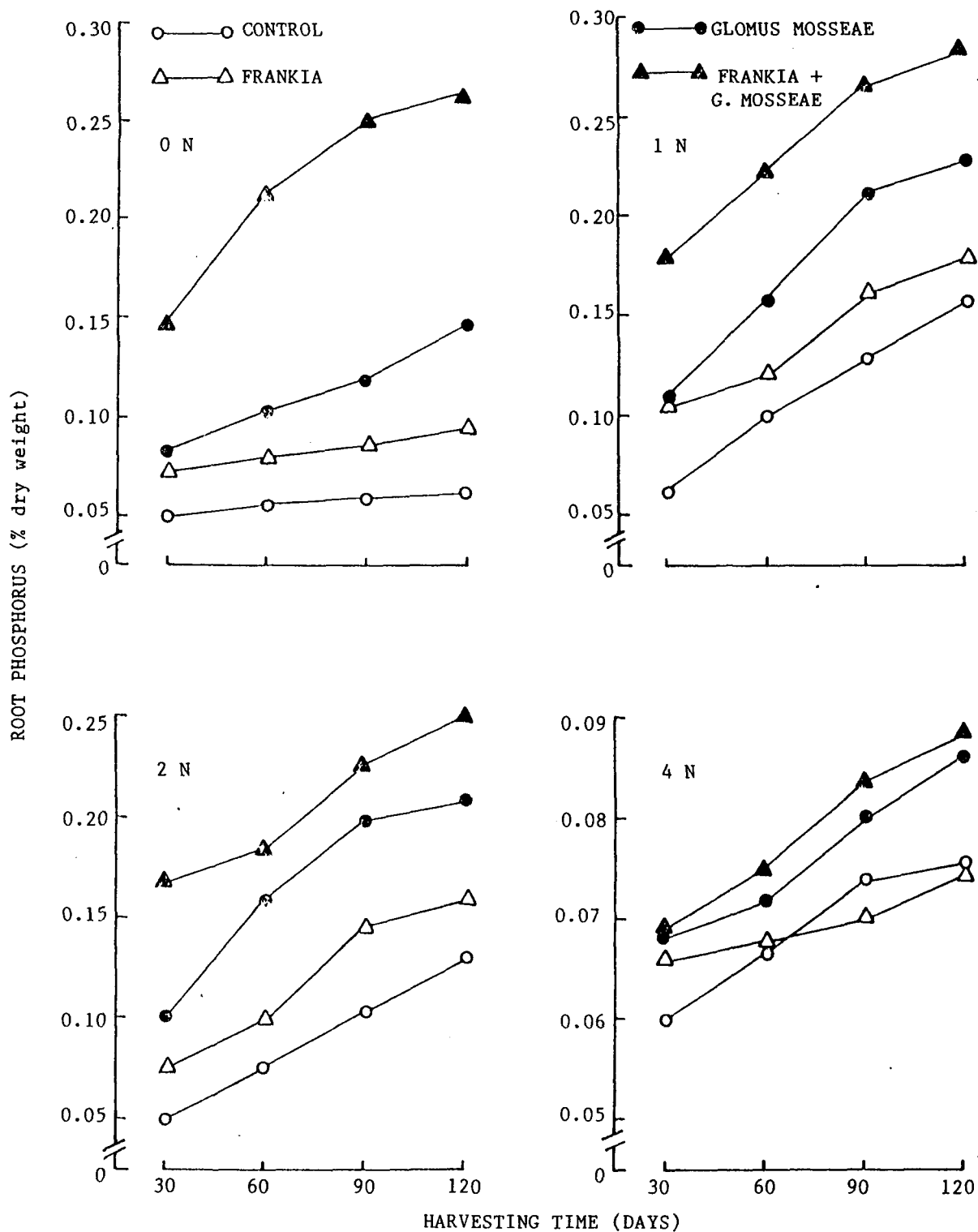


Fig.66b. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root phosphorus content (%) of *A. nepalensis* seedlings grown at different levels of organic matter in soil.

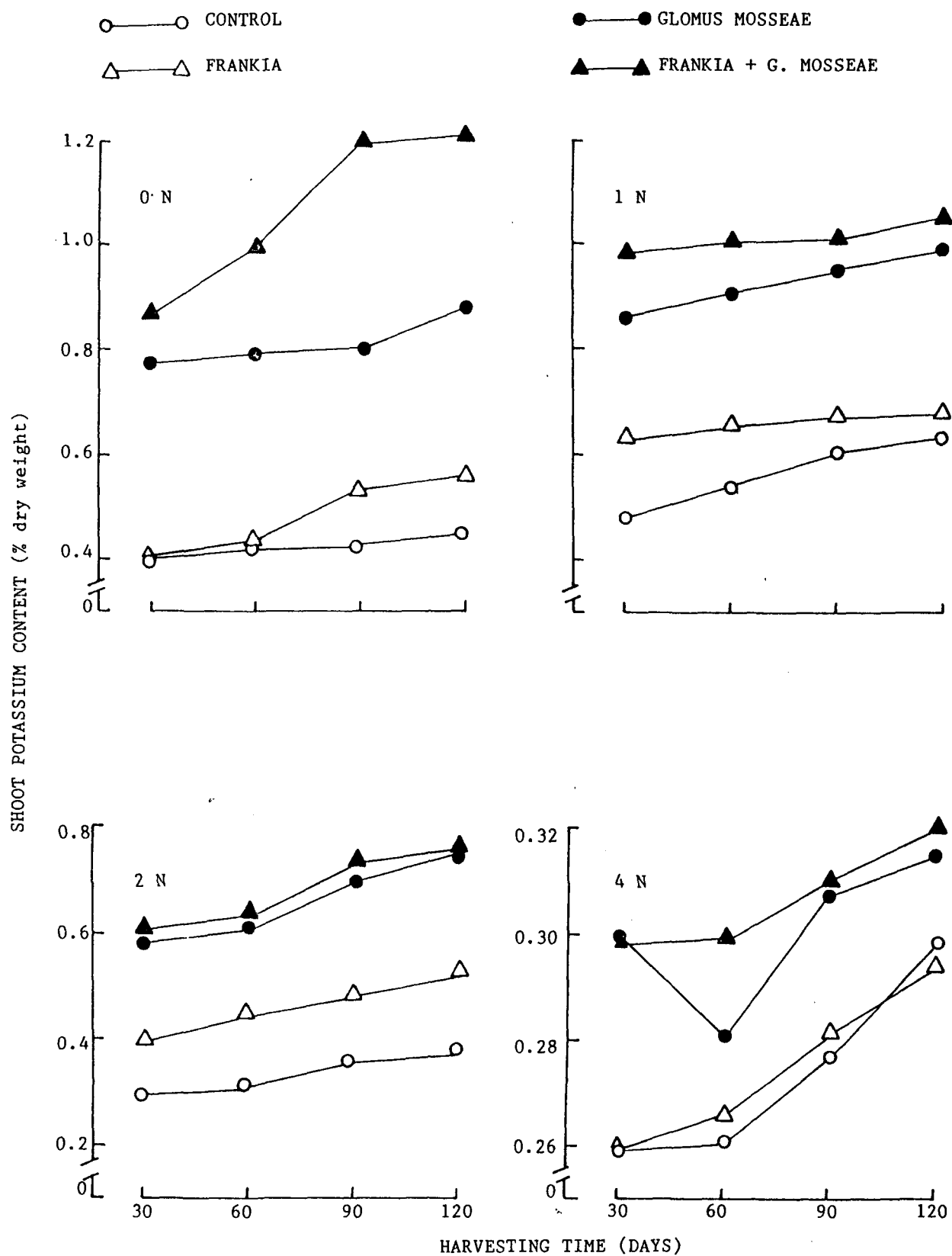


Fig.67a. Effect of Frankia, G. mosseae and Frankia + G. mosseae on shoot potassium content of A. nepalensis seedlings at various levels of soil organic matter.

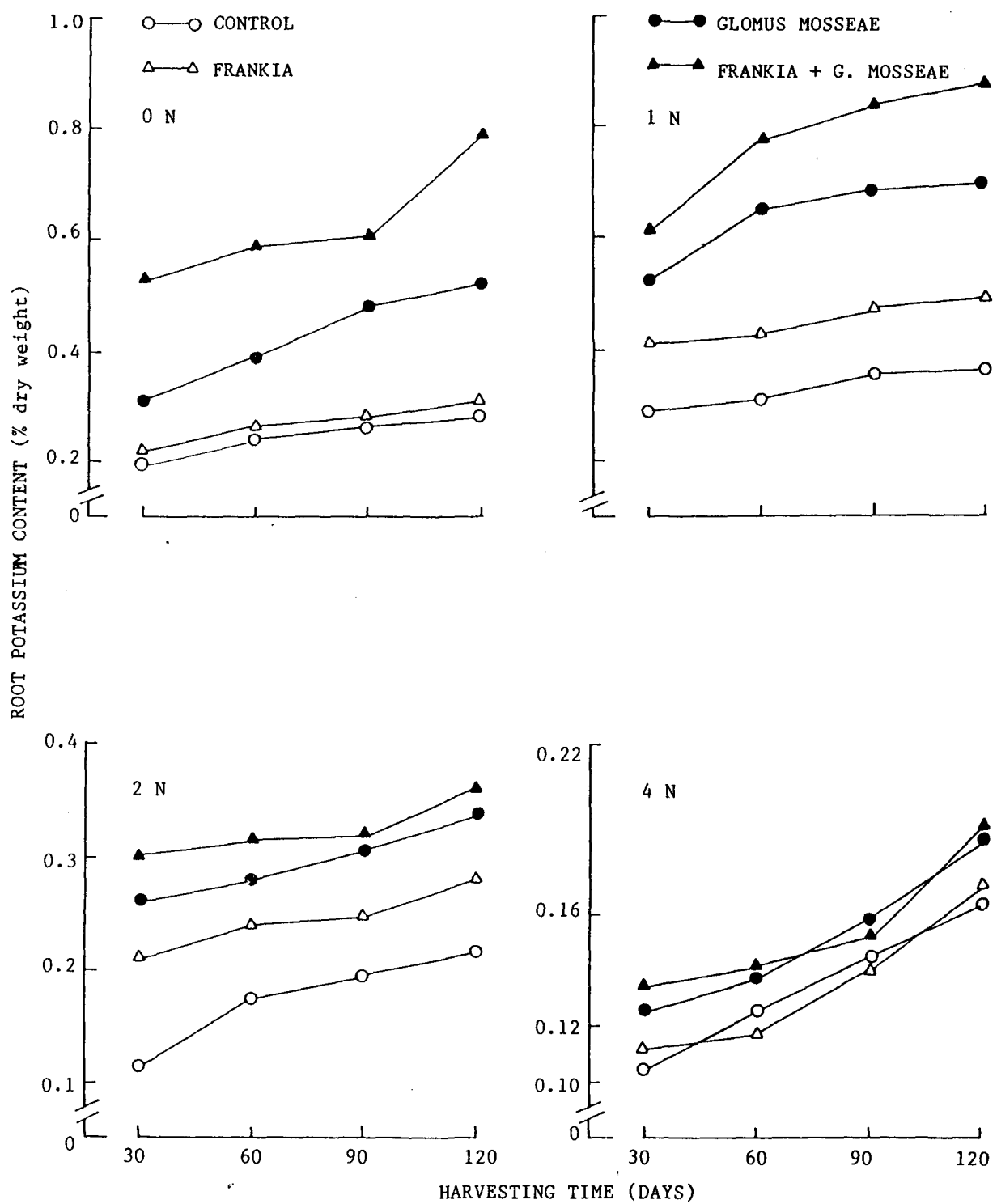


Fig.67b. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root potassium content (%) of *A. nepalensis* at different levels of organic matter.

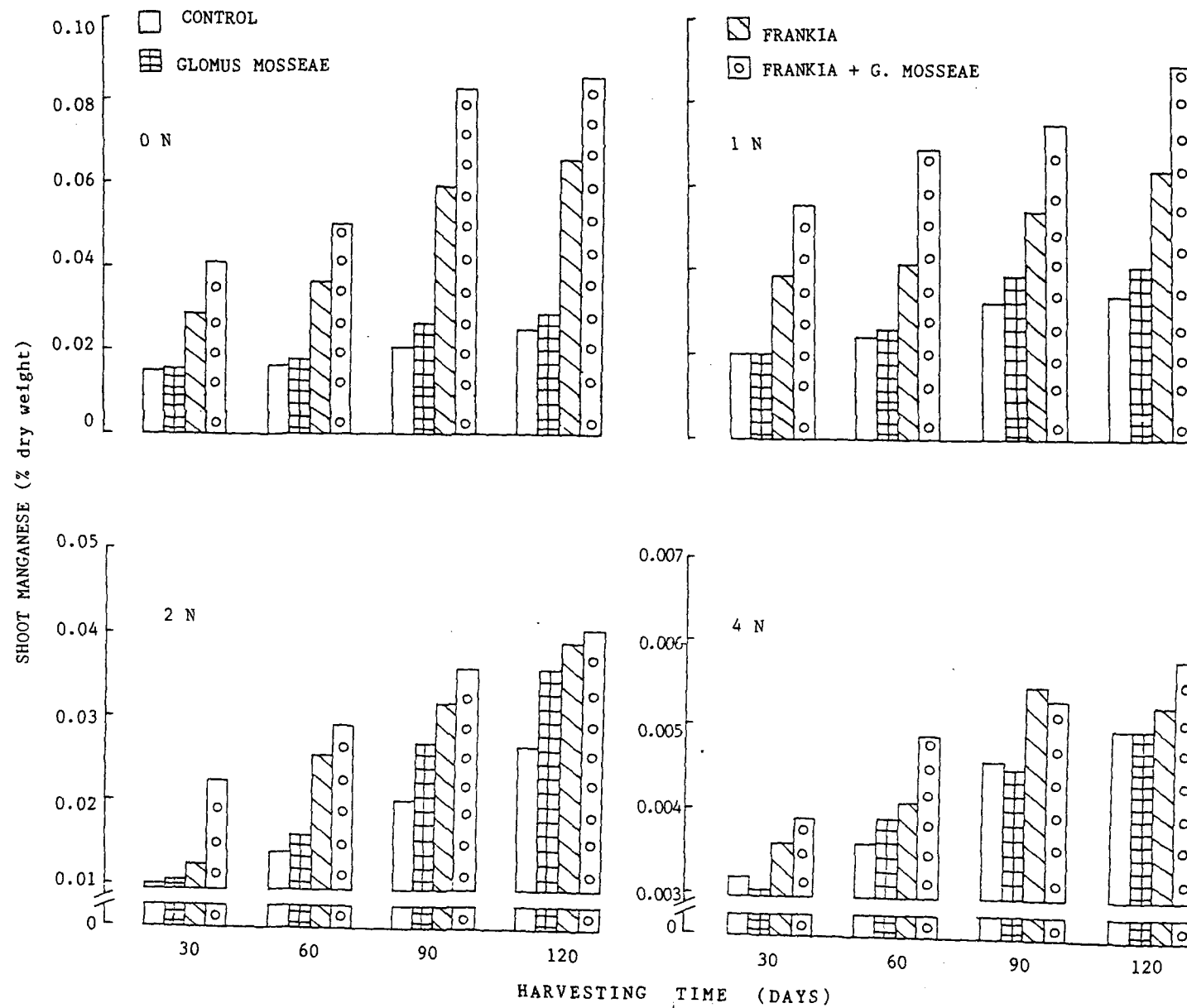


Fig.68a, Effect of *Frankia*, *G. mosseae* and *Frankia + G. mosseae* on shoot manganese content (%) of *A. nepalensis* seedlings at various levels of soil organic matter.

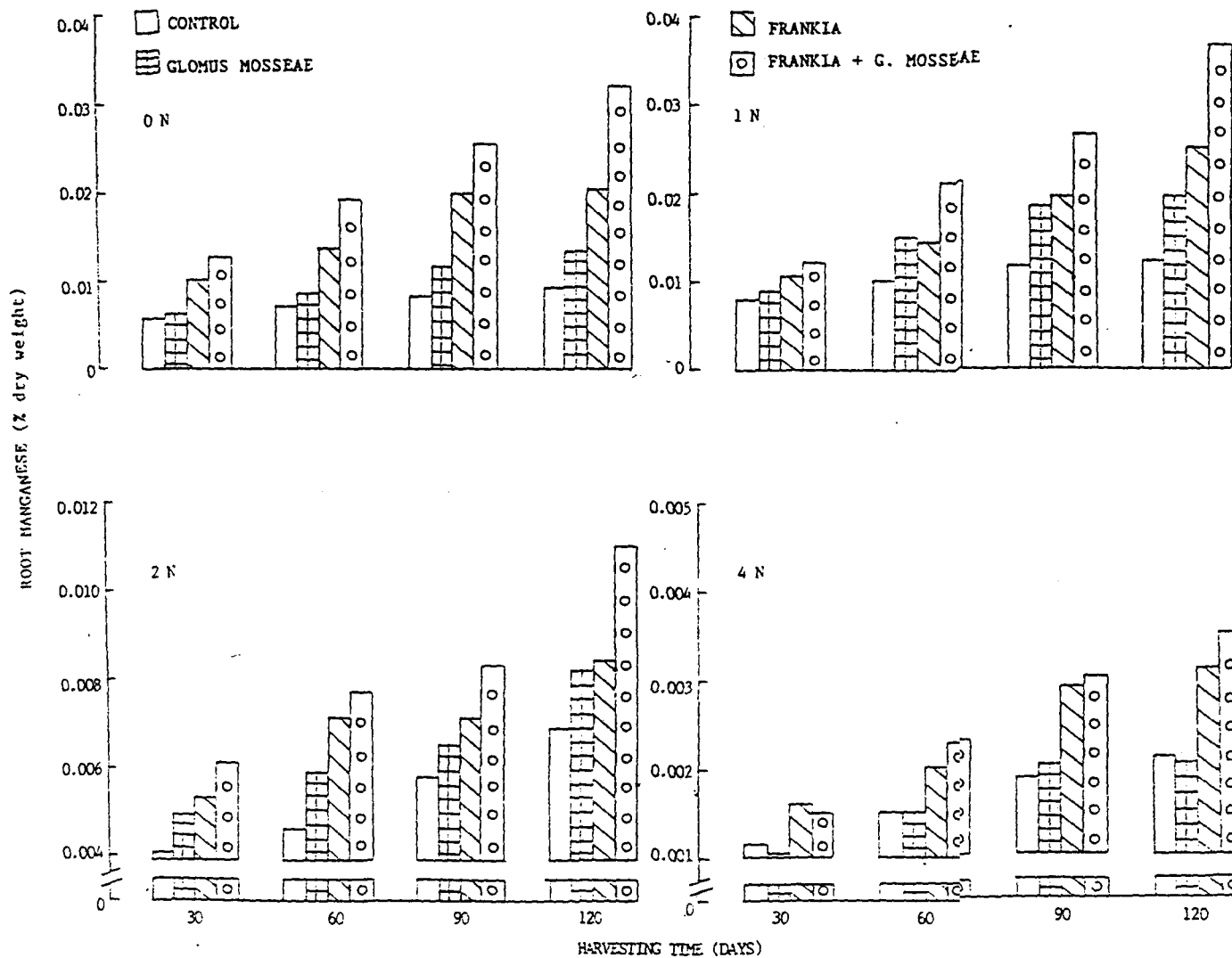


Fig.68b. Effect of *Frankia*, *G. mosseae* and *Frankia + G. mosseae* on root manganese content (%) of *A. nepalensis* seedlings at various levels of organic matter.

68b).

Nutrient contents of both shoot and root tissues were markedly influenced by organic matter levels, harvests and microsymbionts (Table 28).

A significant correlation was established between mycorrhizal infection and nodule number, nodule dry weight, nitrogenase activity and *Frankia* population at low soil organic matter levels (0-1 N) except at 2 N and 4 N doses where mycorrhizal infection was negatively correlated with *Frankia* population (Table 29).

Positive relationships was established between nitrogenase activity and mycorrhizal infection and endogonaceous spore number at all organic matter levels (Table 30). However, nitrogenase activity was correlated insignificantly with arbuscule and vesicle populations (Table 30).

Discussions :

The improved growth of mycorrhizal + nodulated plants in soils amended with organic matter may be attributed

Table 22. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on dry weight of shoot (mg seedling⁻¹) of *A. nepalensis* seedling grown at various levels of organic matter.

Soil organic matter	Harvesting period (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	2.5	3.1	4.1	5.9	4.0	7.1	9.5	14.2	6.3	10.8	14.5	22.8	8.8	14.5	22.0	29.1
1	3.8	4.7	6.2	10.3	6.4	12.3	14.5	28.1	11.5	19.6	24.0	41.0	17.2	28.7	36.0	60.1
2	2.6	4.2	5.1	6.8	4.4	7.3	9.1	12.6	6.2	10.6	12.2	20.5	8.5	13.8	20.6	29.5
4	2.1	2.6	3.8	4.1	2.5	3.0	5.1	5.6	2.7	3.4	5.7	6.9	3.2	4.0	7.2	8.2

1 = Control 2 = *Frankia* 3 = *G. mosseae* & 4 = *Frankia* + *G. mosseae*

Table 23. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root dry weight (mg seedling⁻¹) of *A. nepalensis* seedlings grown at various levels of organic matter.

Soil organic matter	Harvesting periods (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	0.9	0.9	1.2	1.6	1.6	2.5	2.8	3.9	2.8	3.8	4.1	6.2	4.1	5.4	6.3	7.8
1	1.0	1.3	1.4	2.5	2.3	3.5	3.9	5.9	3.5	5.4	5.0	8.1	5.0	8.0	7.6	11.2
2	0.8	1.3	1.5	2.0	1.6	2.4	3.0	4.0	2.4	3.7	3.9	6.2	3.3	5.3	6.5	8.9
4	0.7	0.9	1.2	1.5	0.9	1.1	1.8	1.8	1.1	1.2	1.9	2.3	1.3	1.5	2.4	2.7

1 = Control 2 = *Frankia* 3 = *G. mosseae* & 4 = *Frankia* + *G. mosseae*

Table 24. Effect of *Frankia*, *Glomus mosseae* and *Frankia* + *G. mosseae* on plant dry weight (mg seedling⁻¹) of *A. nepalensis* grown at various levels of organic matter.

Soil organic matter	Harvesting period (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	3.4	4.0	5.3	7.5	5.6	9.6	12.3	18.1	9.1	14.6	18.6	29.0	12.9	19.9	28.4	36.9
1	4.8	6.0	7.6	12.8	8.7	15.8	18.4	34.0	15.0	25.0	29.0	49.1	22.2	36.7	43.6	71.3
2	3.5	5.5	6.6	8.8	6.1	9.7	12.1	16.7	8.6	14.4	16.1	26.7	11.8	19.1	27.5	38.4
4	2.9	3.6	5.0	5.7	3.4	4.2	6.9	7.4	3.8	4.6	7.7	9.2	4.5	5.6	9.7	10.9

1 = Control 2 = *Frankia* 3 = *G. mosseae* and 4 = *Frankia* + *G. mosseae*

Table 25 F values of three way ANOVA test for plant growth characteristics (shoot length, root length, plant height, shoot weight, root weight, plant weight, R/S, stem diameter, leaf number, leaf area) as affected by organic matter level, treatments and harvest.

Sources of variation	Shoot length	Root length	Plant height	Shoot weight	Root weight	Plant weight	R/S	Stem diameter	Leaf number	Leaf area
Organic Matter	137.17 ^{††}	258.58 ^{††}	225.99 ^{††}	112.73 ^{††}	202.66 ^{††}	129.00 ^{††}	95.84 ^{††}	348.49 ^{††}	156.85 ^{††}	103.37 [†]
Harvests	92.84 ^{††}	71.26 ^{††}	107.66 ^{††}	108.33 ^{††}	363.35 ^{††}	141.75 ^{††}	3.74 [†]	236.75 ^{††}	165.42 ^{††}	86.20 ^{††}
Symbionts	98.66 ^{††}	131.67 ^{††}	140.66 ^{††}	84.51 ^{††}	147.39 ^{††}	97.37 ^{††}	53.92 ^{††}	324.92 ^{††}	166.66 ^{††}	163.36 ^{††}
OM x H	12.67 ^{††}	12.17 ^{††}	15.34 ^{††}	14.60 ^{††}	28.57 ^{††}	16.83 ^{††}	1.08	15.53 ^{††}	13.14 ^{††}	8.45 ^{††}
OM x S	9.51 ^{††}	13.58 ^{††}	14.15 ^{††}	9.47 ^{††}	8.20 ^{††}	9.61 ^{††}	7.16 ^{††}	34.66 ^{††}	6.6 ^{††}	11.42 ^{††}
H x S	8.11 ^{††}	2.09 [†]	6.62 ^{††}	7.35 ^{††}	9.66 ^{††}	8.06 ^{††}	0.92	10.18 ^{††}	3.50 ^{††}	6.34 ^{††}

†, †† Significant at P<0.05 and 0.01 levels respectively.

Without asterisk statistically insignificant at these levels.

Table 26. F values of three way ANOVA test for nodule number, nodule weight, nitrogenase activity, *Frankia* population as affected by organic matter levels, symbionts and harvests.

Sources of variation	Nodule number	Nodule weight	Nitrogenase activity	<i>Frankia</i> population
Organic matter	45.13* *	33.97* *	65.93* *	678.04* *
Harvests	14.57* *	14.32* *	16.58* *	12.60* *
Symbionts	3.20	18.30* *	27.11* *	138.66* *
OM x H	15.61* *	4.32*	4.97*	17.03* *
OM x S	2.71	6.57*	11.30* *	8.85* *
H x S	1.81	2.60	2.35	1.51

*, * * Significant at $P < 0.05$ and 0.01 levels respectively.
Without asterisk statistically insignificant at above levels.

Table 27. F. values of three way ANOVA test for VAM infection, arbuscules and vesicles and endogonaceous spore population and total soil phosphatase activity as influenced by soil organic matter levels, symbionts and harvests.

Sources of variation	VAM infection	Arbuscule number	Vesicle number	Endogonaceous spores number	Phosphatase activity
Organic matter	145.61* *	595.63* *	141.30* *	101.28* *	20.48* *
Harvests	45.57* *	1.94	6.85*	131.93* *	40.30* *
Symbionts	44.18* *	81.81* *	9.34* *	46.49* *	78.71* *
OM x H	8.80* *	17.19* *	6.24*	2.75	24.60* *
OM x S	2.80	9.70* *	0.91	7.39* *	9.36* *
H x S	2.20	1.40	0.91	3.45	0.29

*, * * Significant at $P < 0.05$ and 0.01 levels respectively.

Without asterisk statistically insignificant at above levels.

Table 28. F values of three way ANOVA for nutrient contents of root and shoot (nitrogen, phosphorus, potassium and manganese) as influenced by organic matter levels, symbionts and harvests.

Sources of variation	Shoot nitrogen	Root nitrogen	Shoot phosphorus	Root phosphorus	Shoot potassium	Root potassium	Shoot manganese	Root manganese
Organic matter	56.35 ^{† †}	53.40 ^{† †}	545.22 ^{† †}	196.76 ^{† †}	188.15 ^{† †}	568.88 ^{† †}	230.76 ^{† †}	2.38
Harvests	14.49 ^{† †}	15.18 ^{† †}	215.97 ^{† †}	94.24 ^{† †}	12.93 ^{† †}	42.00 ^{† †}	46.82 ^{† †}	3.55 [†]
Symbionts	36.88 ^{† †}	28.33 ^{† †}	746.44 ^{† †}	239.94 ^{† †}	27.10 ^{† †}	233.42 ^{† †}	105.82 ^{† †}	3.76 [†]
OM x H	3.44 ^{† †}	3.87 ^{† †}	11.22 ^{† †}	8.66 ^{† †}	1.19	2.81 [†]	4.77 ^{† †}	1.10
OM x S	9.85 ^{† †}	7.48 ^{† †}	56.21 ^{† †}	26.08 ^{† †}	16.20 ^{† †}	40.38 ^{† †}	20.07 ^{† †}	1.18
H x S	3.18 ^{† †}	2.95 ^{† †}	7.25 ^{† †}	2.05 [†]	1.47	1.41	1.36	3.02 [†]

†, † † Significant at P<0.05 and 0.01 levels respectively.

Without asterisk statistically insignificant at above levels.

Table 29 : Correlation Coefficients (r) between mycorrhizal infection and nodule number, nitrogenase activity, nodule biomass and *Frankia* population at various levels of organic matter.

Sources of Variation	Organic matter level	Nodule number	Nitrogenase activity	Nodule dry weight	<i>Frankia</i> Population
Mycorrhizal infection(%)	0 N	0.999**	0.958*	0.930	0.910
	1 N	0.975*	0.994**	0.976*	0.730
	2 N	0.901	0.992**	0.994**	-0.941
	4 N	-	-	-	-0.740

*,** Significant at P<0.05 and 0.01 levels respectively.
Without asterisk statistically insignificant at above levels.

Table 30. Correlation coefficients (r) between nitrogenase activity and mycorrhizal infection, arbuscule, vesicle and spore populations at different levels of soil organic matter.

Sources of variation	Soil organic matter levels	Mycorrhizal infection	Arbuscule number	Vesicle number	Spore number
Nitrogenase activity	0 N	0.958*	- 0.905	0.466	0.997* *
	1 N	0.994* *	0.993* *	0.894	0.990* *
	2 N	0.992* *	0.086	0.347	0.965*

*, * * Significant at $P < 0.05$ and 0.01 levels respectively.

Without asterisk statistically insignificant at these levels.

to the role of mycorrhizal fungi in nutrient cycling and their uptake thereby reducing the loss of nutrients by leaching (Fogel and Hunt, 1979; Fogel, 1980). Poor performance of nonmycorrhizal seedlings may be assigned to the absence of fungal symbiont which might have resulted in inability of these plants to mobilize and transfer nutrients from the soil.

In the present investigation, improved growth of seedlings in dually inoculated sets was achieved due to improved acquisition of N, P, K and Mn which are directly or indirectly associated with host growth. Similarly, Hayman (1978) Abbott and Robson (1982) also attributed vigorous growth of mycorrhizal plants to efficient uptake of nutrients especially P by mycorrhizal fungi. The role of mycorrhizal fungi in conserving nutrients in their tissues and their release to the host in times of deficiency has also been correlated to the improved plant growth (Trappe and Fogel, 1977). In the absence of mycorrhizal fungi, the immobilization and transfer of nutrients from decomposing soil organic substances to plant roots could considerably be reduced. In fact, mycorrhiza has also been assigned to have role in mineralization of N (Jayachandran *et al.*, 1992) thus may short circuit elemental cycle. These features may have added to optimal growth of plants with dual symbionts under natural

conditions.

The suppressed growth of seedlings at higher level of soil organic matter may be related to release of metabolically active phenolic and acidic compounds from organic matter as well as supra-optimal levels of nutrients which additively may have rendered root symbionts as well as symbiosis ineffective. In fact, the very survival of root endophytes in acidic conditions may have been severely affected at high organic matter level. The poor growth of other *Alnus* species with or without simbionts under acidic peat has also been demonstrated (Hooker, 1987).

Decreased nodulation, nodule biomass and nitrogenase activity at higher level of organic matter may be attributed to higher concentration of nitrogenous compounds in soil which may have rendered *Frankia* symbiosis ineffective. Kohls and Baker (1982) also indicated that exogenous nitrate supply reduced nodulation and nitrogen fixation. In fact at 4 N level the total absence of nodules indicates that *Frankia* propagules might have been rendered dead in soil by phenolic action (Pedersen *et al.*, 1992) or highly acidic pH (Smolander *et al.*, 1988) which is elev_ated by the presence of higher concentration of combined N released from organic matter thus preventing the formation of root nodules (Bond, 1956; Gardner

and Bond, 1957). Further, deleterious effect of phenolics and soil pH on survival, colonization and efficiency of VA endophyte, which is associated with synergistic behaviour on former may have added to depressed nodule characteristics. Decreasing trend in the nitrogen fixing activity of nodule with higher organic matter may be due to inhibition of nitrogenase by combined N (Stewart and Bond, 1961; Bond and Macintosh, 1975; Ingestad, 1980). Besides, at low soil organic matter level, the nodulation, nodule biomass and nitrogenase activity were markedly stimulated by *G. mosseae*. This stimulation may have been accomplished by P mediated effects (Smith *et al.*, 1985) or altered supply of hormones and proteins (Munns and Mosse, 1980; Manjunath and Bagyaraj, 1984; Young *et al.*, 1972; Krishna and Bagyaraj, 1983) as evidenced in leguminous systems. Fraga-Beddiar and Tacon (1991) revealed a synergistic effect of VAM fungus on *Frankia* which may be mediated by unknown mechanism. The ameliorative effect of *G. mosseae* on *Frankia* abundance may be brought about by qualitative and quantitative alteration in the root exudates of mycorrhizal plants (Gitte *et al.*, 1978).

Reduction in mycorrhizal colonization and number of arbuscules, vesicles and spores at higher organic matter amendments may be attributed to supraoptimal levels of

nutrients, release of toxic and phenolic compounds (Pedersen *et al.*, 1981). Sharp decline in soil pH resulting due to release of organic acids might have prevented germination of mycorrhizal spores (Green *et al.*, 1976) thus affecting infection and affectivity of mycorrhizal fungus. This leads to hampered plant growth (Mosse, 1973; Arines and Vilarino, 1990). However, at low level of organic matter, the stimulation of mycorrhizal infection by *Frankia* in dually inoculated seedlings was observed as noted by Berlinger and Torrey (1989). *Frankia* associated with roots, may also be involved in altering hormones qualitatively and quantitatively (Wheeler *et al.*, 1979) thus favouring VAM colonization and activity. Further, direct stimulation of VA endophyte by *Frankia* may be operative by the production of polysaccharides or other active compounds as noted in leguminous systems (Azcon *et al.*, 1980; Paccini *et al.*, 1988). This synergistic behaviour may be important in the uptake of non-labile nutrients which consequently improved nodulation, nitrogen fixation and plant growth.

The stimulation in the production of arbuscules by *G. mosseae* inoculated plants under nutrient deficient conditions may meet the high demand of P of nitrogen fixing organisms. This is an ecological advantage to nodulated + mycorrhizal plants over those with single symbiosis or without it especially under nutrient-depleted soil conditions where

latter groups of plants suffer deficiency of essential elements consequently leading to poor performance by them.

Further, the production of endogonaceous spores by *G. mosseae* in *Alnus-Frankia-G. mosseae* symbiosis was enhanced at lower level of organic matter which may be attributed to stimulatory activity of *Frankia*. The altered metabolic activity of VAM fungus as induced by former may be functional. This synergistic effect may be mediated by alteration of hormones by *Frankia* (Wheeler *et al.*, 1979) or other unknown mechanism. This increases the possibility of infecting more plant roots thereby ensuring the supply of P, which is a critical factor for nitrogen fixation (Asai, 1944) and plant growth.

The detection of phosphatase activity in mycorrhizal and nodulated + mycorrhizal seedlings and its absence in nonmycorrhizal *Frankia* and uninoculated control counterparts signifies that the enzyme may possibly be fungal specific (Gianinazzi-Pearson and Gianinazzi, 1978; Tisserant *et al.*, 1993). The ecological implication of this may be important in the acquisition and transfer of P to nitrogen fixers (Asimi *et al.*, 1980). Improved phosphatase activity in nodulated mycorrhizal plants as compared to mycorrhizal ones may be attributed to synergistic effect of *Frankia* on *G. mosseae* activity by providing organic nitrogen to the latter

which in return renders more P for nitrogen fixation and plant growth. Phosphatase activity of soil increased at beginning after amendment of organic matter and gradually decreased with time. This observation is supported by the findings of Appiah *et al.*, (1964) and Vlasyuk and Lisoval (1964). Further a reduction in the activity at high organic matter level may be related to detrimental effect of phenolics and acid substances on mycorrhizal fungus, *G. mosseae* which is responsible for enzyme activity. Increase in the level of P with increased application of organic matter may also have contributed to it.

Generally nutrient contents i.e. N, P, K and Mn in the shoot and root tissues of nodulated + mycorrhizal plants were markedly higher than singly or non-inoculated plants at all levels of organic matter, which may be assigned to greater exploration of soil volume and uptake of nutrients by mycorrhizal symbiont. Increased acquisition of nutrients such as N, P and Ca in tripartite symbioses has previously been demonstrated (Rose and Youngberg, 1981; Gardner *et al.*, 1984; Russo, 1989; Jha *et al.*, 1993). This combined effect of symbiotic root endophytes in the acquisition of nutrients in nutrient (organic matter) depleted soil conditions may be instrumental in the improved survival and better performance of plants with dual symbioses (Harley, 1969; Daft and Hacskeylo, 1976).

Chapter	:	Interaction between <i>Frankia</i> and <i>Glomus mosseae</i> on
VI	:	P uptake, nodulation, nitrogen fixation and their
	:	influence on seedling growth of <i>Alnus nepalensis</i>
	:	at different levels of rock phosphate amended
	:	soil.

Introduction :

Phosphorus is the most important nutrient for growth, nodulation (McLacelan and Norman, 1961; Gibson, 1976) and nitrogen fixation by legumes (Demeterio *et al.*, 1972). However, in natural conditions P forms the most limiting nutrient due to its short circuit and rapid fixation in many soils of tropical and subtropical regions. In such circumstances nitrogen fixation and growth of nitrogen fixing legumes may severely be affected (Mosse *et al.*, 1976; Mosse *et al.*, 1981).

However, colonization of legumes by VAM fungi can increase P uptake in infertile and high P-fixing soils (Mosse, 1973; Barrow, 1975; Powell and Daniel, 1978) mainly

due to capacity of mycorrhizal fungi to absorb P from the soil and transfer it to the host root (Starks, 1971; Mosse, 1973; Mosse *et al.*, 1976). It has been suggested that mycorrhiza in addition, aids in the acquisition of other vital nutrients such as S, Cu, Co, Zn, and Mn (Gray and Gerdemann, 1973; Rhodes and Gerdemann, 1978; Zaag *et al.*, 1979; Kothari *et al.*, 1991) which may directly or indirectly lead to enhanced nodulation, nitrogen fixation and host growth (Crush, 1974). Hewitt (1958) reported that under field conditions P or Cu concentration in plant tissue influences the infectivity and the rate of nitrogen fixation by legumes. Further, intensity of mycorrhizal colonization positively influences development of nodules in legumes and favours effective symbiosis (Crush, 1974; Carling *et al.*, 1978).

The increased application of soluble phosphate in soils may inhibit mycorrhizal infection (Menge *et al.*, 1978; Ratnayake *et al.*, 1978) and its spread in host root which may lead to detrimental effect on nodulation, nitrogen fixation and plant growth (Asimi *et al.*, 1980). It is evident that P source for both mycorrhizal and non-mycorrhizal plants remains the same (Sanders and Tinker, 1971; Hayman and Mosse, 1971; Tinker, 1975; Powell, 1976) however, mycorrhizal plants absorb more P than non-mycorrhizal one. Improved utilization

of rock phosphate by mycorrhizal fungi leading to improved nitrogen fixation by *Rhizobium* is evident (Mosse *et al.*, 1976; Pairunan *et al.*, 1980). In tropics and subtropics, however, many P-deficient soils occur in developing and under-developing countries where cost and rapid fixation of soluble P fertilizers preclude their use. Therefore, the application of cheaper and efficient rock phosphate fertilizer may serve as alternative to soluble fertilizers. Most of the investigations on the role of P source/s on tripartite symbiosis have been restricted to leguminous systems due to agronomic importance whereas same on non-legume-*Frankia*-VAM association is limited (Russo, 1989; Jha *et al.*, 1993). Little is known on the uptake of P and growth performance of actinorhizal host on insoluble P sources (Jha *et al.*, 1990) which may be important from the view point of reclamation of denuded lands of North-East India where traditional cultivation practice (jhum) and various human activities have resulted in the degradation of fragile ecosystem. This man-made catastrophe may consequently lead to desertification of land if timely action on afforestation of same is not initiated.

Alnus nepalensis, a fast growing actinorhizal species which forms symbiosis with VAM endophytes colonizes nutrient-deficient habitats in Eastern Himalaya and North-

East India, may be important from the viewpoint of revegetation programme.

Therefore, the present experiment has been designed to investigate the interaction between *Frankia* and VAM fungus in alder-*Frankia*-VAM fungus symbioses on P uptake, nitrogen fixation and seedling growth of *A. nepalensis* under various levels of rock phosphate.

Materials and methods

Collection and sterilization of soil:

Sandy loam laterite soil from Botanical garden of the North Eastern Hill University, Shillong, with following physicochemical properties i.e. CEC 25 meq/ 100 g, sand 66 %, silt 24 %, clay 10 %, pH - 5.34, organic matter - 3.41 %, total N 0.343 %, available P - 0.022 %, exchangeable K - 0.121 %, Ca - 0.094 %, Mg - 0.072 % and Mn - 0.0013 % was collected, mixed with builder's sand (2:1), moistened to field capacity and sterilized as described in chapter IV. The sterile soil was allowed to stand for 15 days.

Soil amendment with Mussourie rock phosphate:

Mussourie rock phosphate contained total P 8.1 %; 0.5 % NaHCO_3 soluble P, and 2 % citric acid soluble P. MRP is present as carbonate-fluorapatite which constitutes about 69 % of the total MRP. The rate of rock phosphate application varies from few hundred to 1000 kg (generally between 25 and 150 kg of phosphorus) per hectare (Thomson and Troeh, 1985). Taking above facts into consideration, 5 levels of RP i.e. 1. 0 N (minus RP amendment), 2. 1/2 N (20 kg P/ha soil) 3. 1 N (40 kg P/ha) 4. 2 N (80 kg P/ha) and 5. 4 N (160 kg P/ha) were maintained.

The soil amended with above levels of RP was mixed thoroughly, filled (700 g approx.) in 1 L capacity conical flasks and stoppered with sterilized cotton plugs.

Collection of seeds and raising of seedlings:

Seeds of *A. nepalensis* were collected in the month of March, 1992 from Himalayan alder stand situated at Upper Shillong. They were husked, surface sterilized and germinated in 1 % water agar medium as described in chapter IV.

Preparation of *Frankia* inoculum:

Ten week old culture of *Frankia* AnF₁ (abbreviated as *Frankia*) growing in DPM liquid medium was harvested, washed and inoculum was prepared accordingly as given in Chapter IV.

Preparation of VA mycorrhizal inoculum:

Glomus mosseae was cultured and maintained on maize under greenhouse condition. The inoculum was prepared as described in chapter IV.

Inoculation of microsymbionts and plant growth conditions:

Healthy 1 month old Himalayan alder seedlings growing on 1 % water agar medium were harvested, washed several times in sterile distilled water and 5 seedlings were transplanted in each conical flask containing soil amended with various level of rock phosphate. For each level of RP, four inoculation regimes were maintained i.e.

1. Uninoculated control (-*Frankia* - *G. mosseae*)
 2. + *Frankia*
 3. + *G. mosseae* and
 4. + *Frankia* + *G. mosseae*.
- Control plants received twice autoclaved inoculum of *Frankia* and *G.*

mosseae. The seedlings were watered and grown in similar condition as stated in chapter IV.

Harvesting:

Harvests of seedlings were conducted on 30, 60, 90, and 120 days after inoculation of microendophyte/s in similar manner as in chapter IV.

Growth performance of the seedlings:

Various parameters for the assessment of seedling growth were used. The details of these are given in Chapter IV.

Nodulation, nodule biomass and nitrogen fixation:

Thoroughly washed root system of seedlings was excised and transferred to 10 ml serum bottles. It was stoppered with suba seal and sealed with parafilm. Then 1 ml of air was withdrawn from the bottle and replaced with 1 ml of C_2H_2 gas thus imparting 10% C_2H_2 in the serum bottle. The details of acetylene reduction technique used for the determination of nitrogenase activity are described in Chapter I. The activity was expressed in nmoles C_2H_4 h^{-1} seedling $^{-1}$. The nodules were detected under the

stereomicroscope and was expressed as seedling⁻¹. Thereafter, nodule dry weight was determined by drying them in a hot air oven at 60°C till constant weight was achieved.

Population of rhizospheric *Frankia* :

Frankia population of the rhizospheric soil was determined following method of Baker and O'Keefe (1984) as described in chapter I.

Assessment of VAM infection, arbuscules and vesicles:

On each sampling, the root system was washed and cut into 1 cm long segments, cleared and stained following the method of Koske and Gemma (1988). VAM infection was determined following the method of Read *et al.* (1976) outlined in chapter I. Number of arbuscules and vesicles was recorded using stained root segments and expressed as cm⁻¹ root length.

Determination of endogonaceous spore population:

Air dried rhizosphere soils were used for the isolation and enumeration of endogonaceous spores by wet

sieving and decanting technique (Gerde mann and Nicolson, 1963) as described in chapter I.

Determination of rhizospheric phosphatase activity :

Total phosphatase activity of the rhizosphere soil was assayed following the method of Tabatabai and Bremner (1969). One g air dried and sieved (0.2 mm) soil was taken in a 50 ml conical flask. Then 4 ml of modified universal buffer (pH 6.5), 0.25 ml of toluene, 1 ml of 0.115 M p-nitrophenyl phosphate solution were added to the flask containing soil. It was cotton plugged and incubated at 37° C for 1 h. The details of the method have been given in chapter I.

Analysis of nutrient contents in seedlings:

Oven dried root and shoot samples were ground separately and sieved. Fine ground plant tissue (<0.2 mm) was used for the analysis of N, P, K and Mn.

For the estimation of N, 0.2 g ground fine plant material was digested in Kjeldahl flask along with 2 g of K₂SO₄ -HgO catalyst and 3 ml of Conc. H₂SO₄. N content in tissues were determined following semi-microKjeldahl method

(Allen, 1974). The details of the technique are described in chapter I.

Plant P was estimated following the molybdenum blue method (Allen, 1974). Fine plant material ^{0.2g} was digested in triacid mixture, diluted to 50 ml with distilled water and proceeded as described in chapter IV.

For the determination of K, same aliquot used for analysis of P, was employed whose absorbance was read at 766.5 nm in atomic absorption spectrophotometer using K lamp (Allen, 1974).

Plant Mn content was determined using same aliquot as used for K and taking the absorbance of aliquot at 279.5 nm in atomic absorption spectrophotometer using Mn lamp (Allen, 1974). The procedures are described in detail in chapter I.

Statistical analysis:

Data for plant growth, VAM and nodule characteristics were subjected to 3 way ANOVA. Coefficient of correlation was employed to assess the relationship

between root microendophytes.

RESULTS

Plant growth:

Positive growth response of *A.nepalensis* seedling to increasing levels of RP was observed which varied among different treatments. Mycorrhizal plant was superior in almost all levels of RP while *Frankia* nodulated and uninoculated plants grew well only at higher level of RP. However, growth response of nodulated + mycorrhizal plants exceeded others at most soil P levels.

Shoot height, root length, total seedling height and stem collar diameter increased with increasing level of RP. Highest growth response by *Frankia* and noninoculated control seedlings was observed at highest level of RP whereas mycorrhizal plants performed better at 2 N RP level. *Frankia* + *G. mosseae* infected plants grew better than either *G. mosseae* or *Frankia* inoculated ones at all RP levels and harvests (Fig. 69, 70, 71 & 72).

The rate of seedling growth also followed above trend in which nodulated + mycorrhizal plants accumulated

more biomass than singly or noninoculated ones at all RP amendments and harvests (Table 31, 32, 33 & 34).

Number of leaves and leaf area increased with time and increasing RP level. Mycorrhizal plants produced more leaves and leaf area over the nonmycorrhizal *Frankia* and noninoculated ones. However, *Frankia* + *G. mosseae* infected plants showed more vigourousity than singly inoculated or uninoculated ones at any level of RP and harvests (Fig. 73 & 74).

Seedling growth features like shoot height, root length, seedling height, stem collar diameter, root, shoot, and plant biomass, R/S ratio, leaf number and leaf area were significantly (ANOVA $P < 0.01$) influenced by RP, harvests and endophytes (Table 35).

Nodulation, nodule biomass and nitrogen fixation:

Nodules were developed only by *Frankia* and *Frankia* + *G. mosseae* inoculated plants. The root nodules appeared after 30 days of inoculation in both singly and dually inoculated plants. The nodule number increased exponentially with increasing concentration of soil P. After 30 days of growth, dually inoculated seedlings produced 2-3 times more

nodules as compared to *Frankia* inoculated ones (Fig. 75).

Nodule biomass also increased in time and with increase in soil P level. In *Frankia* nodulated plants, nodule growth was poor and the rate of biomass accumulation lagged behind that of nodulated + mycorrhizal seedlings at most RP levels (Fig. 76).

The activity of nitrogenase enzyme of root nodules was markedly higher in *Frankia + G. mosseae* than in *Frankia* inoculated one at all level of RP amendment (Fig. 77).

Rhizospheric *Frankia* population :

Linear growth of *Frankia* was observed at different P levels in the rhizosphere soil of singly (*Frankia*) and dually (*Frankia + G. mosseae*) inoculated plants. Marked difference in its population was noticed between nodulated and nodulated + mycorrhizal seedlings (Fig. 78).

Most of the nodule characteristics by *Frankia* and its population were significantly (ANOVA $P < 0.01$) affected by RP, harvests and root symbionts (Table 36).

VAM infection and dynamics of arbuscules and vesicles :

Generally, VA mycorrhizal infection increased in time at all RP levels. Nodulated + mycorrhizal plants were more mycotrophic than mycorrhizal plants alone at corresponding level of RP and harvest (Fig. 79).

Number of arbuscules was more than vesicles at 30 days but the ratio decreased with the increase in time. The decrease in ratio of arbuscules to vesicle was more pronounced at lower level of RP than at higher level (Fig. 80 & 81). A variation in the population of arbuscules between *G. mosseae* and *Frankia* + *G. mosseae* infected plants was detected especially at higher level of RP application (Fig. 80).

Endogonaceous spore number :

The abundance of endogonaceous spores increased in time and with increasing level of RP upto 2 N level. Plants inoculated only with *G. mosseae* produced less spores than that by *Frankia* + *G. mosseae* symbiosis at most RP levels (Fig. 82).

Soil phosphatase activity :

Phosphatase activity decreased with increasing level of RP application. It was detected lowest at high RP amended soil. The activity was detected only in the rhizosphere soil of mycorrhizal and nodulated + mycorrhizal plants. Interestingly, a marked variation in the enzyme activity in above groups of plants was evident (Fig. 83).

VAM infection, arbuscule, vesicle and spore number was evidently (ANOVA $P < 0.01$) affected by RP levels, harvests and root endophytes (Table 37). Further, RP, harvests and treatment had profound influence (ANOVA $P < 0.01$) on phosphatase activity (Table 37).

N, P, K and Mn concentrations of seedlings :

Root and shoot tissue of nodulated + mycorrhizal plants harboured maximum N, P, K and Mn as compared to singly inoculated or noninoculated counterparts (Fig. 84, 85, 86 & 87). However, roots contained less nutrients than shoot.

Nutrient contents of root and shoots were significantly (ANOVA $P < 0.01$) influenced by RP, harvests and

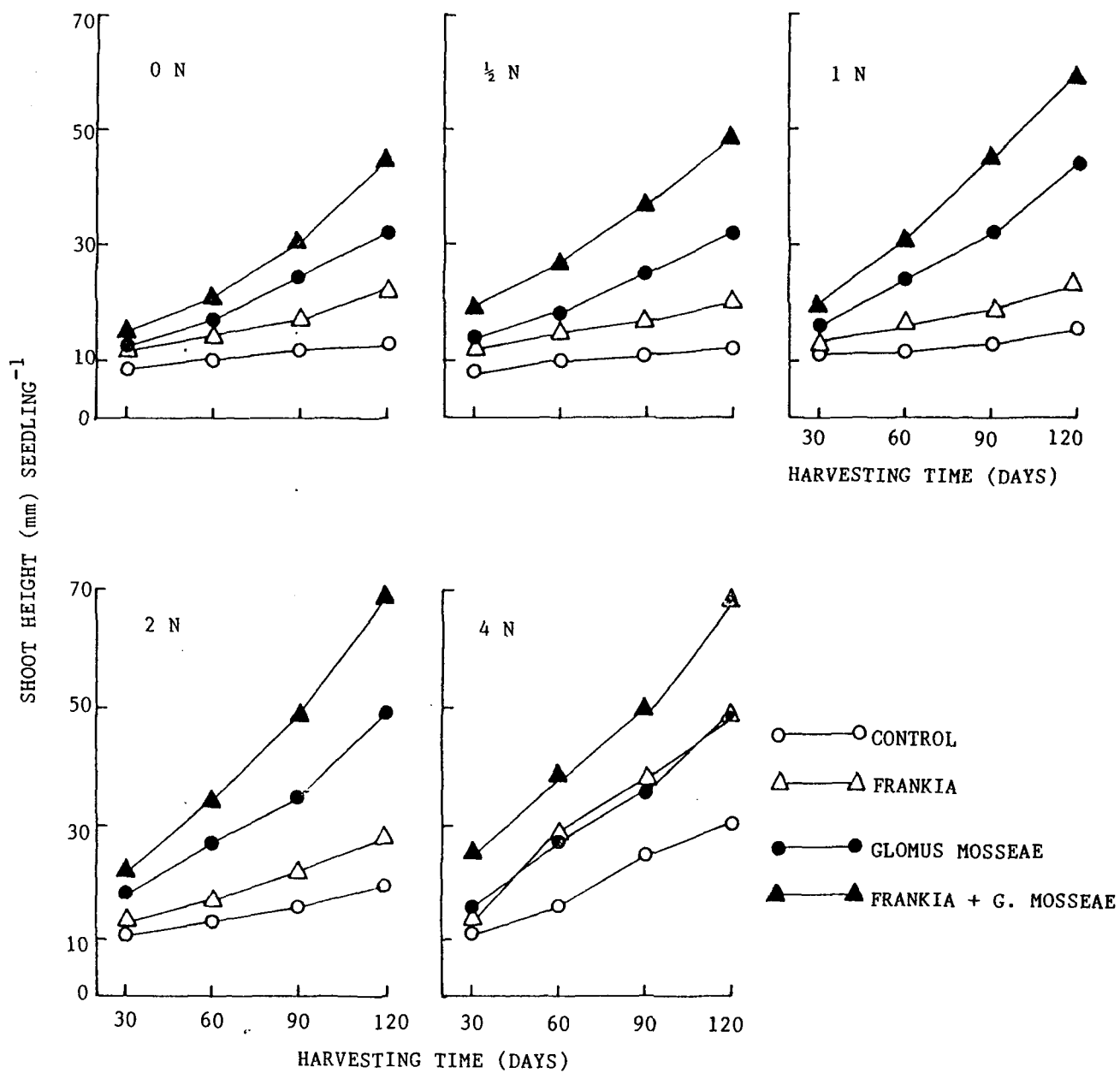


Fig.69. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on shoot height of *A. nepalensis* seedlings grown at various levels of rock phosphate.

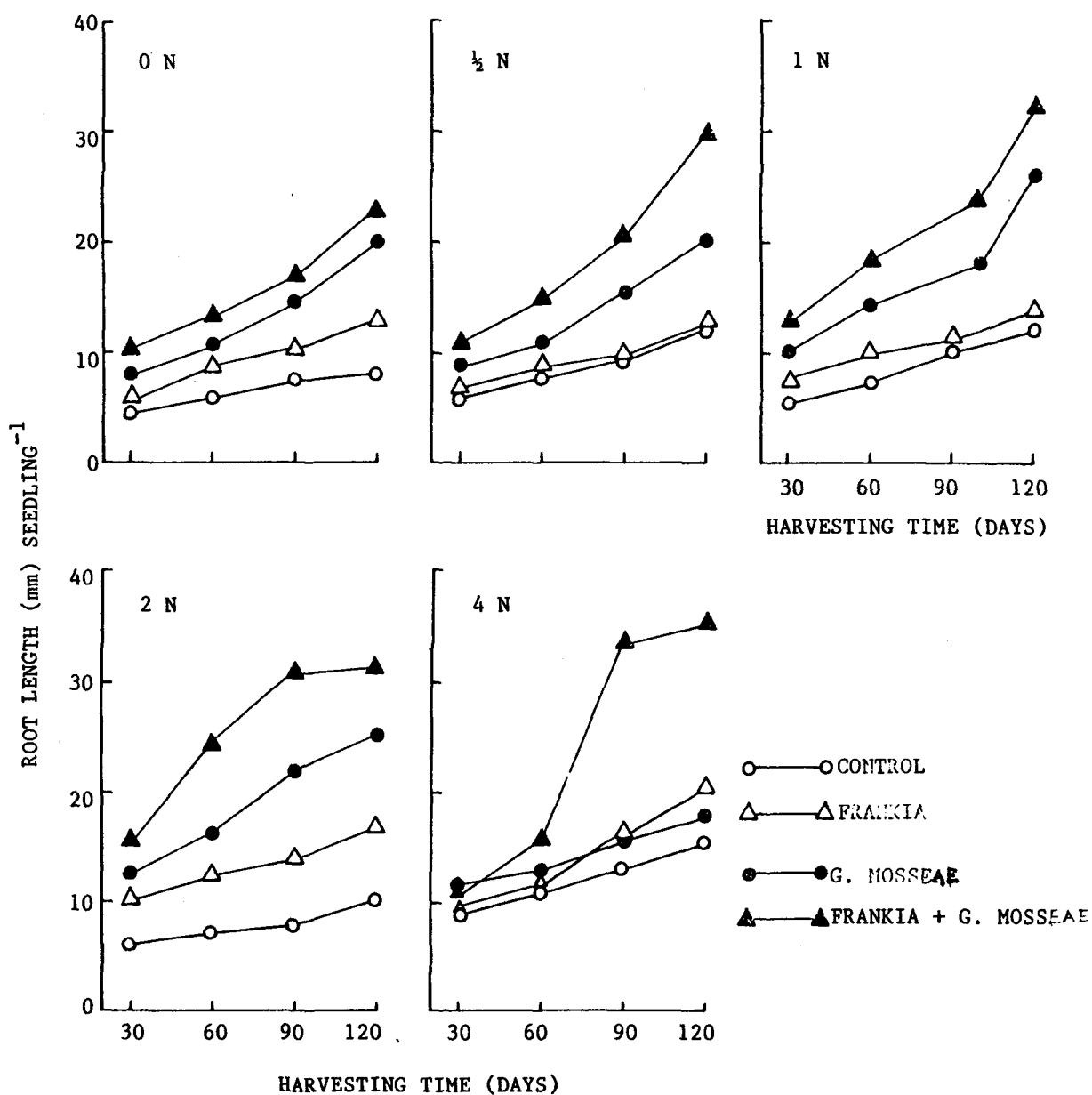


Fig.70. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root length of *A. nepalensis* seedlings grown at various levels of rock phosphate.

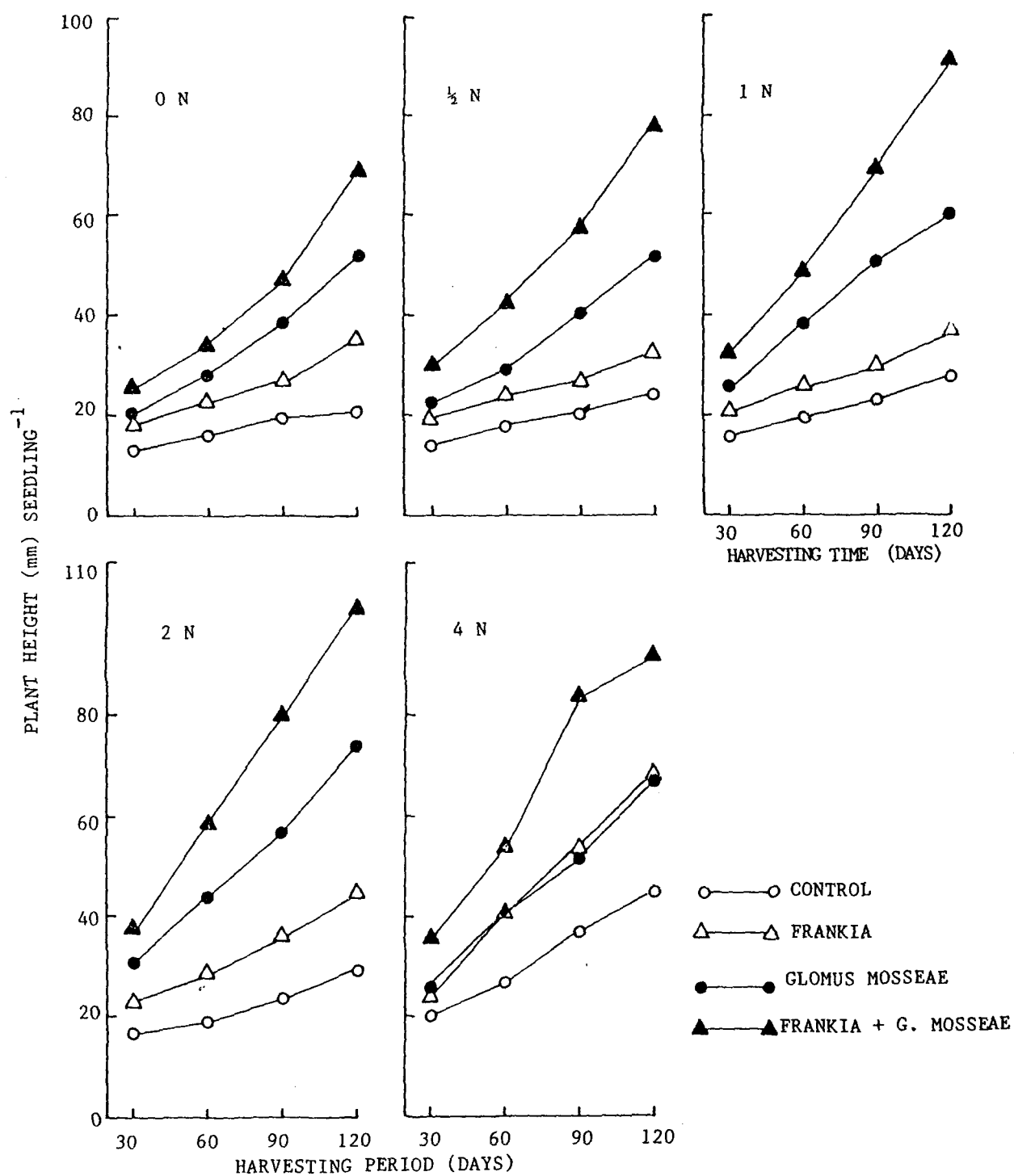


Fig. 71. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on total height of *A. nepalensis* seedlings grown at different levels of rock phosphate.

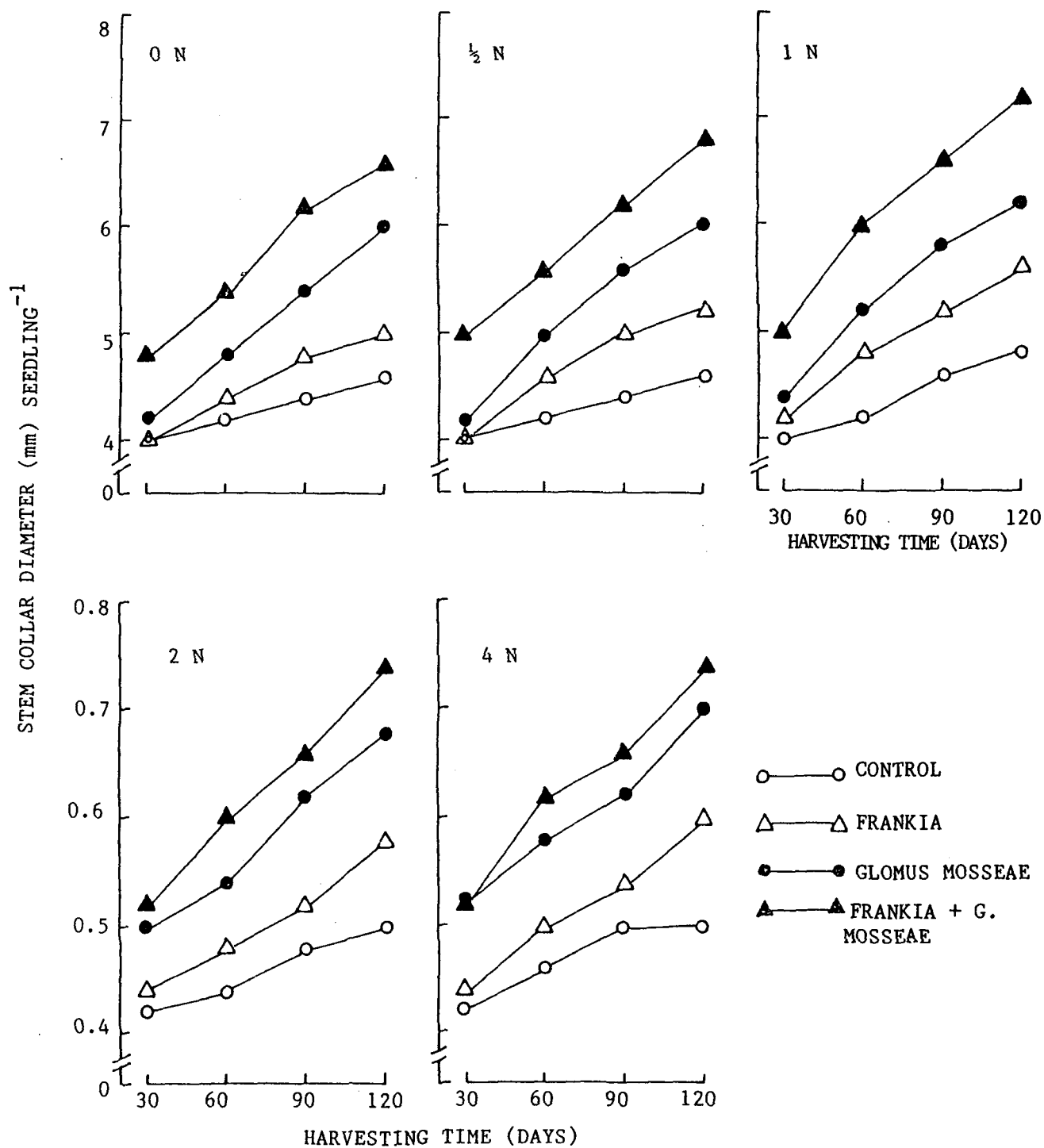


Fig. 72 Effect of Frankia, G. mosseae and Frankia + G. mosseae on stem collar diameter of A. nepalensis seedlings grown at different levels of rock phosphate.

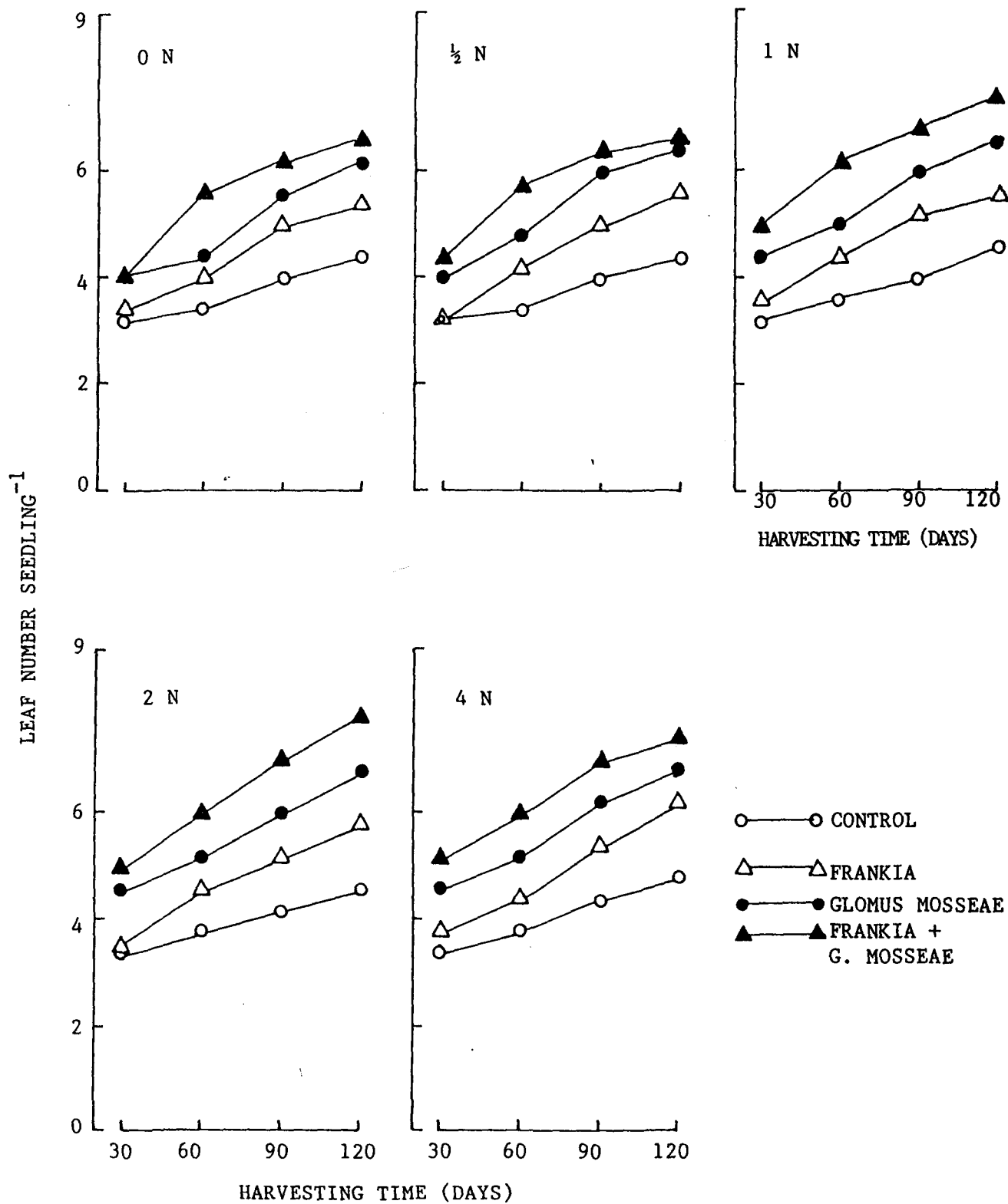


Fig. 73 Effect of Frankia, G. mosseae and Frankia + G. mosseae on leaf number of A. nepalensis seedlings at different levels of rock phosphate.

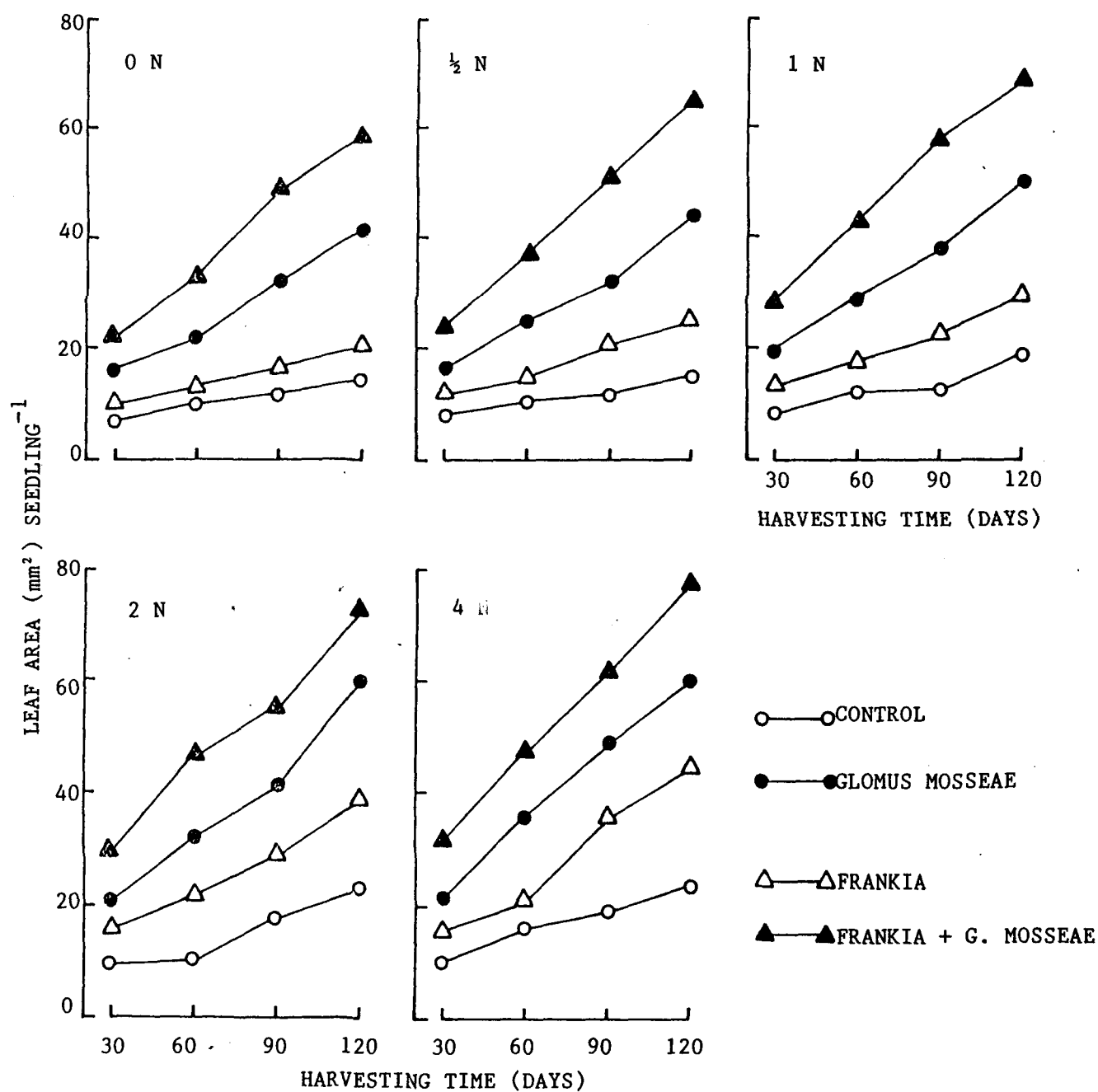


Fig.74. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on leaf area of *A. nepalensis* seedlings grown at various levels of rock phosphate.

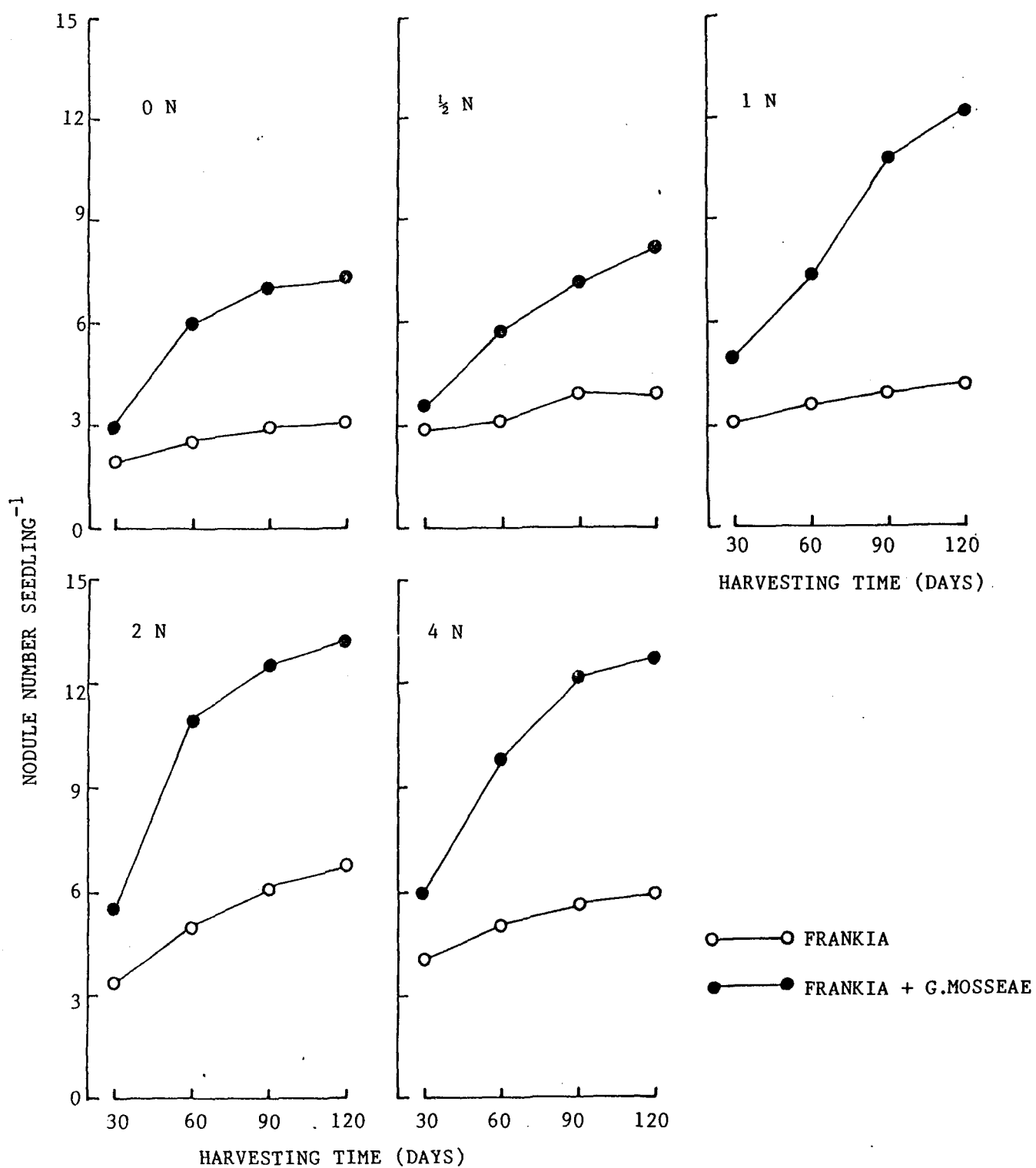


Fig.75.Effect of *Frankia* and *Frankia* + *G. mosseae* on nodule number of *A. nepalensis* grown at different levels of rock phosphate.

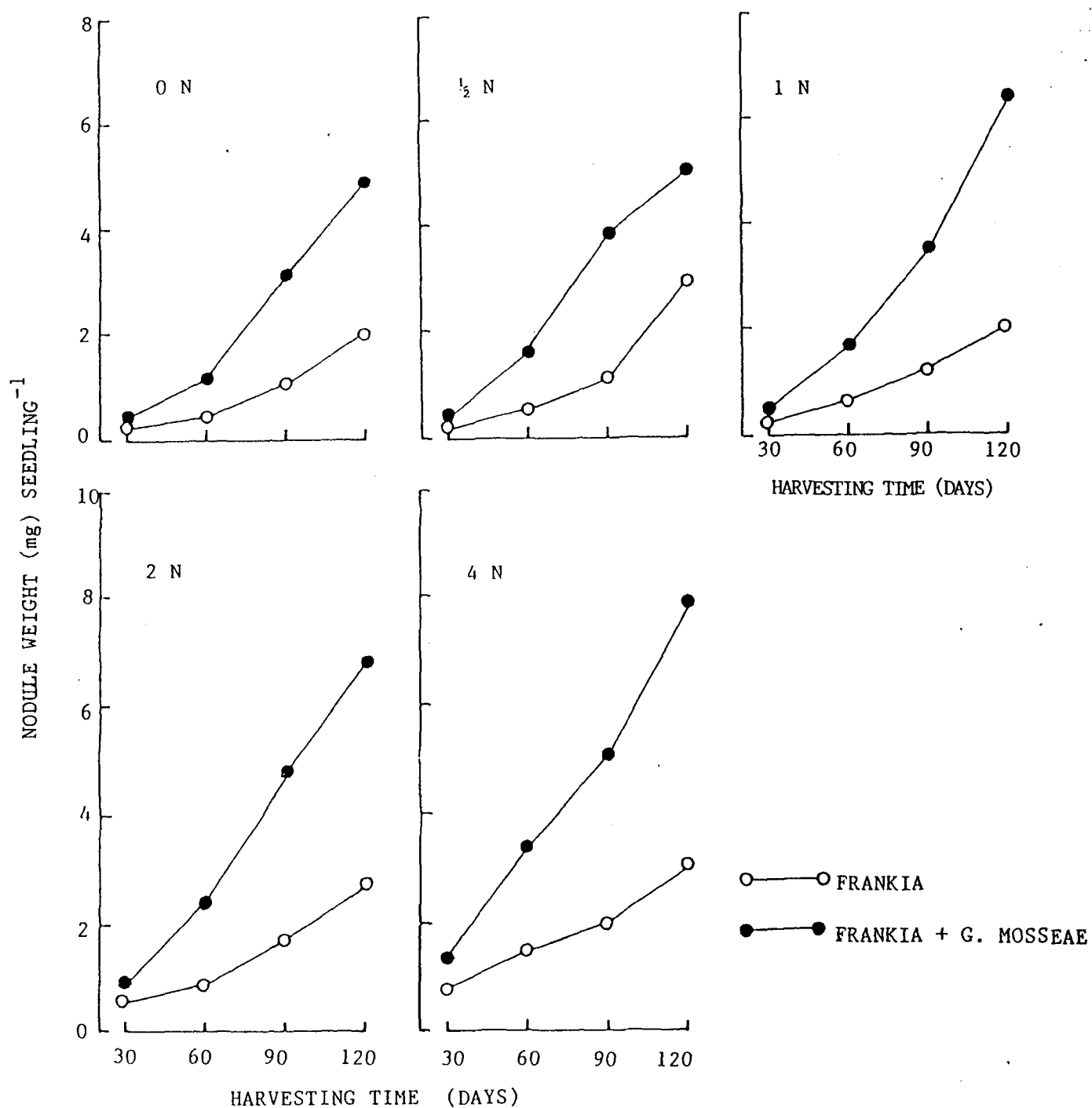


Fig.76. Effect of Frankia and Frankia + G. mosseae on dry weight of nodules of A. nepalensis seedlings grown at different levels of rock phosphate.

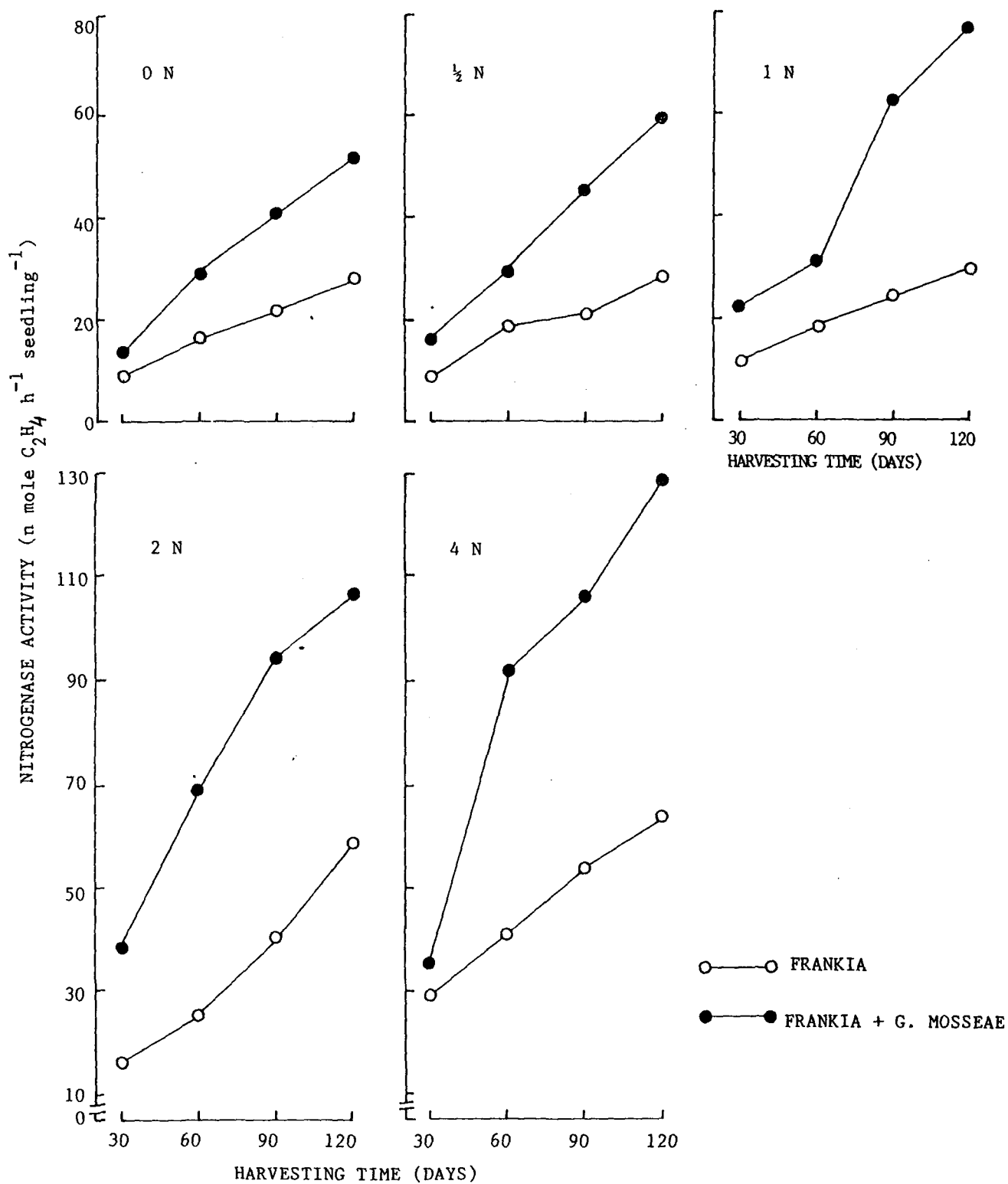


Fig.77. Effect of Frankia and Frankia + G. mosseae on nitrogenase activity of nodule of A. nepalensis seedling grown on soil with different levels of rock phosphate.

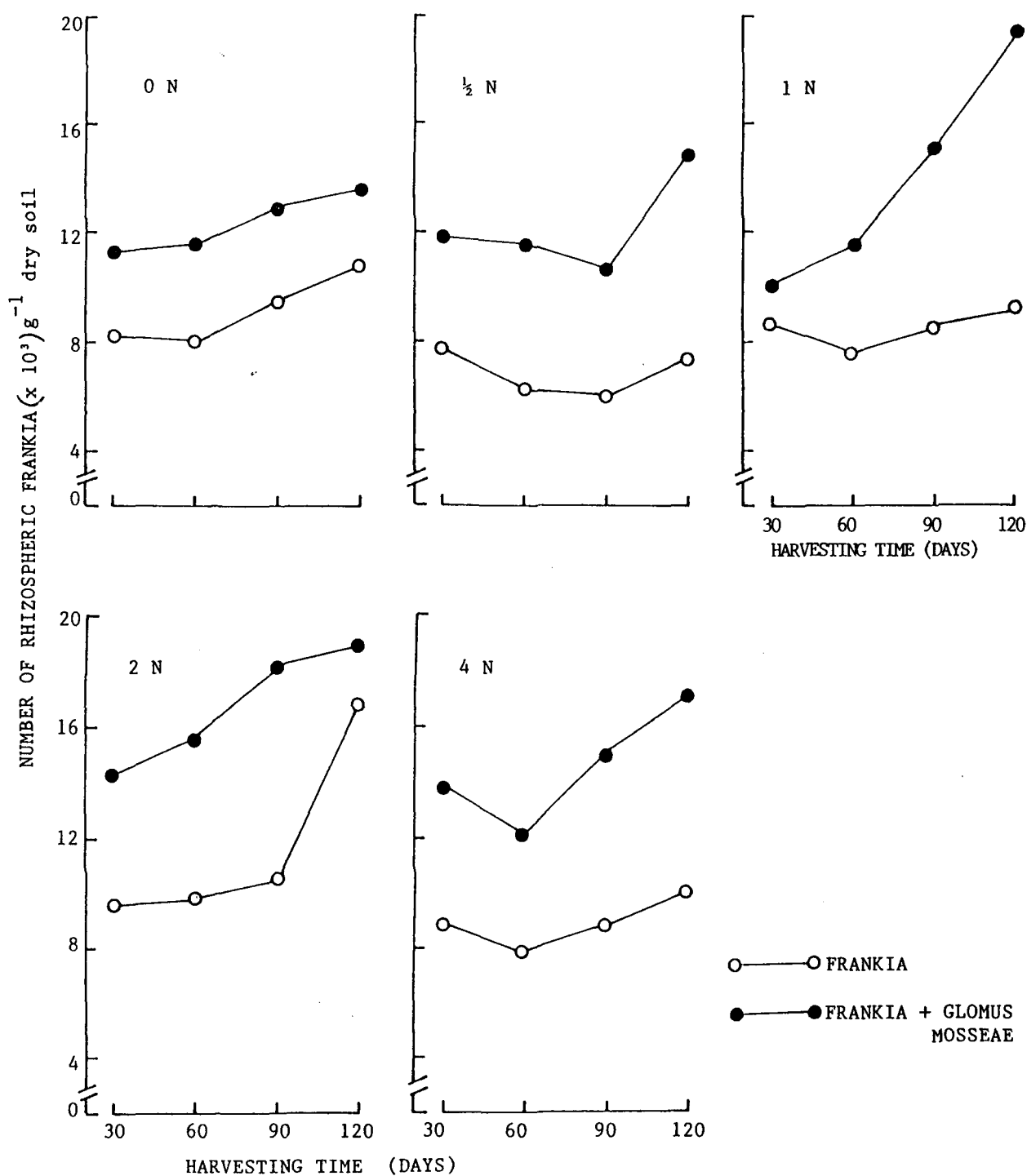


Fig.78. Population of *Frankia* in rhizosphere soil of *A. nepalensis* with *Frankia* and *Frankia* + *G. mosseae* at various levels of rock phosphate.

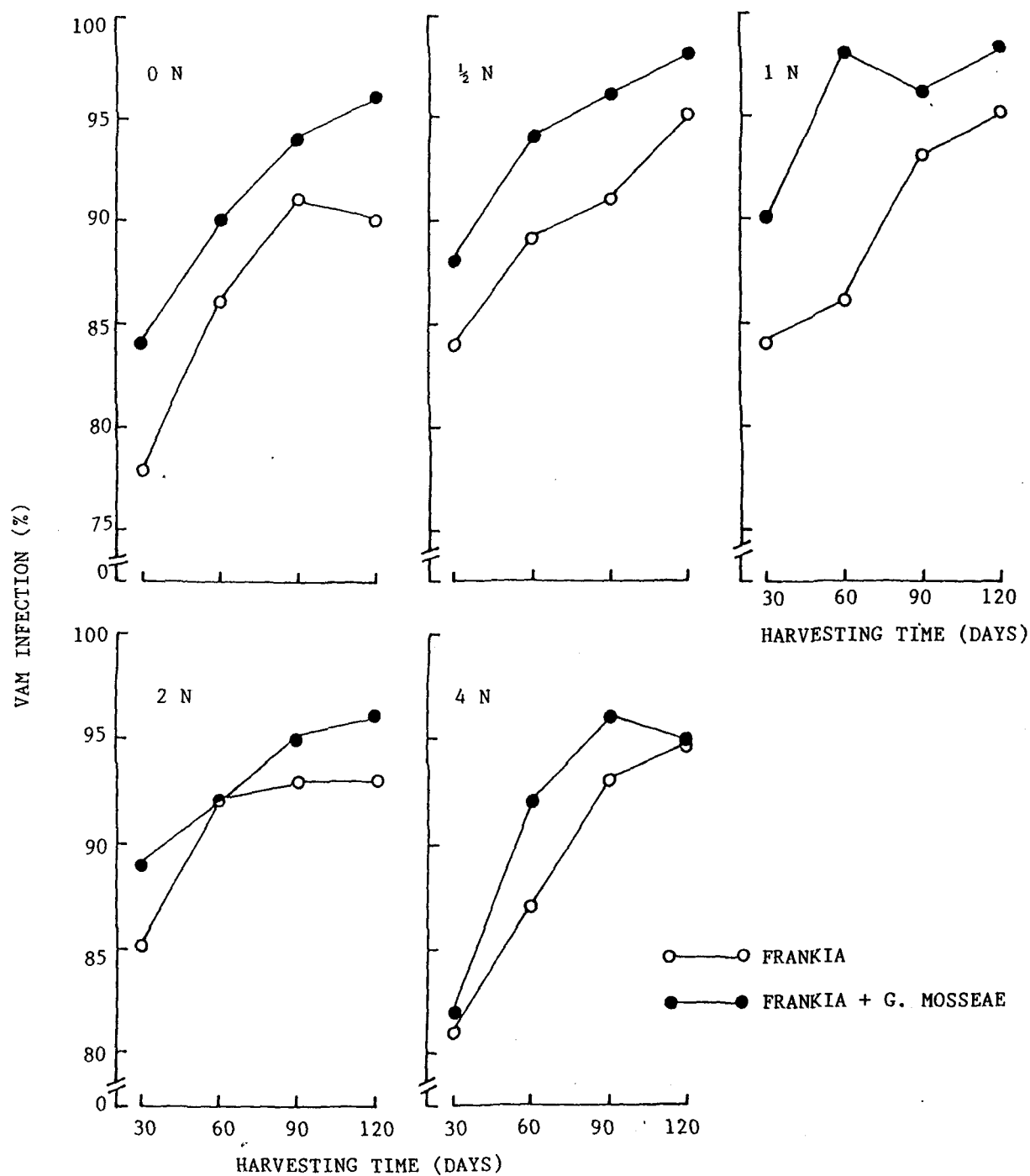


Fig.79.VA mycorrhizal infection of A. nepalensis root inoculated with G. mosseae and Frankia + G. mosseae at various levels of rock phosphate.

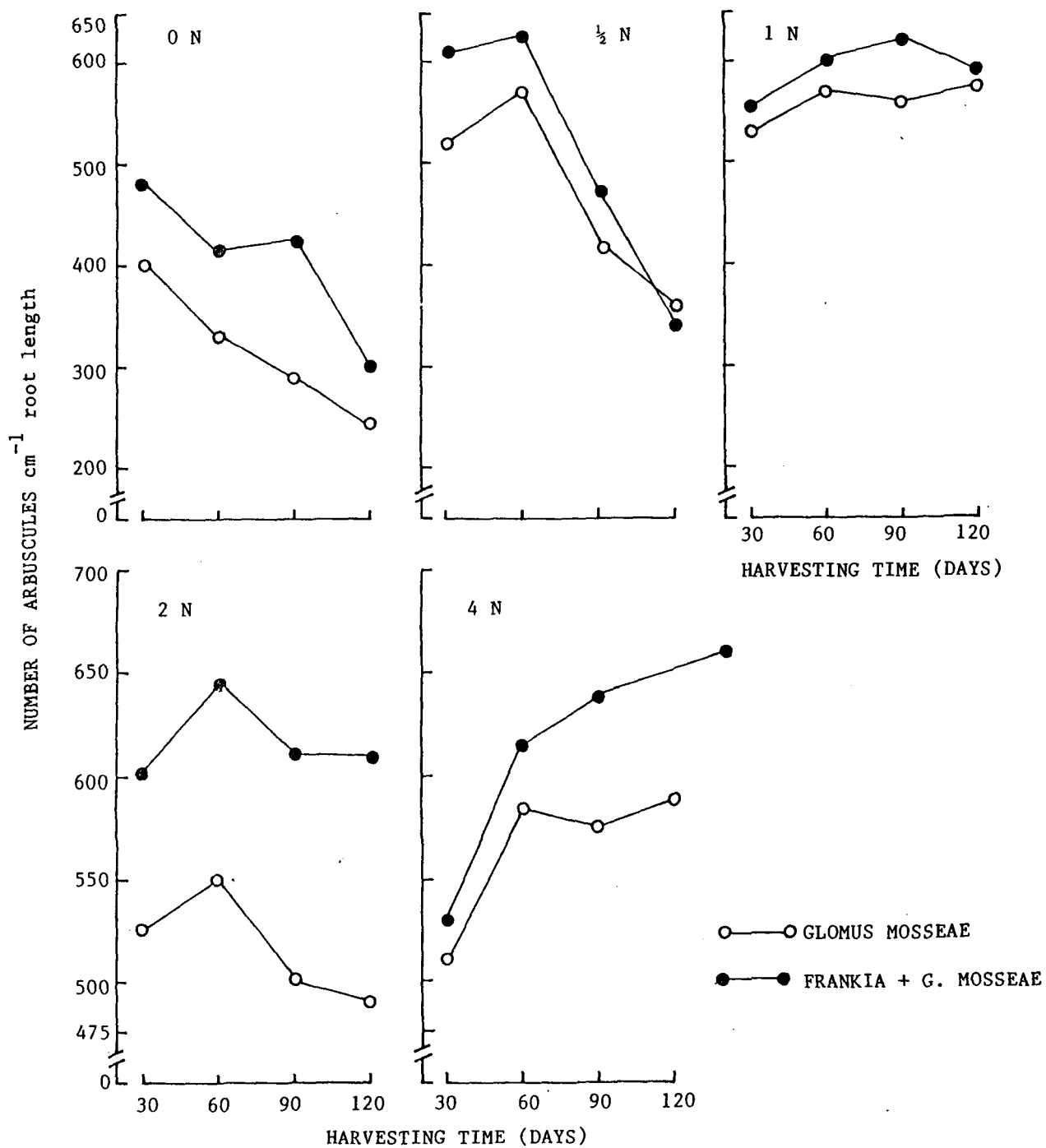


Fig.80. Effect of *G. mosseae* and *Frankia + G. mosseae* on the production of arbuscule in mycorrhizal root of *A. nepalensis* at different levels of rock phosphate.

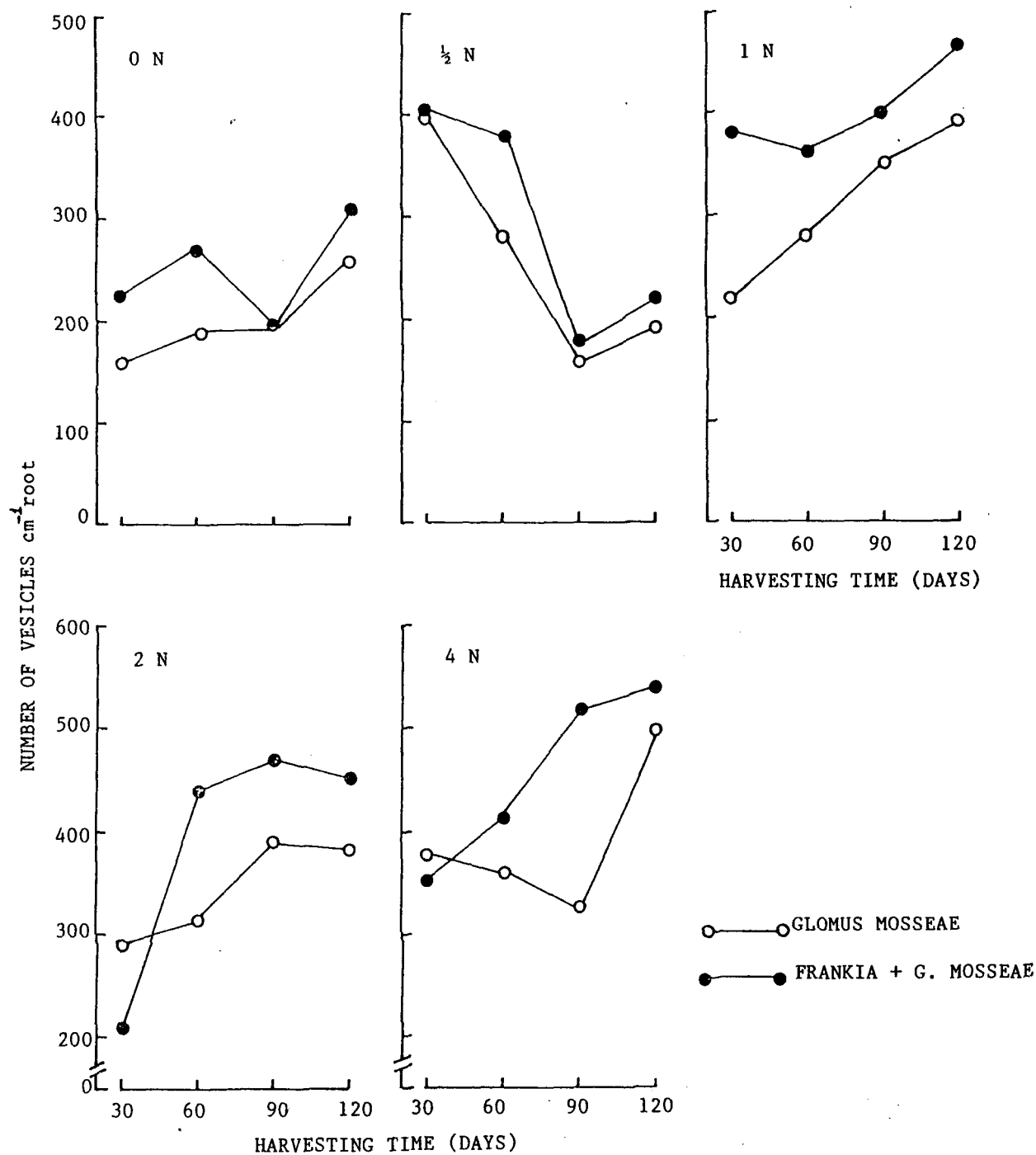


Fig.81. Number of vesicles in mycorrhizal roots of *A. nepalensis* inoculated with *G. mosseae* and *Frankia + G. mosseae* at various levels of rock phosphate.

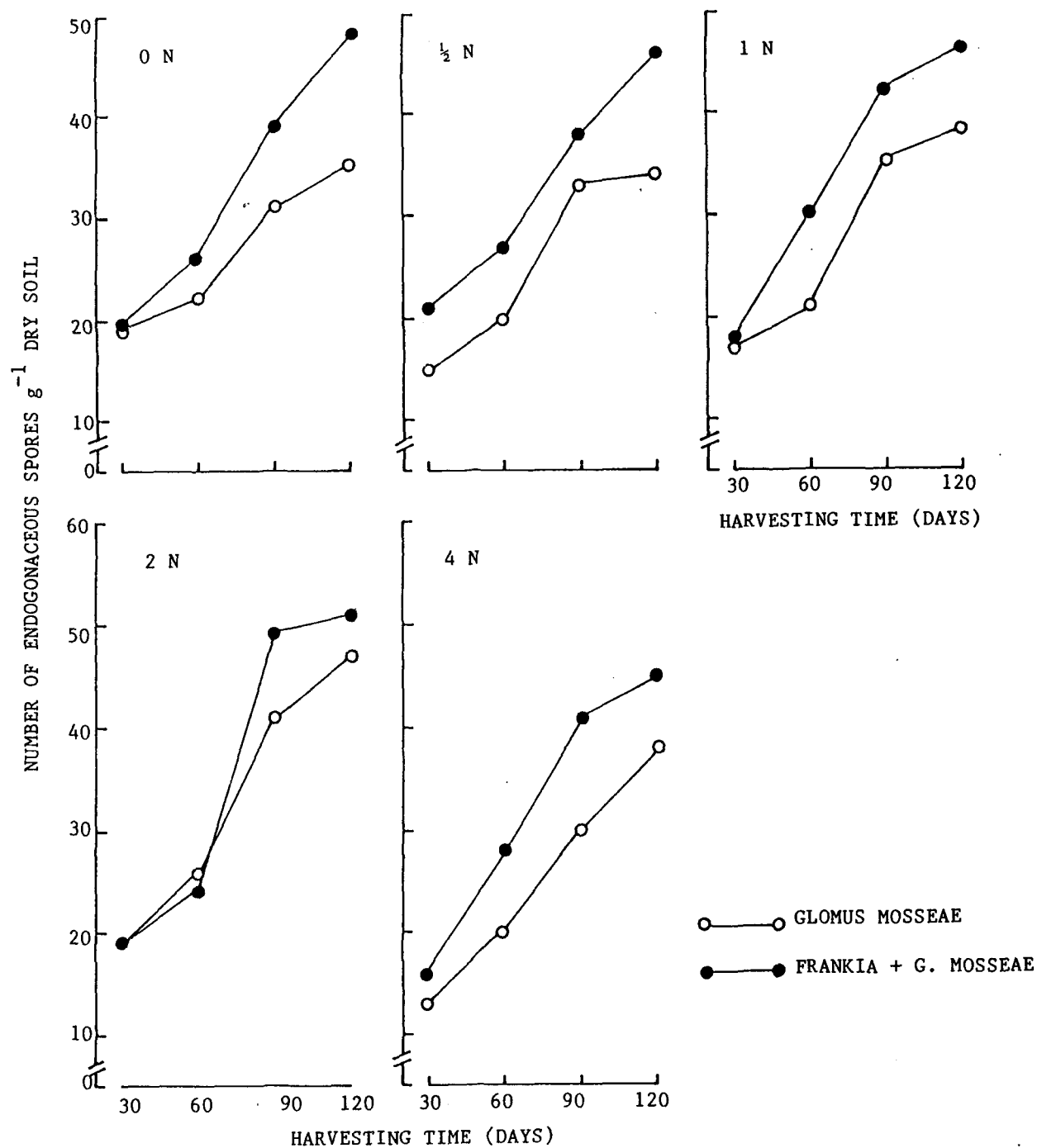


Fig.82. Population of endogonaceous spores in rhizosphere soil of A. nepalensis inoculated with G. mosseae and Frankia + G. mosseae at different levels of rock phosphate.

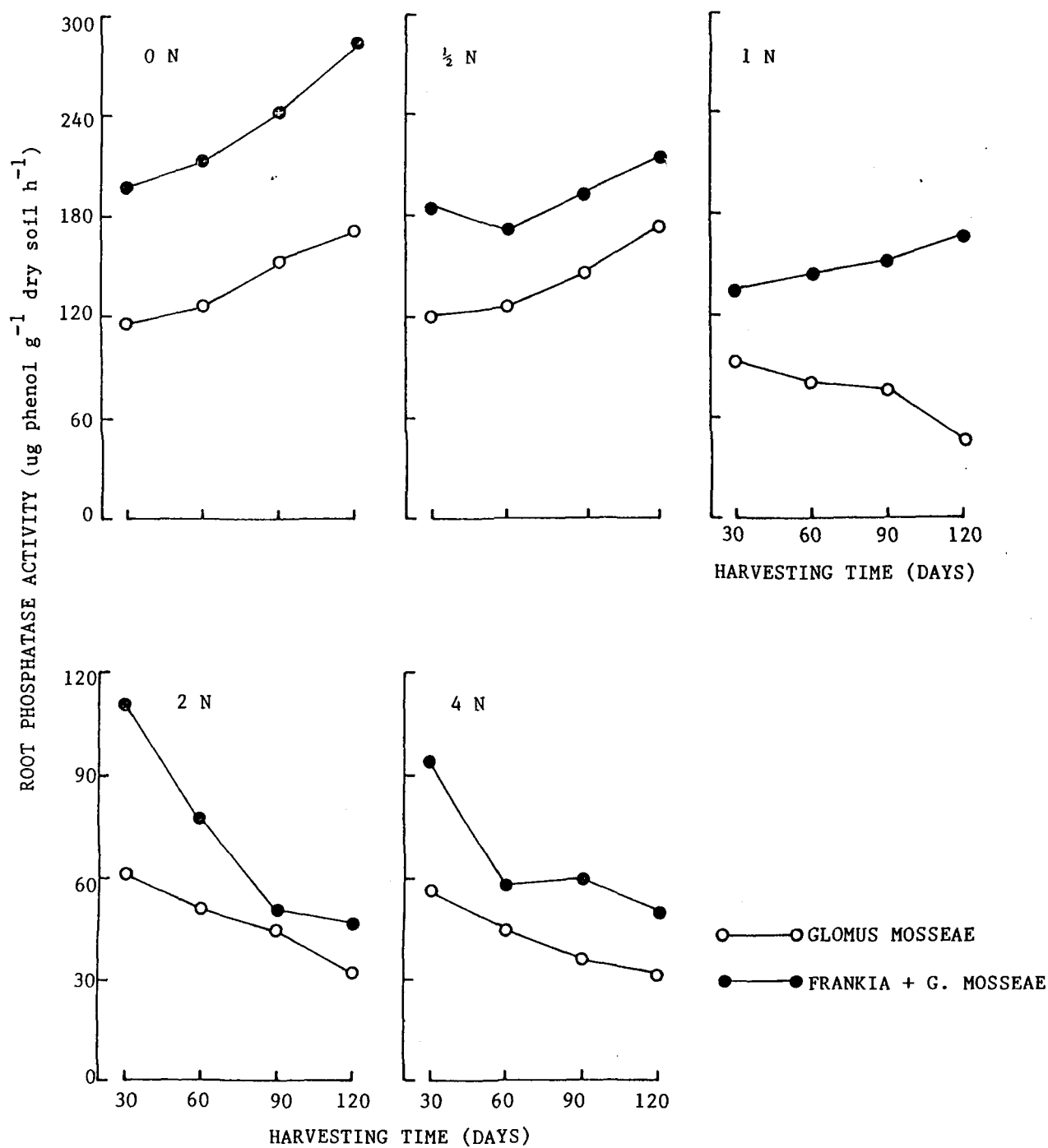


Fig.83. Effect of *G. mosseae* and *Frankia + G. mosseae* on phosphatase activity of rhizosphere soil of *A. nepalensis* at various levels of rock phosphate.

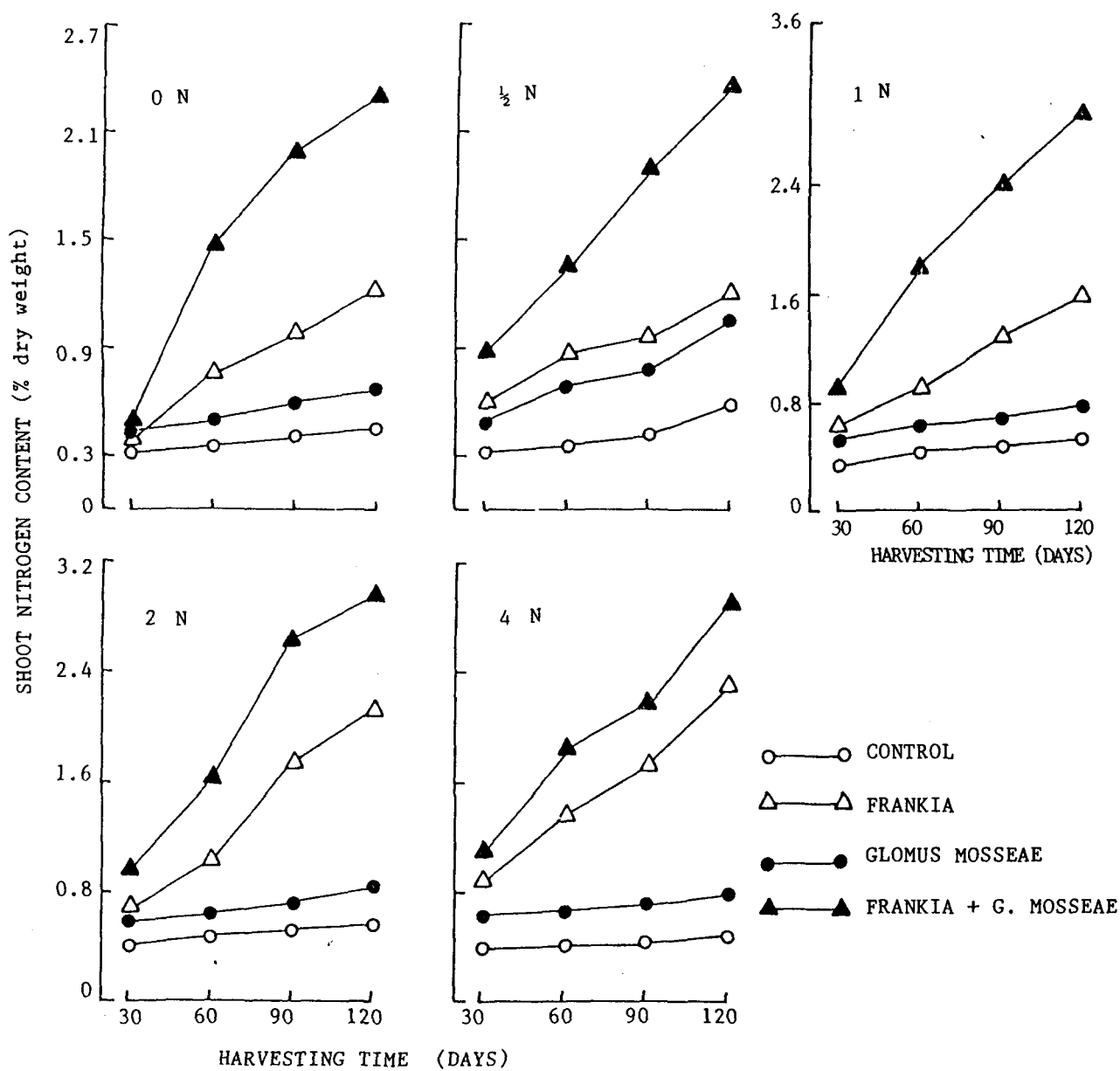


Fig.84a.Effect of Frankia, G. mosseae and Frankia + G. mosseae on shoot nitrogen content (%) of A. nepalensis seedlings grown at various rock phosphate levels.

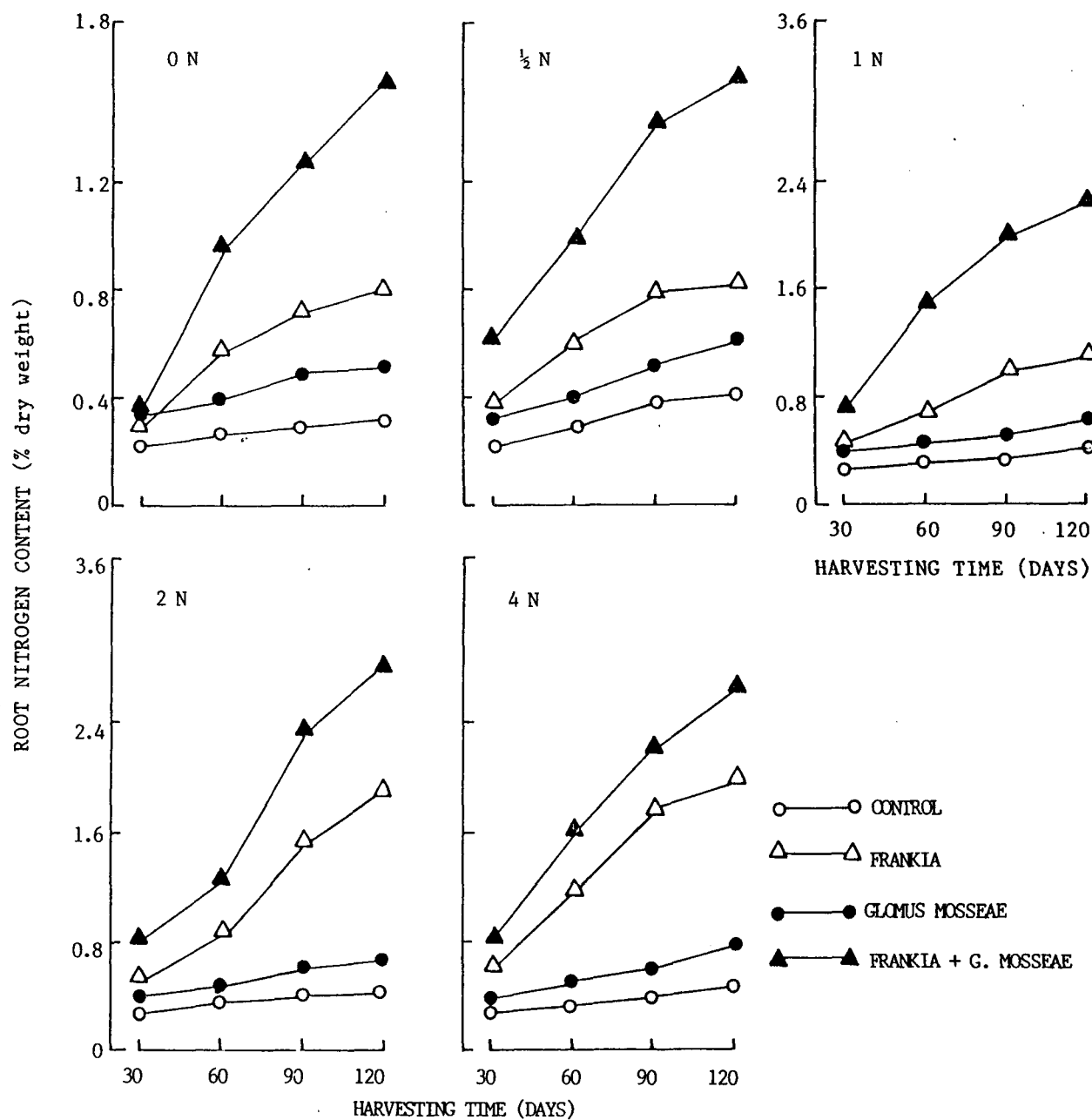


Fig.84b. Effect of Frankia, G. mosseae and Frankia + G. mosseae on root nitrogen content (%) of A. nepalensis seedlings grown at various levels of rock phosphate.

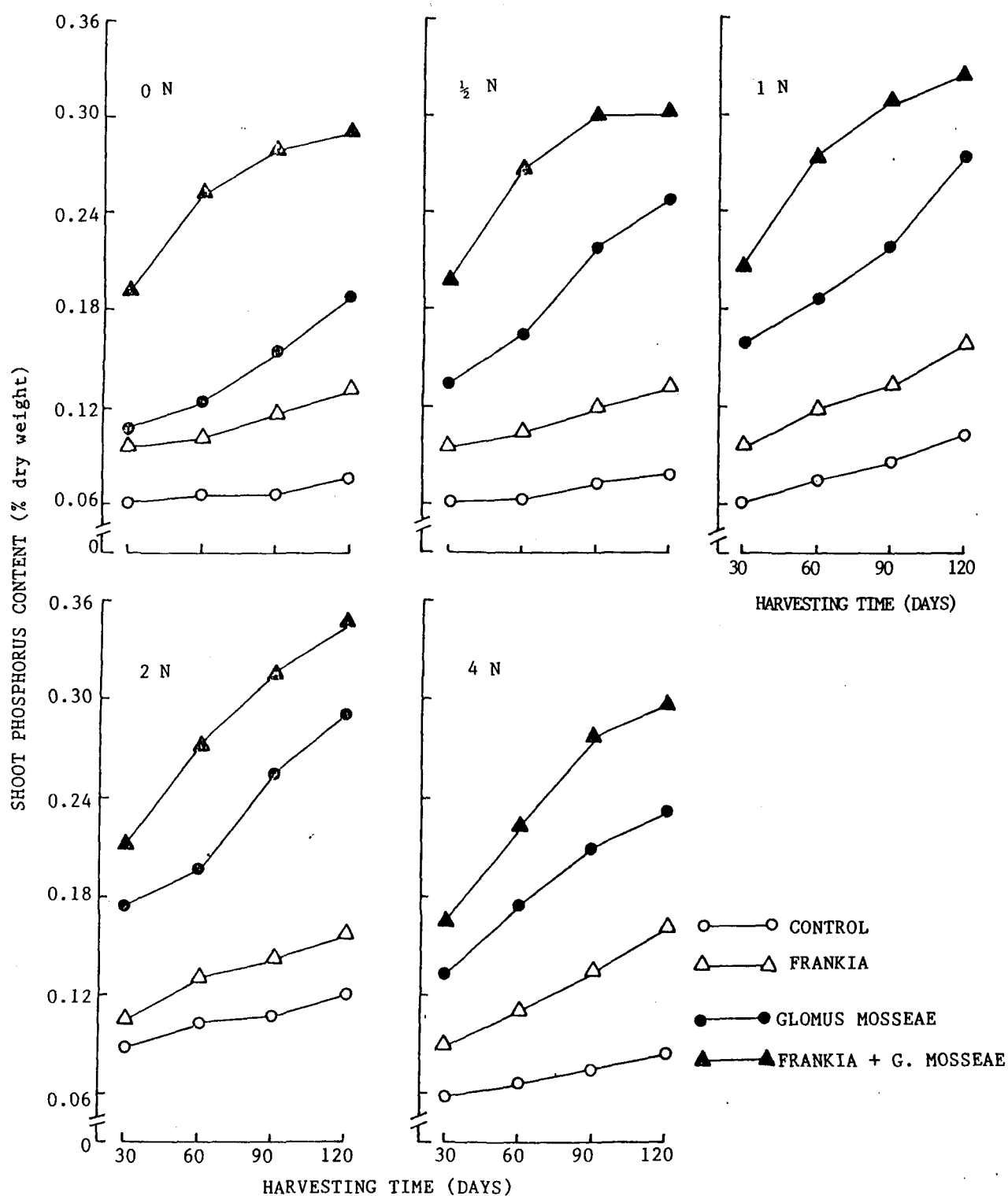


Fig. 85a Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on shoot phosphorus content (%) of *A. nepalensis* seedlings grown at different levels of rock phosphate.

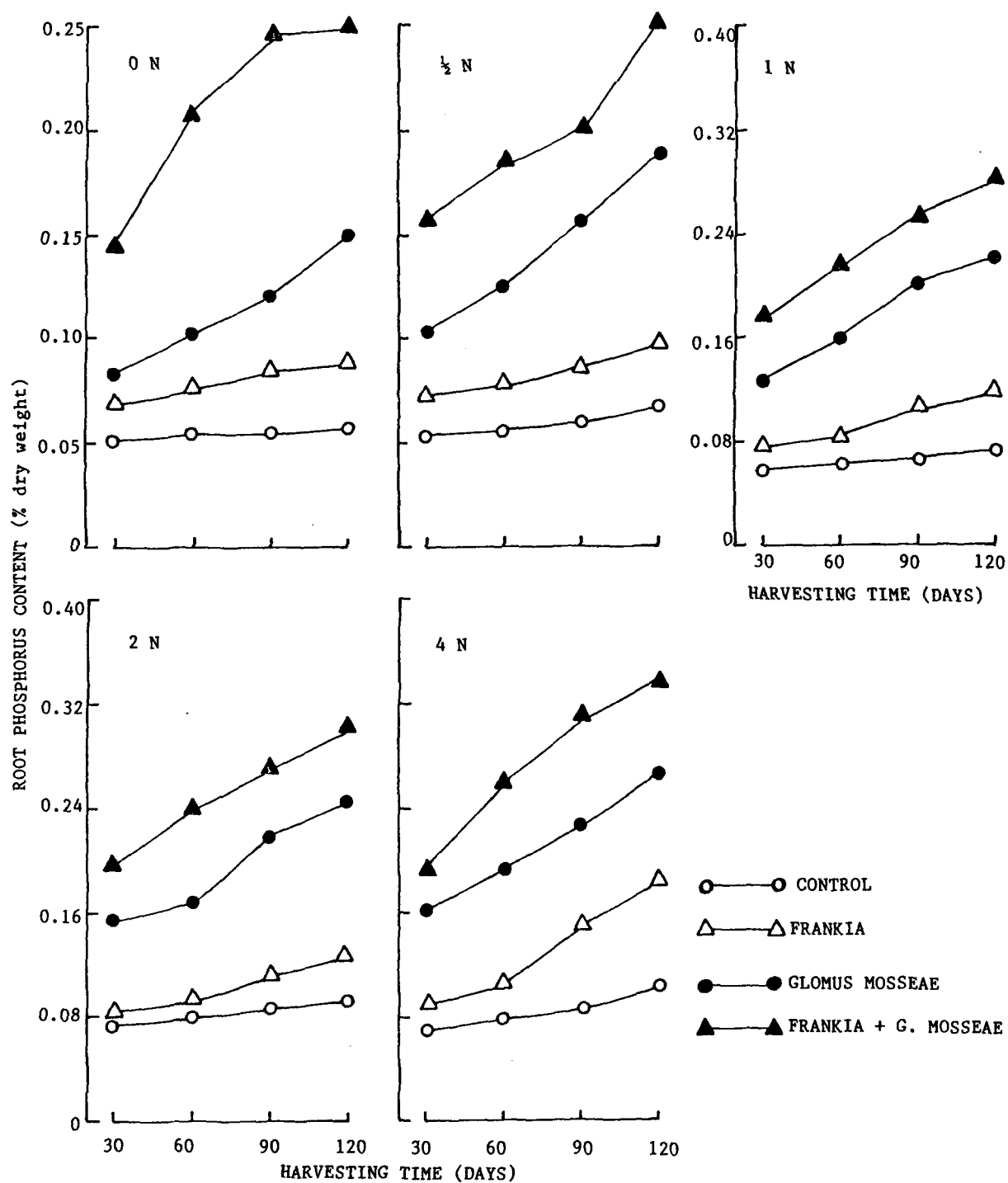


Fig.85b. Effect of *Frankia*, *G. mosseae* and *Frankia + G. mosseae* on shoot phosphorus content (%) of *A. nepalensis* seedlings at various levels of rock phosphate.

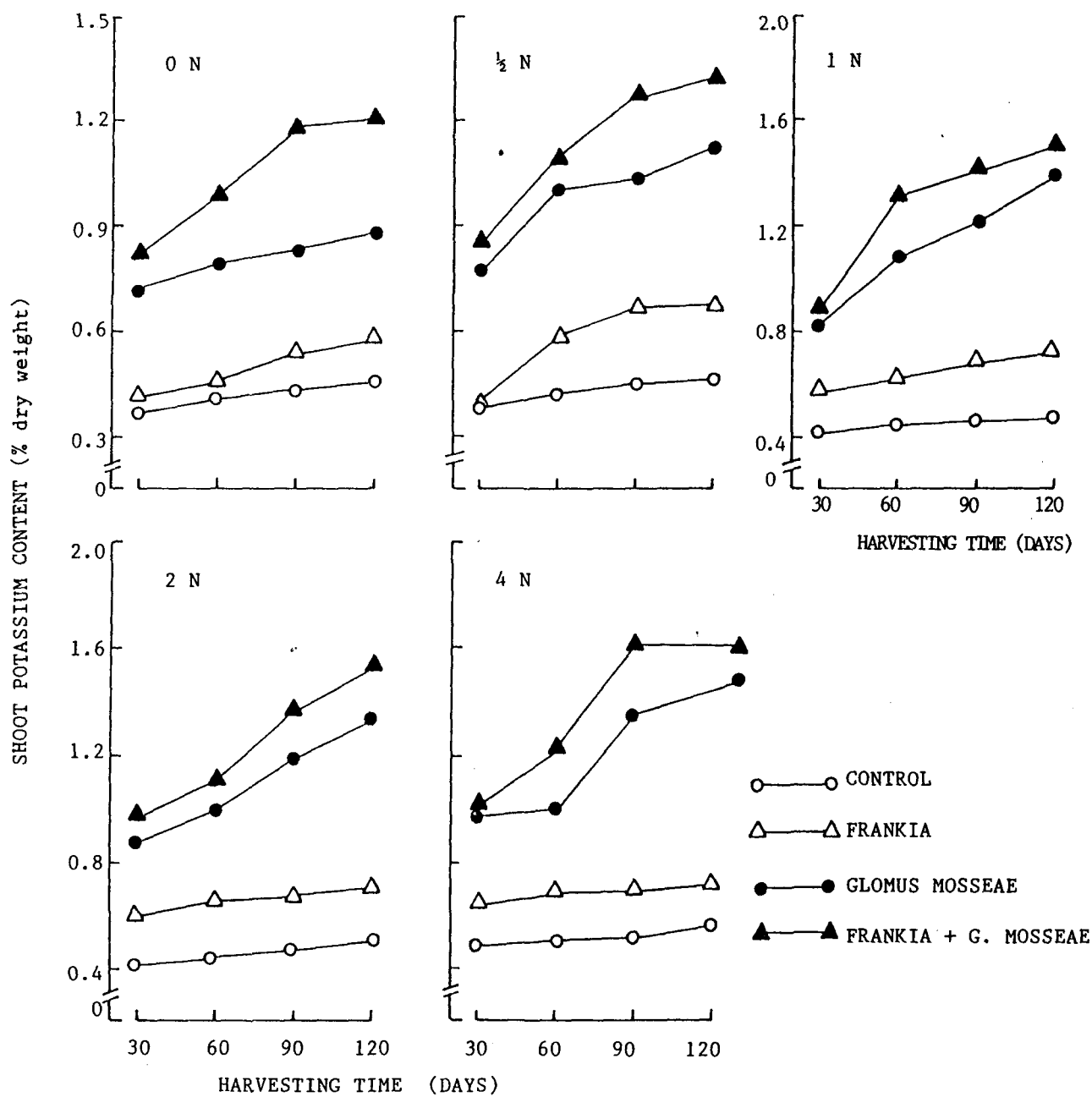


Fig.86a.Effect of Frankia, G. mosseae and Frankia + G. mosseae on shoot potassium content (%) of A. nepalensis seedlings at different levels of rock phosphate .

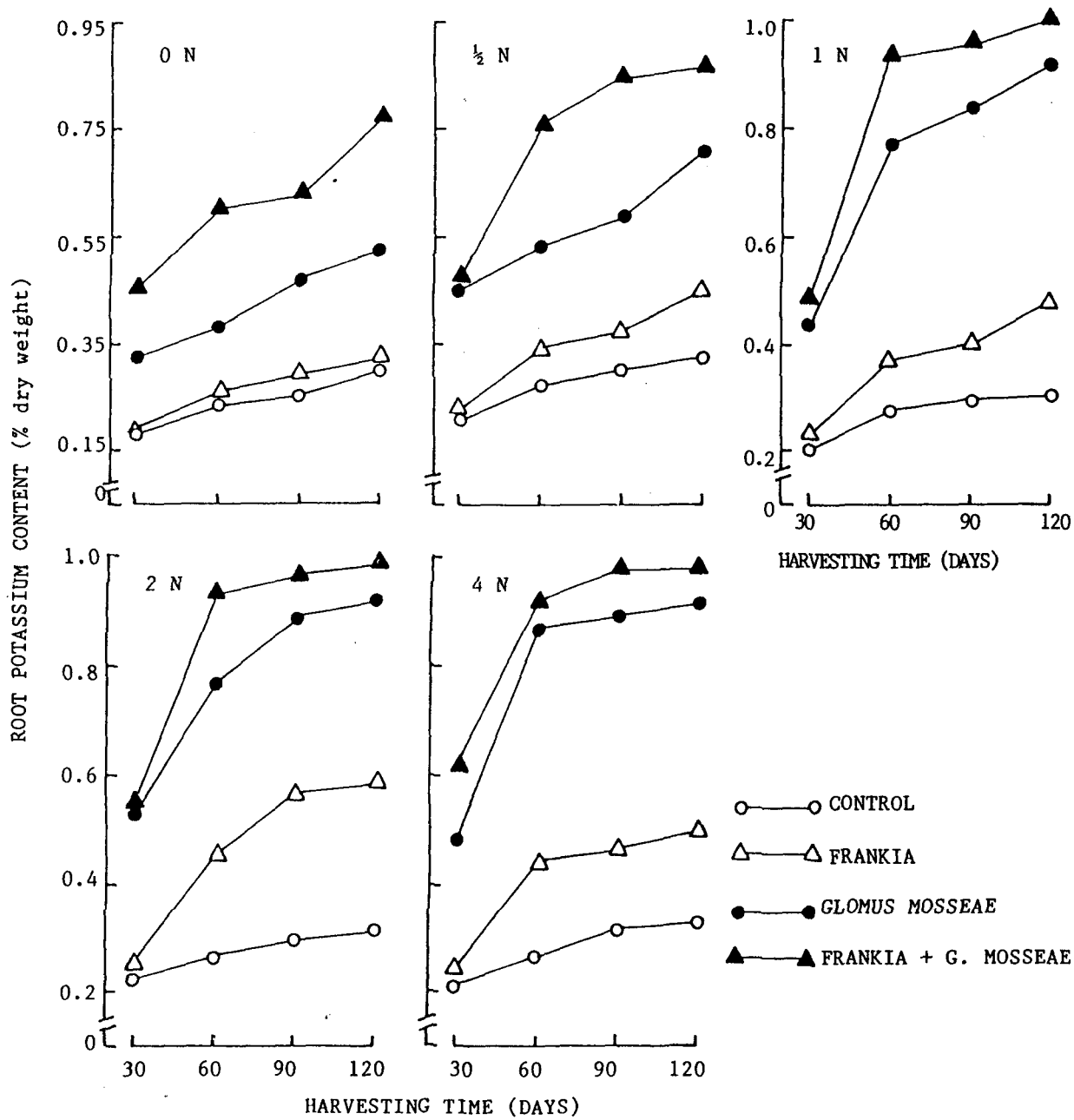


Fig.86b. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root potassium content (%) of *A. nepalensis* seedlings grown at different levels of rock phosphate.

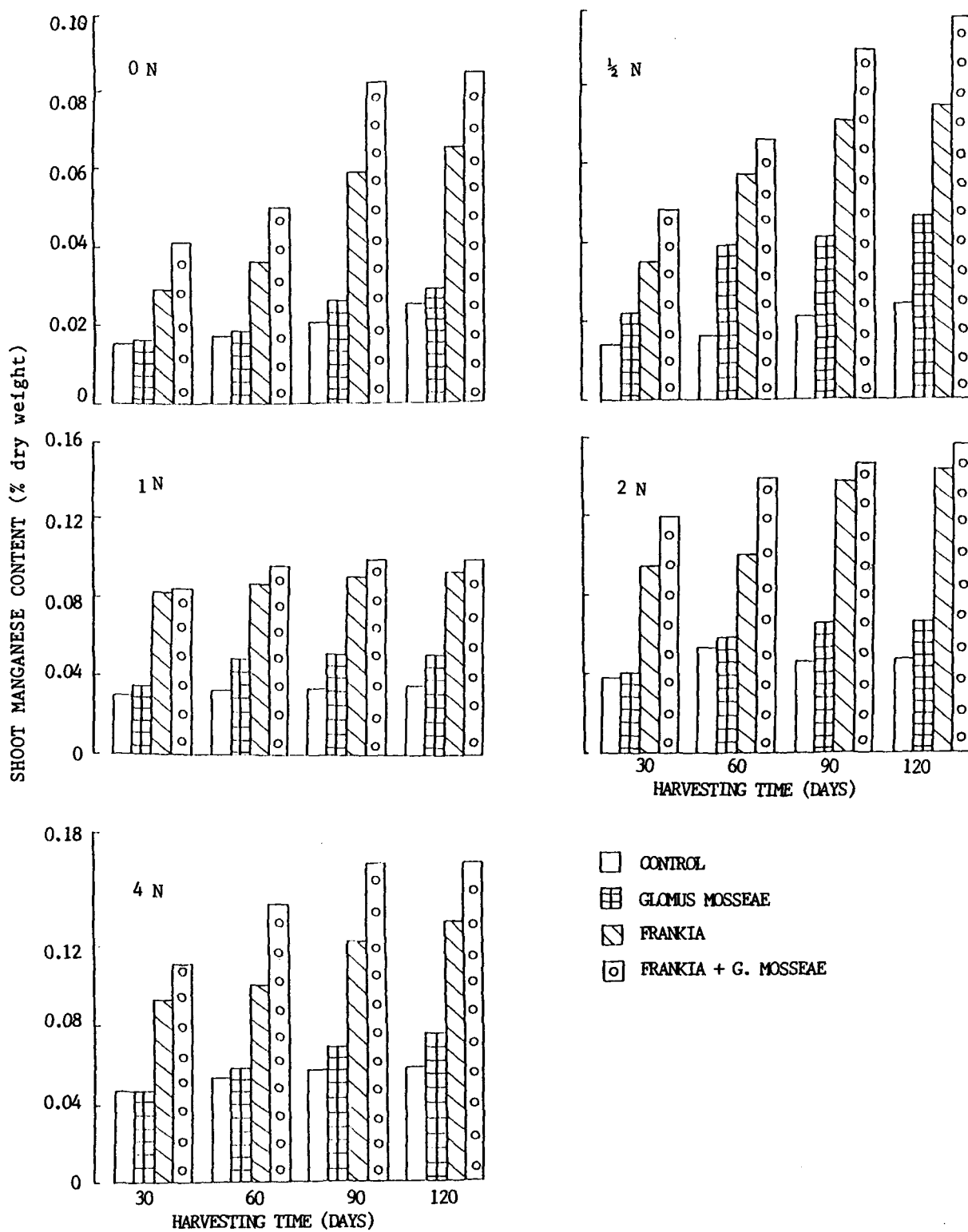


Fig.87a, Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on shoot manganese (Mn) content of *A. nepalensis* seedlings grown on soils with various levels of rock phosphate.

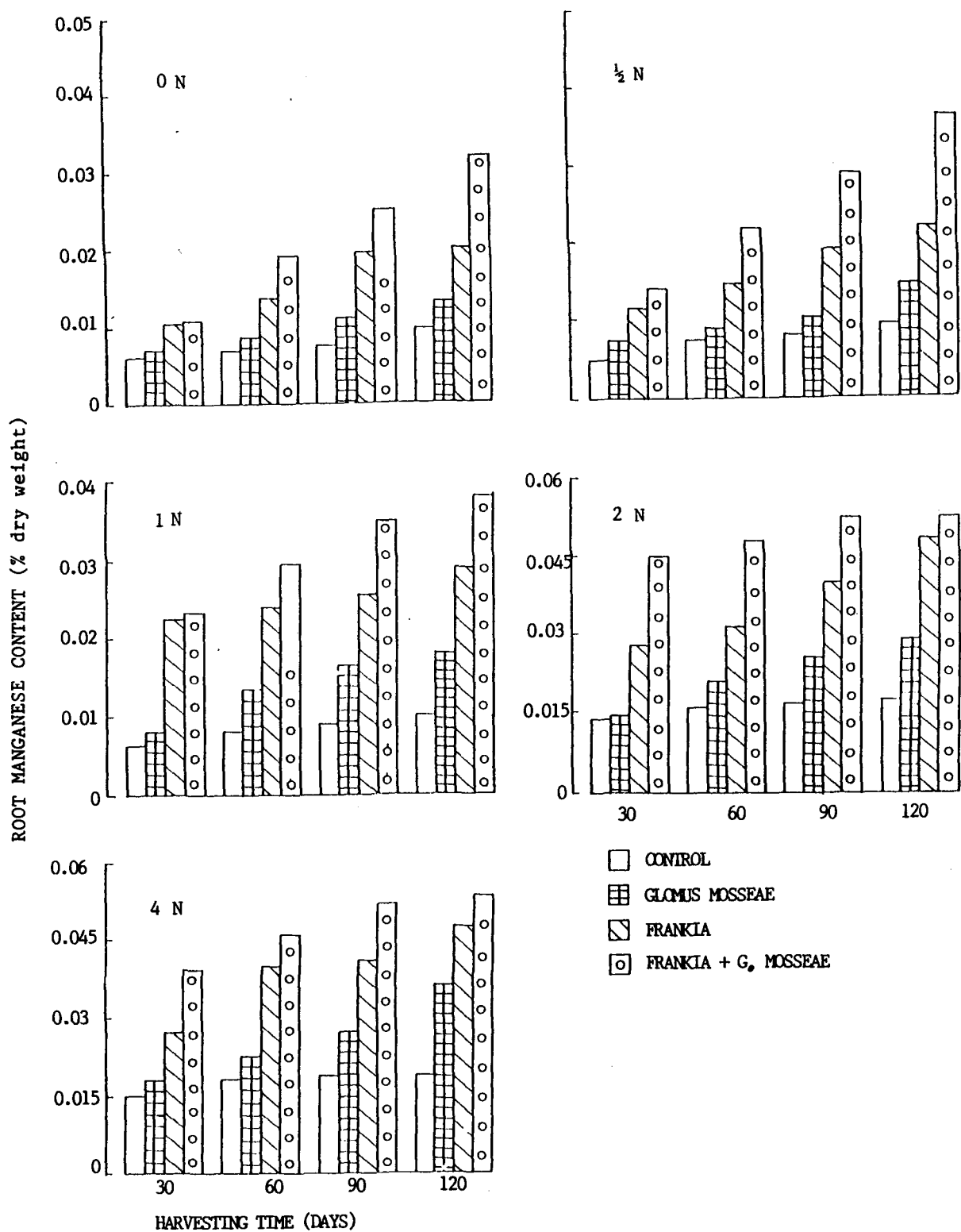


Fig.87b Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root manganese (%) content of *A. nepalensis* seedlings grown in soil with different levels of rock phosphate.

microendophytes (Table 38).

Mycorrhizal infection exhibited a significant positive correlation with number of nodules, nitrogenase activity and nodule biomass (Table 39). Further, a erratic relationship was established between VAM infection and *Frankia* population (Table 39).

Correlation between nitrogenase activity and mycorrhizal infection was positive at moderate level of RP. Insignificant positive correlation was established between nitrogenase activity and arbuscule and vesicle populations (Table 40).

Discussion :

The results showed that VA mycorrhizal association has profound influences on nitrogen fixation and growth of alder seedlings which has widely been supported from the investigations in mycorrhizal legumes especially growing on soils with low P availability (Hayman 1982; Tinker, 1982; Smith *et al.*, 1982; Barea *et al.*, 1989).

Better growth response of mycorrhizal plants at

Table 31. Effect of Frankia, *G. mossae* and Frankia + *G. mossae* on Shoot dry weight (mg seedling-1) of *A. nepalensis* grown at various levels of rock phosphates.

RP levels	Harvesting period (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	2.5	3.1	4.1	5.9	4.0	7.1	9.5	14.2	6.3	10.8	14.5	22.8	8.8	14.5	22.0	29.1
1/2	2.6	3.7	4.8	7.1	4.0	7.3	10.2	15.5	6.3	11.0	15.2	23.4	9.0	15.3	22.7	33.5
1	2.8	4.1	4.9	7.8	4.6	7.8	11.4	16.8	7.1	12.2	17.0	27.3	10.2	16.8	25.1	36.7
2	3.9	5.2	6.	10.6	5.9	9.5	13.9	23.9	8.8	14.3	19.5	34.8	12.5	19.3	28.8	45.7
4	4.8	6.8	7.7	13.4	7.9	14.2	16.1	25.5	11.0	20.3	21.8	36.7	15.1	25.1	30.9	47.9

1 = Control 2 = Frankia 3 = B. mesoae and 4 = Frankia + B. mesoae

Table 32. Effect of *Frankia*, *G. mosseae* and *Frankia* + *G. mosseae* on root dry weight (mg seedling⁻¹) of *A. nepalensis* seedlings grown at different levels of rock phosphate.

RP levels	Harvesting period (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	0.9	0.9	1.2	1.6	1.6	2.5	2.8	3.9	2.8	3.8	4.1	6.2	4.1	5.4	6.3	7.8
1/2	0.9	1.1	1.3	1.9	1.5	2.5	2.8	4.2	2.6	3.9	4.0	6.1	3.8	5.0	5.9	8.4
1	0.9	1.2	1.3	1.8	1.6	2.7	3.0	4.3	2.8	4.5	4.4	7.0	4.0	6.1	6.3	9.1
2	1.2	1.5	1.4	2.4	2.1	2.9	3.2	5.2	3.1	4.8	4.8	7.6	4.5	6.8	6.5	9.7
4	1.4	1.9	1.8	3.0	2.4	4.1	3.6	5.4	3.7	5.8	4.9	7.8	5.0	7.2	6.8	10.1

1 = Control 2 = *Frankia* 3 = *G. mosseae* & 4 = *Frankia* + *G. mosseae*

Table 33 . Effect of *Frankia*, *Glomus mosseae* and *Frankia* + *G. mosseae* on plant dry weight (mg seedling⁻¹) of *A. nepalensis* grown at various levels of rock phosphates (RP).

RP levels	Harvesting Period (Days)															
	30				60								90			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	3.4	4.0	3.3	7.5	5.6	9.6	12.3	18.1	9.1	4.6	18.6	29.0	12.9	19.9	28.4	36.9
1/2	3.5	4.8	5.1	9.0	5.6	9.9	13.0	19.7	8.9	14.9	19.3	29.5	12.9	20.3	28.6	41.9
1	3.8	5.4	6.2	9.6	6.3	10.5	14.5	21.2	10.0	16.7	21.4	34.3	14.2	22.9	31.4	45.9
2	5.1	6.7	7.6	13.0	8.0	12.4	17.1	29.1	11.9	19.1	24.3	42.4	17.1	26.1	35.4	55.4
4	6.2	8.7	9.6	16.4	10.3	18.3	19.8	31.0	14.7	26.1	16.7	44.5	20.1	32.3	37.7	58.0

1 = Control 2 = *Frankia* 3 = *G. mosseae* and 4 = *Frankia* + *G. mosseae*

Table 34. Effect of Frankia, *G. mosseae* and Frankia + *G. mosseae* on root/shoot ratio of *A. nepalensis* seedlings grown at different levels of rock phosphate.

RP levels	Harvesting period (days)															
	30				60				90				120			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	0.3	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.4	0.3	0.2	0.2	0.4	0.3	0.2	0.2
1/2	0.3	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.4	0.3	0.2	0.2	0.4	0.3	0.2	0.2
1	0.3	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.4	0.3	0.2	0.2	0.3	0.3	0.2	0.2
2	0.3	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.3	0.3	0.2	0.2
4	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.3	0.2	0.2	0.2

1 = Control 2 = Frankia 3 = *G. mosseae* & 4 = Frankia + *G. mosseae*

Table 35. F values of three way ANOVA for plant growth characteristics (shoot height, root length, plant height, shoot weight, root weight, plant weight, R/S ratio, stem collar diameter, leaf number and leaf area) as influenced by rock phosphate levels, root symbionts and harvests.

Sources of variation	Shoot height	Root length	Plant height	Shoot weight	Root weight	Plant weight	R/S ratio	Stem diameter	Leaf number	Leaf area
Rock P	80.47 ^{††}	1.09	89.32 ^{††}	256.49 ^{††}	134.76 ^{††}	278.41 ^{††}	161.48 ^{††}	151.66 ^{††}	46.11 ^{††}	138.49 ^{††}
Harvests	283.01 ^{††}	3.88 [†]	392.52 ^{††}	1727.01 ^{††}	2831.05 ^{††}	2284.44 ^{††}	28.90 ^{††}	1004.17 ^{††}	657.55 ^{††}	900.05 ^{††}
Symbionts	341.82 ^{††}	5.17 ^{††}	498.54 ^{††}	1550.97 ^{††}	1044.66 ^{††}	1731.05 ^{††}	908.90 ^{††}	1297.53 ^{††}	761.16 ^{††}	1662.35 ^{††}
Rock P x H	6.81 ^{††}	0.94	4.49 ^{††}	9.32 ^{††}	3.81 ^{††}	9.26 ^{††}	1.93	2.56 ^{††}	0.69	6.99 ^{††}
Rock P x S	4.88 ^{††}	0.97	8.56 ^{††}	17.73 ^{††}	6.53 ^{††}	17.70 ^{††}	5.42 ^{††}	6.71 ^{††}	3.04 ^{††}	4.18 ^{††}
H x S	21.01 ^{††}	0.35	30.33 ^{††}	120.67 ^{††}	67.89 ^{††}	131.06 ^{††}	21.15 ^{††}	37.77 ^{††}	13.01 ^{††}	66.09 ^{††}

†† Significant at P<0.01.

Without asterisk, statistically insignificant at above level.

Table 36. F values of three way ANOVA for Nodule number, Nodule dry weight, Nitrogenase activity and rhizospheric *Frankia* population as affected by rock phosphate, symbionts and harvests.

Sources of variation	Nodule number	Nodule weight	Nitrogenase activity	<i>Frankia</i> population
Rock Phosphate	79.62* *	23.50* *	66.46* *	70.75* *
Harvests	111.44* *	220.88* *	81.75* *	12.95* *
Symbionts	659.97* *	306.60* *	200.61* *	111.18* *
RP x H	2.21	0.77	2.60	0.85
RP x S	10.97* *	3.65* *	8.22* *	0.93
H x S	35.47* *	43.63* *	12.50* *	1.20

* * Significant at $P < 0.01$ level.

Without asterisk statistically insignificant at above level.

Table 37. F values of three way ANOVA for VAM infection, arbuscule and vesicle populations, spore abundance and rhizospheric phosphatase activity as influenced by rock phosphate levels, symbionts and harvests.

Sources of variation	VAM infection	Arbuscule number	Vesicle number	Endoganeous spore population	Total phosphatase activity
Rock Phosphate	6.83 [*] [*]	183.76 [*]	19.13 [*] [*]	8.92 [*] [*]	142.66 [*] [*]
Harvests	74.97 [*] [*]	24.55 [*] [*]	2.56	296.76 [*] [*]	1.22
Symbionts	51.01 [*] [*]	98.38 [*] [*]	16.43 [*] [*]	78.76 [*] [*]	133.80 [*] [*]
RP x H	1.09	20.54 [*] [*]	4.81 [*] [*]	2.09	4.66 [*] [*]
RP x S	1.79	4.63 [*]	0.54	1.79	9.0 [*] [*]
H x S	0.80	1.59	0.75	4.77 [*]	0.56

^{*}, ^{*} ^{*} Significant at P<0.05 and 0.01 level respectively.

Without asterisk statistically insignificant at above levels.

Table 38. F values of three way ANOVA for nutrient contents of root and shoot (nitrogen, phosphorus, potassium and manganese) as influenced by rock phosphate, symbionts and harvests.

Sources of variation	Shoot N	Root N	Shoot P	Root P	Shoot K	Root K	Shoot Mn	Root Mn
Rock Phosphate	28.60 ^{† †}	23.78 ^{† †}	131.98 ^{† †}	30.62 ^{† †}	36.41 ^{† †}	65.87 ^{† †}	3.95 [†]	165.70 ^{† †}
Harvests	175.35 ^{† †}	86.10 ^{† †}	298.77 ^{† †}	50.22 ^{† †}	73.38 ^{† †}	176.64 ^{† †}	0.41	63.28 ^{† †}
Symbionts	578.35 ^{† †}	259.16 ^{† †}	2045.1 ^{† †}	338.84 ^{† †}	715.64 ^{† †}	737.80 ^{† †}	13.35 ^{† †}	315.44 ^{† †}
RP x H	1.51	2.05 [†]	3.70 ^{† †}	1.26	1.47	3.90 ^{† †}	0.76	0.91
RP x S	8.58 ^{† †}	7.43 ^{† †}	10.40 ^{† †}	2.37 [†]	3.55 ^{† †}	9.77 ^{† †}	0.92	6.74 ^{† †}
H x S	43.60 ^{† †}	19.93 ^{† †}	30.01 ^{† †}	5.70 ^{† †}	12.58 ^{† †}	10.67 ^{† †}	0.65	3.79 ^{† †}

†, † † Significant at P<0.05 and 0.01 levels respectively.

Without asterisk statistically insignificant at above levels.

Table 39. Correlation coefficients (r) between mycorrhizal infection and number of nodule, nitrogenase activity, nodule dry weight and rhizospheric *Frankia* population at various rock phosphate levels.

Sources of variation	Rock phosphate amendments	Number of nodules	Nitrogenase activity	Nodule dry weight	Rhizospheric <i>Frankia</i> number
	0 N	0.986**	0.961**	0.930*	0.913*
	1/2 N	0.991**	0.918*	0.933*	0.382
Mycorrhizal infection (%)	1 N	0.726	0.672	0.584	0.615
	2 N	0.960*	0.993**	0.971**	0.986**
	4 N	0.976**	0.955*	0.832	0.438

*, ** Significant at $P < 0.05$ and 0.01 respectively.

Without asterisk statistically insignificant at these levels.

Table 40. Correlation coefficients (r) for nitrogenase activity, mycorrhizal infection, arbuscule, vesicle number of *A. nepalensis* and rhizospheric spore populations at different rock phosphate levels in soil.

Sources of variation	Rock P levels	Mycorrhizal infection	Arbuscule number	Vesicle number	Spore number
	0 N	0.988*	- 0.879	0.461	0.982*
	1/2 N	0.943	- 0.944	- 0.879	0.998**
Nitrogenase activity	1 N	0.618	0.531	0.861	0.965*
	2 N	0.998**	0.197	0.883	0.941
	4 N	0.955*	0.996**	0.928	0.962*

*, ** significant $P < 0.05$ and 0.01 respectively.

Without asterisk statistically insignificant at these levels.

to improved P uptake by mycorrhizal fungi (Hayman 1975; Powell and Daniell, 1978) mainly due to their capacity to absorb P from deficient soils and transfer it to host root (Rhodes and Gerdemann, 1978; Li *et al.*, 1991a & b). Mycorrhizal roots have been shown to take up several times more P than nonmycorrhizal ones (Harley, 1969). Higher biomass of mycorrhizal plants even at low soil P indicates the efficiency of mycorrhizal fungi at P stress conditions where non-mycorrhizal plants failed to grow which may be considered as an ecological advantage to these plants in colonizing soils of low fertility. Mosse *et al.* (1976) attributed superior growth of mycorrhizal plants to low threshold value of absorption compared to nonmycorrhizal ones. Thus the symbiosis enables plants to acquire optimal P with enhanced exploration of soil by external hyphae beyond the depletion zone (Hayman and Mosse, 1971 and Rhodes and Gerdemann, 1978).

The improved nodulation, nodule biomass, nitrogen fixation and consequently superior plant growth may be attributed to synergistic effect of VA mycorrhizal fungi on *Frankia* as observed in nodulated mycorrhizal legumes (Rendente and Reeves, 1981; Saif, 1987; Medina *et al.*, 1988; Barea *et al.*, 1989) which may be achieved by increased P uptake from P-deficient soils. Similar view is shared by

Carling *et al.* (1978), Asimi *et al.* (1980) and Bethlenfalvay and Yoder (1981). Thus improvement of P status may also influence other factors involved in the nitrogen fixation by *Frankia* such as trace elements, essential hormones and supply of photosynthates (Munns and Mosse, 1980; Manjunath and Bagyaraj, 1984). A stimulation in the *Frankia* abundance by VAM fungi in dually inoculated plants may be explained by the fact that root exudates of dually inoculated plants may highly favour proliferation of *Frankia*. This is substantiated by the findings of Gitte *et al.* (1978).

Higher level of rock phosphate was not found to depress the mycorrhizal status of *A. nepalensis* seedlings which was attributed to sparingly soluble nature of it. The findings of Barea *et al.* (1980) supported the results. However, increasing level of soluble P in growing medium has been shown to adversely affect mycorrhizal infection (Menge *et al.*, 1978).

Significant improvement in mycorrhizal infection by *G. mosseae* in dually inoculated (*Frankia* + *G. mosseae*) was attributed to synergistic behaviour of *Frankia* on infection by *G. mosseae*. This may be mediated by hormonal action as noted in legumes (Azcon *et al.*, 1978; Barea and Azcon-Aguilar,

1983) polysaccharides (Azcon *et al.*, 1980), or directly affecting fungal metabolism (Paccini *et al.*, 1988). It may also involve alteration of proteins (Young *et al.*, 1972; Krishna and Bagyaraj, 1983). Further higher population of arbuscules and in dually inoculated seedlings may be practically important in the higher exchange of nutrients (Cox *et al.*, 1975) consequently leading to higher nitrogen fixation and plant growth. Enhanced production of endogonaceous spores by VAMfungi in nodulated + mycorrhizal plants may have implications in the achievement of higher mycorrhizal infection of host root thereby stimulating nodulation and nitrogen fixation.

The presence of phosphatase activity only in mycorrhizal and nodulated mycorrhizal plants indicate that majority of the activity is fungal specific as noted by Gianinazzi-Pearson and Gianinazzi (1978). Improvement in mycorrhiza-specific phosphatase activity in nodulated + mycorrhizal plants may be assigned to stimulated activity of *Frankia* in order to provide more P and other nutrients to the host thereby increasing nitrogen fixation and predisposing host to grow luxuriously in nutrient (P) deficient situations. The role of phosphatase in the conversion of unavailable P into available form is well known. Higher

activity in little RP amendment/unamended soil signifies available P stress, which triggers enzyme synthesis.

Improvement in the nutrient contents i.e. N, P, K and Mn of nodulated + mycorrhizal plants may be assigned to greater exploration of soils by VAM hyphae leading to increased uptake of these elements. Improved acquisition of these nutrients by VA mycorrhizal fungi in nonlegumes has also been previously demonstrated (Rose and Youngberg, 1981; Gardner *et al.*, 1984). This improved nutritional status of tripartite symbiosis may be achieved by beneficial interactions of microendophytes and thus be responsible for the increased growth of the host plant in degraded habitats where those with single or without symbiosis fail to establish (Harley, 1970; Harley, 1973; Daft and Hacskeylo, 1976).

GENERAL DISCUSSION

Seasonality in the activity of nitrogenase enzyme (nitrogen fixing rate) in root nodules of *Alnus nepalensis* D. Don under field condition was observed. It also ^{has} been noted by other investigators (Akkermans, 1971; Pizelle, 1975; Tripps, 1979; Sharma and Ambasht, 1984; Sharma, 1988). Seasonal changes in nitrogen fixing activity of nodules were due to the complex interaction between a variety of soil, climatic, host and endophyte (*Frankia*) factors. The peak enzyme activity during rainy season may be attributed to conducive moisture, temperature, soil pH and soil nutrients which were directly related to nitrogen fixation by Himalayan alder. Sharma (1988) and Huss-Danell *et al.* (1989) established a significant relationship between former and soil temperature. Soil variables like soil moisture also exert marked influence on nodulation and nitrogen fixation (Righetti *et al.*, 1986; Sharma 1988).

Apart from role of P which is indispensable

for nitrogen fixation (Russo, 1989; Jha *et al.*, 1993), other essential nutrients like Ca (Tisa and Ensign, 1987) and Mo (Zhiznevskaya *et al.*, 1982) are also intimately associated with former. Onset of nitrogen fixation coincided with sprouting of leaf buds in spring and a peak activity during rainy season signify the importance of photosynthate in the regulation of atmospheric fixation of nitrogen by *Frankia* induced alder nodules. Wheeler (1971), Wheeler *et al.* (1983), Wheeler and Lawrie (1976), Huss-Danell (1978) and Huss-Danell and Sellstedt (1985) also have demonstrated the role of photosynthate in nitrogenase activity in many actinorhizal species. The metabolic state of nodule endophyte i.e. *Frankia* itself is important which exhibits pleomorphic characteristics (Lalonde and Calvert, 1979) and the vesicular form is mainly associated with nitrogen fixation (Akkermans, 1982; Akkermans and Houwers, 1983; Norridge and Benson, 1986; Meester, 1987). Similarly decrease in the enzyme activity during winter may be related to water stress (Dalton and Zobel, 1977; Seilor and Johnson, 1984), low temperature (Winship and Tjepkema, 1985; Huss-Danell *et al.*, 1987), soil acidity (Guofan and Tingxiu, 1987), nutrient stress and competition for metabolic factors (Pizzelle, 1984).

The proportion of active (young and mature

aged) nodules to old and senescing ones varied among various seasons which may be attributed to continuous transition of nodules of one age class into another with increase in time factor. This finding is in conformity with the investigation of Sharma and Ambasht (1986). Restriction of root nodules to uppermost 15 cm soil depth (D_1) indicated the importance of aeration in nodulation. Sharma (1988), Rytter (1989) and Kurdali *et al.* (1991) have also observed a similar vertical pattern of nodule distribution in various alder stands. In the present investigation *A. nepalensis* produced sufficiently more nodule biomass unit area⁻¹ during rainy season and least during winter which agree with the data of Akkermans and Van Dijk (1976) and Sharma and Ambasht (1986), which coincided with seasonal fixation of atmospheric nitrogen. This implies that nodule tissue is directly associated with nitrogen fixation by Himalayan alder.

High abundance of vesicles of *in vivo Frankia* during rainy season was directly correlated with nitrogen fixation which indicated their importance in the process. Mian and Bond (1978) also found that nitrogenase activity was induced concurrently with spontaneous production of vesicles by endophyte in the root nodules of *Alnus nitida*. Symbiotic *Frankia* also existed in the form of hyphal masses (microcolonies) and sporangia whose intensity ^{was} different in _^

different zones of cortical tissue and seasons. High abundance of sporangia in the cortical cells of middle and basal zones of nodules coincided with cessation of nitrogenase activity during winter. This may be ecologically important from the view point of nitrogen fixing activity by *Frankia* and latter's dispersal into soil after nodule death and decay (Akkermans and Van Dijk, 1981).

Population of soil *Frankia* exhibited a marked seasonality. Climatic, soil and host factors were mainly responsible for it. Conducive climatic and edaphic regimes were instrumental for high abundance of *Frankia* during rainy season. Rodriguez-Barrueco (1968) and Houwers and Akkermans (1981) too have indicated poor nodulation capacity in acidic soil which increased positively with increase in medium/soil pH. Further, the amount of host root exudate supporting extranodular *Frankia* in soil may be important from the view point of its independent proliferation. During rainy season, a part of total photosynthate fixed by host may be secreted in the rhizosphere soils in the form of root exudates which may highly favour its growth. The quantity and the nature of exudates may also regulate its vertical distribution in soil. In this investigation *Frankia* propagules were found to be mostly confined to soil depth D₁

where a majority of fine roots were present, which decreased exponentially with increasing soil depth. Our finding is supported by Lalonde *et al.* (1981) who showed the stimulatory effect of host on *Frankia* abundance in its rhizosphere. Besides, aeration and other unidentified factors (Van Dijk, 1984) may be operative in the vertical distribution of soil *Frankia*.

Production of more vesicles in the nodule cortex by *Frankia* during rainy season coincided positively to the nitrogenase activity and nodule N content. This indicates the fact that vesicles are in fact genuine site of nitrogen fixation in root nodules. However, varied intensity in the nodule cortex may be related to the metabolic state of host cell (Akkermans and Van Dijk, 1976).

Mycorrhizal association which may be considered as an adaptive strategy of higher plants in nutritionally stressed conditions was a rule rather than exception. Actinorrhizal *A. nepalensis* harboured mycorrhizal symbionts thus forming formidable tripartite association in colonizing nutrient poor soil conditions. High dependence of non-leguminous host on VAM association (Danielson and Visser, 1990) evaluated in terms of mycorrhizal intensity which was seldom less than 90 %, is thus evident. This association

helps host in the uptake of growth-limiting nutrients especially non-labile ones like P and other essential elements consequently leading to higher nitrogen fixation and plant productivity in forest ecosystem where P cycle is closed (Mosse and Philips, 1971).

Availability of soil P and its concentration in host tissue may be important to determining its intensity (Mosse, 1973; Braunberger *et al.*, 1991). In addition, intensity of infection may be related to other soil factors (Hayman, 1970; Furlan and Fortin, 1973) and host characteristics (Mosse, 1973; Thompson *et al.*, 1991). A low level of mycorrhizal intensity during rainy season may have accomplished due to increase in tissue P content which may be instrumental in regulating VA colonization (Ross, 1971; Mosse, 1973; Menge *et al.*, 1978; Robson and Abbott, 1986). In addition, an increased level of soil moisture during rainy season may have depressed infection by VA endophyte. Khan (1974) ~~also~~ observed a similar reduction in root infection at high soil moisture content. Besides, a reduced level of VAM infection under field conditions may have been accomplished as a result of mutual microendophytic understanding in terms of tolerance of environmental conditions ensuing prolonged survival and benefit to host and

endophytes .

Production of more arbuscules by VAMfungi in mycorrhizal roots of *A. nepalensis* during rainy season may have some ecological implication as ^{the}former was positively related to nitrogen fixation by *Frankia*. This signifies that arbuscules are the site of exchange of metabolite and P between host and VA endophyte which may be beneficial for nodulation and nitrogen fixation. Cox *et al.* (1975) and Gardner *et al.* (1984) have also demonstrated the role of arbuscule in the exchange of materials between fungus and the host. Further, an increased abundance of vesicles during winter (dormant) season may be important in the release of stored nutrients to host during period of nutrient deficiency (Gerde mann and Trappe, 1974), which contribute as well to inoculum potential of fungus (Gerde mann and Trappe, 1974; Biermann and Linderman, 1983).

A high abundance of endogonaceous spores during winter unlike its coendophyte *Frankia*, was observed. Gemma and Koske (1988) also recorded high spore density during winter. This may be due to differential response of VAMendophyte to prevailing soil and climatic conditions. Low soil temperature and low soil moisture during winter may be

responsible for the induction of sporulation by endophyte fungus which enable it to tide over unfavourable conditions ensuing renewed infection and growth ^{on} following growing season. However, this spacing in time of peak endophytic abundance may be a predetermined strategy by them to avoid interendophytic competition in space and in time. The host root exudates may play an important role in the confinement of majority of endogonaceous spores in upper soil depth (Gianinazzi-Pearson *et al.*, 1989). Abundance of VAM fungal species which varied in time and in space may be related to adaptive capacity of fungal symbionts to a particular environment. Stahl and Christensen⁽¹⁹⁸²⁾ noted adaptation of VAM species to the particular soil conditions. The result of McGee (1989) also suggests that different species of VAM fungi have different survival strategies for existence. The dominance of *G. mosseae* in time and in space over other fungal species may be related to vigourousity of the species which in addition, may also be influenced by selective influence by host plant species on ^a particular site. Besides, a ephemeral nature of *Gigaspora* species in present investigation may be explained by selective influence of soil factor like temperature which may favour its growth. The ^{very} support is afforded by the investigations of Verma (1982).

Occurrence of more than one fungal species in

a specific site may indicate the non-specificity of VAM fungi which may be substantiated by occupation of host root by fungal species with varied hyphal dimension. McGonigle and Fitter (1988) and Rosendahl *et al.* (1990) in their investigations also showed that under field conditions a single host was colonized simultaneously by many VAM species.

The success of microdissection with double agar layer technique in the isolation of symbiotic *Frankia* from root nodules of *A. nepalensis* growing under field conditions may be attributed to efficient elimination of contaminating organisms by H_2O_2 (Diem *et al.*, 1983) or OsO_4 (Lalonde *et al.*, 1981) treatment. Further, peeling of surface epidermis, microaerophilic conditions as afforded by double agar layer which impart efficient separation of residual contaminants in space may be instrumental in the isolation of *Frankia*. Modified sucrose fractionation gradient technique coupled with pour plate and double agar layer was also effective in the isolation of nodule endophyte in pure culture. Baker and O'Keefe (1984) also successfully isolated numerous *Frankia* strains from nodules and soil using ^{the} above technique. In addition, modified crushing technique was employed successfully in the isolation of *Frankia* from *A. nepalensis* nodules in liquid media. This may

have been accomplished by efficient removal of contaminating organisms and release of *Frankia* propagules in sufficient amount to effect its growth in synthetic media. The amount of inoculum has also been considered to influence successful isolation of nodule endophyte in axenic culture (Rosbrook, 1990).

A high sporulation by *Frankia* isolates may be a phenomenon related to C-source as noted by Tisa *et al.* (1983).

Symbiotic *Frankia* isolated from the nodule of *A. nepalensis* were categorised into 3 groups on the basis of morphological, physiological and host nodulation characteristics, which differed markedly from each other. This observation agrees with the findings of other investigators who isolated and characterized more than one *Frankia* strains from root nodules of actinorhizal plants growing over a single locale (Benson, 1982; Lechevalier *et al.*, 1983; Hafeez *et al.*, 1984; Baker, 1987).

The fact that most of the *Frankia* isolates utilized propionate as carbon source indicates that these endophytes possess the enzyme system to utilize above carbon source. The findings of Murry *et al.* (1984) substantiate

above result. However, a different response of *Frankia* isolates in their capacity to metabolize other carbon source/ for the growth may be explained by the presence or absence of glycolytic pathway activity. Similar variation in the utilization of carbon source by *Frankia* strains isolated from same host has previously been reported (Blom, 1982; Murry et al., 1984).

Suggest

Utilization of ~~varied~~ carbon sources by *Frankia* strains suggests that they could act as secondary colonizers utilizing simple breakdown products released through the enzyme activities of other soil microbes. Many organic acids are commonly found in the aerobic and anaerobic soils. Root exudates contain a range of organic acids, amino acids and many simple carbohydrates which include those most commonly found to be utilizable by them (Rovira, 1965) suggesting that constituents for the growth of extranodular *Frankia* are commonly present in soils.

Differences in the growth performance of *Frankia* isolates in medium supplemented with different nitrogen sources was observed in which NH_4Cl , in majority of cases, was better utilized by *Frankia* isolates. Preferences of *Frankia* to NH_4Cl as N source have also been noted by Shipton

and Burggraaf (1982), Lechevalier *et al.* (1983) and Shipton and Burggraaf (1983). Poor growth on yeast of *Frankia* isolates may be related to lack of uptake system.

Suitable growth of majority of *Frankia* at a temperature range between 28-30°C as found earlier (Burggraaf and Shipton, 1982; Hafeez *et al.*, 1984) reveals that temperature maxima for all strain overlap. However, the competition for infection, nodulation and effective symbiosis by them in addition, may also involve competitive and adaptive ability of the strains (Kurdali *et al.*, 1990).

Higher versatility of AnF₁ and AnF₂ at pH around 6 and 7 indicates that these strains are neutrophilic and possibly may form effective symbiosis with host at above soil pH range. On ^{the} contrary, better growth response of AnF₃ at lower pH than other strains suggests their potentiality to grow on low pH soils where other coendophytes are unable to survive and form symbiosis.

Varied nodulation speed of *Frankia* isolates on its original host was evident which may be ascribed to the degree of infectivity and compatibility of host-endophyte combination. Greater vigor^uosity of *Frankia* AnF₁ compared to

other endophytes may provide additional assets to it in the formation of association with its host.

Further, *in vivo* differences in induction and quantity of nitrogen fixation by root nodules of Himalayan alder as induced by various *Frankia* strains may be attributed to differences in effectivity and compatability with their host. Dillon and Baker (1982) also observed a marked variation in the nitrogenase activity of various host-endophyte combinations.

Higher rate of nitrogenase activity of *Frankia* isolate especially in medium amended with propionate as carbon-source may be explained by the fact that ^{the} latter may be suitable energy source which may stimulate endophyte to produce more enzyme. Conversely, depression in nitrogenase activity of NH_4^+ grown cells (Callaham *et al.*, 1978; Fontaine *et al.*, 1984; Murry *et al.*, 1984) may be related to suppression of enzyme nitrogenase in the presence of exogenous N.

The	dominance of <i>Frankia</i> AnF ₁ in most of the
seasons of the	year may be attributed to suitable

environmental conditions for extranodular growth. In addition, it also may be ascribed to compatibility with its host and selectivity of latter as well (Faure-Raynaud *et al.*, 1992).

The results have shown that tripartite association involving *Alnus-Frankia-Glomus* is more efficient in terms of seedling growth ^{than} ~~as compared to~~ those with single or without symbionts over a range of soil pH. This enhancement of host growth may be achieved by positive interaction between microendophytes in terms of uptake and accumulation of nutrients especially P and N leading to optimal host performance. This ameliorative growth in dually inoculated plants was maximum at soil pH 6, which may be attributed to availability of majority of nutrients essential for plant growth and their effective utilization by endophytes. The effectivity of latter may also depend upon pH range of growing medium as both the endophyte were effective in slightly acidic soil. This may be attributed to the adaptation of both *Frankia* and *G. mosseae* in *A. nepalensis* stand where average soil pH ranged from 5.3 - 5.7 during January (winter) and July (rainy) respectively. Poor performance of seedlings infected with *Frankia* and *G. mosseae* in soil with lower pH than 5.0 may be explained by detrimental effects of soil acidity on survival and/or

efficiency of microsymbionts either in bipartite or tripartite association involving *Alnus*. Low survival and nodulation capacity of *Frankia* in acid soils/environment have been shown by Burggraaf and Shipton (1982), Shipton and Burggraaf (1983) and Smolander *et al.* (1988). Similarly Hayman and Mosse (1971) observed negligible infection of host root by VAM fungi in acid soil of pH 3.3 - 4.6. Further, toxicity of Al and other micronutrients at low pH also may be responsible for depressed growth of *A. nepalensis* seedling either with or without symbiosis. Similar instances of Al toxicity on root and overall plant growth under acidic conditions were observed by other investigators

(Sheppard and Floate, 1984). The uptake and translocation of P, Ca, Mg, K, Fe, Zn, Cu, and Mn by plants may severely be affected by Al toxicity (Hutchinson *et al.*, 1971; Mugwira, 1980).

Ameliorative nodulation, nodule number, nodule weight, nitrogen fixation and *Frankia* abundance in tripartite symbiosis at a pH range around and above 5.0 may be important from the view point of performance of Himalayan alder seedlings in harsh habitats. This effect of *G. mosseae* on *Frankia* may have been accomplished by improved supply of P. The support comes from the fact that nonmycorrhizal actinorhizal (*Alnus*) plants lagged that of former in most of

nodule aspects which explain the beneficial role of VAM fungus on it. However, the role of hormones or unknown mechanism (Munns and Mosse, 1980; Fragabeddiar and Tacon, 1990) may not be excluded, which requires further investigation. However, depression in nodule characteristic by *Frankia* even in the presence of VAM fungi at pH 4 may be related to detrimental effects of acidity on survival of both microendophytes (Burggraaf and Shipton, 1982; Smolander *et al.*, 1988; Kruckelmann, 1973; Green *et al.*, 1976) as both the root endophytes were isolated from soil with pH range 5.3-5.7. Under such (acidic soil) conditions, mycorrhizal symbiosis, if formed may also be ineffective in imparting benefits to the host (Danielson and Visser, 1989; Arines and Vilarino, 1991).

VAM infection, arbuscule and spore population of *G. mosseae* were remarkably improved in the presence of *Frankia* at pH range of 5-7 thus indicating a direct positive interaction. Induced increase in VAM characteristics by *Frankia* may be related to synergistic effect of latter on former (Berlinger and Torrey, 1989). Increased population of rhizosphere spores of *G. mosseae* in dually inoculated plants was evident. Qualitative and quantitative changes in the root exudates and hormones selectively promotory to VA

endophyte by *Frankia* (Wheeler *et al.*, 1979) may be responsible for the ameliorative effects of latter on former. However, decreased synergistic effect of nodule endophyte on VAM at highly acidic soils may be attributed to harmful effect of latter on *Frankia* survival (Rodriguez-Barrueco, 1968; Smolander *et al.*, 1988) which may consequently hamper ameliorative effect on VA mycorrhizal endophyte.

Presence of phosphatase activity only in *G. mosseae* and *Frankia + G. mosseae* inoculated plants suggests that the enzyme/s system may be of fungal origin (Gianinazzi-Pearson and Gianinazzi, 1978). Increased activity of the enzyme at acidic pH may be instrumental in colonization of P deficient harsh habitats by these groups of plants (Daft and Hacskeylo, 1976).

Analysis of nutrients in seedlings indicated that uptake and assimilation of N, P, K and Mn in most of the occasions was greater in tripartite symbiosis than in bipartite or those without symbiosis. Similar observation has been made by Rose and Youngberg (1981), Gardner *et al.* (1984) and Russo (1989). The increased uptake of nutrients by dually inoculated plants under infertile soil conditions may be related to synergistic interactions between *Frankia* and *G. mosseae*, which is inevitably related to robust growth

of the host.

The improved growth of *A. nepalensis* seedlings with *Frankia* + *G. mosseae* over singly inoculated (either with *Frankia* only or *G. mosseae*) and uninoculated controls at most levels of organic matter may be attributed to improved nutritional status especially N and P nutrition. The role of *G. mosseae* was evident in improving P status by extramatrical mycelial network beyond the depletion zone which remained absent in nonmycorrhizal counterparts (Rhodes and Gerdemann, 1975; Xiao *et al.*, 1991). Besides, the nutrient conserving capacity of mycorrhizal fungi in their tissue and its release whenever in short supply may also afford considerable advantage over nonmycorrhizal plants especially in nutrient -deficient habitats.

The difference in growth response of dually, singly and uninoculated however, decreased with increasing level of soil organic matter which may be due to availability of growth -limiting nutrients. The role of mycorrhizal and nodule symbionts in improving plant growth is evident especially under nonfertile conditions (Mosse, 1973; Asimi *et al.*, 1980) which, however, does not confer benefit under the situations where nutrients are plentiful.

Further, dramatic reduction in growth parameters of all groups of plants at 4 N level of organic matter may be attributed to supraoptimal level of nutrients which may consequently affect colonization and beneficial role by microendophytes. The decrease in soil pH and increase in phenolic substances with organic amendment may have caused harmful effects on microendophytes (Smolander *et al.*, 1988; Peterson *et al.*, 1991).

A complete inhibition of nodulation and nitrogen fixation at 4 N organic matter level may have resulted due to supraoptimal level of nutrients especially combined N and partly be attributed to detrimental effects of plant phenolics on *Frankia* and *G. mosseae*. Perradin *et al.* (1983) and Vogel and Dawson (1985) showed that plant phenolic compounds have deleterious effect on *Frankia* survival while Kohls and Baker (1989) also have demonstrated the suppression of nodulation by high nitrate level in the growth medium.

In contrast, the enhancement of nodulation, nodule biomass and nitrogen fixation by *Frankia* in the presence of *G. mosseae* especially at lower doses of organic matter may be attributed to synergistic effects of endophytic fungus on *Frankia* activity which may be brought about by improved P nutrition (Gardner *et al.*, 1984; Russo, 1989; Jha

et al., 1993) which also may influence other factors like supply of photosynthate, trace elements and essential hormones (Munns and Mosse, 1980; Manjunath and Bagyaraj, 1984). Inability of optimal nutritional conditions in stimulating nodule characteristics in the present investigation, may partly support the hypothesis that VAM fungus, in addition to P supply, may also act by means of hormonal action or some unknown mechanism (Fraga-Beddiar and Tacon, 1990). In fact, VA endophyte preferentially influencing nodule function as noted in *Glycine-Rhizobium-Glomus* association (Barea and Azcon-Aguilar, 1983; Van Nuffelsen and Schenck, 1984) may be expected. However, the actual mechanism of action remains to be elucidated. Higher abundance of rhizosphere *Frankia* in tripartite association involving *Alnus-Frankia-Glomus* especially at low soil organic matter level may be attributed to qualitative and quantitative changes in the root exudates of mycorrhizal plants (Gitte *et al.*, 1978). In fact, hormones and compounds essential for the extranodular growth of *Frankia* may also be involved in synergistic effect of *Glomus* on earlier (Munn and Mosse, 1980; Krishna and Bagyaraj, 1983).

An ameliorated mycorrhizal intensity, arbuscules and endogonaceous spore abundance in dually

inoculated (*Frankia* + *G. mosseae*) seedlings at low organic matter doses are ecologically important. This synergistic effect may be related to improvement in uptake and transfer of P and other essential nutrients directly involved in nitrogen fixation and overall plant growth. However, this may be mediated by nutritional, hormonal or other unknown mechanism. Paccini *et al.* (1988) concluded from their studies that rhizobial effect on VA symbiont may be direct by affecting fungal metabolism which does not occur via TCA cycle. However, possibility of hormonal action may not be excluded as quantitative changes in the hormonal content of *Frankia* colonized plants are evident (Wheeler *et al.*, 1979).

The result of phosphatase activity of rhizosphere soil indicates that enhancement of the activity in dually inoculated plants. This synergistic effect of *Frankia* on *G. mosseae* at low pH soil may be important from the viewpoint of acquisition of P by plants which becomes limiting to plants with single symbiont or without it.

Superiority of dually inoculated plants over singly or uninoculated seedlings in terms of nutrient accumulation at low fertility level clearly indicates that both the endophytes help in acquisition of above

nutrients and predispose host to establish in nutrient deficient hostile habitats where those with single association or without it fail to survive (Daft and Hacskeylo, 1976).

Improvement in the growth response of dually inoculated (*Frankia* + *G. mosseae*) over singly or control seedlings over a range of rock phosphates may be attributed to proper exploitation, utilization of rock phosphate and assimilation of atmospheric nitrogen. The mycorrhizal plants performed ^{better than} nonmycorrhizal ones with the virtue of possession of extramatrical hyphae which provide extra absorbing organ beyond the depletion zone. As P is critical factor for plant growth and nitrogen fixation, presence of VAM fungi may be important in the productivity of tree seedlings in forest ecosystems where P is in short supply. However, the capacity of mycorrhiza in the mineralization of organic phosphate sources (Jayachandran et al., 1992) will provide mycorrhizal plants an additional asset over nonmycorrhizal one. The improvement in growth of dually inoculated plants at elevated level of rock phosphate unlike soluble P sources, indicates that sparingly soluble source of P may be beneficial in the productivity of tree seedlings. Moreover, with further increase in rock phosphate level, the growth of either *Frankia* and noninoculated plants

also increased. This may be due to increased availability of P which otherwise was insufficient to them at lower doses of rock phosphate application. Mycorrhizal + nodulated plants were however, more effective even at lower level of rock phosphate which may be attributed to efficient utilization of P source by *G. mosseae*. Less root to shoot ratio of dually inoculated seedlings over singly or noninoculated ones indicated more proportionate growth. Rose and Youngberg (1981) have also indicated similar results.

Ameliorative effects of *G. mosseae* on *Frankia* induced nodule characteristics were evident which may be attributed to improved uptake and utilization of rock phosphate by the earlier one. The present investigation revealed that the highest level of rock phosphate application did not inhibit the nodulation and nitrogen fixation where application of soluble P source at high level caused detrimental effect on mycorrhizal and nodule characteristics as in leguminous system (Asimi *et al.*, 1980). Apart from the indirect influence of *G. mosseae* on *Frankia*, qualitative and quantitative alteration of root exudates and hormones by VAM fungus stimulating proliferation, entry and effectivity of *Frankia* may also be true. The support comes from the fact that even highest P amendment in the form of rock phosphate did not stimulate

Frankia.

An increment in mycorrhizal infection and other characteristics of *G. mosseae* in tripartite over bipartite i.e. *Alnus-G. mosseae* association was clear which may be attributed to qualitative and quantitative changes in hormones by *Frankia* (Wheeler *et al.*, 1979) or other mechanism involving endophyte-endophyte interactions. This feature of endophyte interactions involving synergistic role on activities of each other may be important which consequently leads to robust growth of their hosts under nutrient deficient conditions where plants with single symbiosis or without it fails to perform well.

Results of phosphatase activity of rhizospheric soil showed that only mycorrhizal i.e. *Frankia* + *G. mosseae* and *G. mosseae*) inoculated plants possessed the activity which may be related to the fact that the enzymes may be fungal specific (Gianinazzi-Pearson and Gianinazzi, 1978). The activity of the enzymes was stimulated by *Frankia*. Further, higher activities in little RP amended soil signify the P stress, which triggers enzyme synthesis by *G. mosseae*. The investigation of Tarafdar *et al.* (1989) supported above finding. Excretion of the enzyme may be instrumental in the efficient utilization of nonlabile soil P

by mycorrhizal plants. Ecological implication of the possession of the enzyme by *G. mosseae* leading to improved P supply may directly predispose host to fix more nitrogen and to grow luxuriantly.

A significant improvement in the nutrient contents of dually inoculated seedlings over singly or uninoculated counterparts signifies that both *Frankia* and *G. mosseae* interact positively in the acquisition of nutrients in nutrient-deficient conditions. This interaction may be important in plant productivity in abandoned lands where P is a growth-limiting factor. Hence, it may be exploited in the revegetation of wastelands.

SUMMARY

Present investigation was carried out to evaluate the interaction between symbiotic *Frankia* and vesicular-arbuscular mycorrhizal (VAM) fungi on nutrient uptake, nitrogen fixation and growth of *Alnus nepalensis* D. Don .

Significant changes in the nitrogen-fixing (nitrogenase) activity of root nodules of *A. nepalensis* under field conditions in different seasons were observed. A marked higher activity was detected during rainy and low during winter season. Seasonal standing nodule biomass was high during rainy and low during winter season. Former was intimately related ^{with} _Λ nitrogen fixation rate. Root nodules, however, were restricted only to the uppermost soil depth i.e. D₁ (0-15 cm). Qualitatively, active nitrogen-fixing nodules i.e. young and medium aged ones formed a significant proportion of population during rainy season. However, the ratio of active to old and senescing nodule decreased exponentially in winter season. Total nodule population per unit soil volume too showed a marked seasonality. Nitrogen

content of root nodules showed a significant positive correlation with nitrogen-fixing capability of nodules.

Population of *Frankia* under Himalayan alder (*A. nepalensis*) forest soil exhibited a marked seasonality. Abundance of *Frankia* was recorded highest during rainy and low during winter months. Irrespective of seasons, uppermost soil depth (D_1) harboured maximum *Frankia* propagules which decreased exponentially with increase in soil depth.

Ultrastructural and light microscopic studies of nodules revealed that the proportion of nodule cortical cells containing vesicular form of *Frankia* varied remarkably during various seasons and was characteristic of the seasons. Highest value during rainy and lowest during winter seasons was recorded. However, in a given season occurrence of vesicles in the cortical cells of apical, middle and basal zones of nodules varied. In addition, the abundance of nodule cells with hyphal and sporangial form of micro endophyte in apical, middle and basal zones differed among various seasons.

Intensity of mycorrhizal infection varied to less extent in different seasons which was high and low

during winter and rainy seasons respectively. Mycorrhizal roots were highly aerobic as they were confined to 0-15 cm soil depth i.e D₁. The seasonal assay of chitin in the mycorrhizal roots of Himalayan alder also showed a similar trend as that of mycorrhizal infection. The biomass of mycorrhizal roots, however, was more during winter and low during rainy seasons. The morphology of VAM endophyte in mycorrhizal roots was distinctive of the seasons. VAM produced more arbuscules than vesicles during rainy season whose ratio decreased with increase in time and reached minimum during winter months. In many cases, roots were found to be colonized by finer VA endophytes (in terms of hyphal diameter) along with other fungal endophyte.

Majority of endogonaceous spores were confined to D₁ and D₂ soil layers which declined considerably with further increase in soil depth. Maximum number of spores were recorded during winter and minimum during rainy season. 7.0 different species of VAM fungi were identified whose abundance varied in space and in time. *Glomus mosseae* was dominant species encountered in most of the seasons and soil depths.

Nitrogenase activity of nodules showed a positive and significant correlation with soil temperature, soil

moisture, pH, organic matter, total N, available P, exchangeable Mg and Mn.

Generally correlation between VAM infection and above soil physio-chemical characteristics was negatively established.

Further a strong negative relationship was established between nitrogenase activity and mycorrhizal infection, chitin content and vesicle number. Nodule N content was negatively correlated with mycorrhizal infection.

Besides a clear cut positive and significant relationship was noted between nitrogenase activity and nodule N, cortical vesicular cells, but was negatively correlated with sporangial and hyphal (microcolonies) cells.

Amongst various isolation techniques tested, microdissection coupled with double agar layer, modified sucrose gradient density technique and modified nodule crushing technique proved to be most efficient for the isolation of symbiotic *Frankia* from the root nodules of *A. nepalensis* in solid and liquid cultures respectively.

Three *Frankia* isolates i.e. AnF₁, AnF₂ and AnF₃ were isolated and characterised on the basis of morphological, physiological and host nodulation characteristic. All *Frankia* strains showed a characteristic cushion-like branched, septate hyphae ranging from 0.5-1.2 μ m in diameter. Vesicles were produced by all isolates especially in N-free medium, whose diameter varied to some extent. A marked variation in the dimensions of sporangia produced by various *Frankia* was evident.

Optimal growth of all three *Frankia* isolates was observed between temperature range 28-30^o C. None of them could perform well above 35^o C. However, AnF₃ grew better at lower temperature than other isolates. Growth response of *Frankia* strains to various pH exhibited an interesting feature. *Frankia* AnF₁ and AnF₂ grew better at pH 6-7 and AnF₃ at pH 5.0. No isolate could grow at pH 4.0.

In vivo nitrogenase activity differed for each of the isolate when grown on either propionate or ammonium nitrate amended medium. The activity was stimulated by earlier source and repressed by the latter one.

The nodulation period of *Frankia* was characteristic for each strain. AnF₁ and AnF₂ could nodulate

its original host i.e. *Alnus nepalensis* D. Don after 30 days and and AnF₃ after 45 days of inoculation. Nodule actinomycetes reisolated from the root nodules of host resembled original isolates. The activity of nodules formed by AnF₁ was significantly higher than with AnF₂ and AnF₃ strains.

Three *Frankia* isolates exhibited an interesting seasonal dominance under field condition as assessed by isolation from root nodules. *Frankia* AnF₁ was dominant throughout the year while AnF₃ was active only during winter season.

The soils supporting *A. nepalensis* harboured ~~7~~ VAM fungal species which belonged to *Glomus*, *Gigaspora* and *Acaulospora* genera.

Seedlings of Himalayan alder inoculated either with *Frankia*, *Glomus mosseae* singly or in combination and noninoculated one responded differently to various soil pH levels. The dually inoculated (*Frankia* + *G. mosseae*) seedlings showed higher growth than singly or uninoculated counterparts specially at slightly acidic (pH 5) to neutral soil pH (pH 7). However, the difference in growth performance between singly or dually inoculated plants

narrowed at pH 4. *Frankia* + *G. mosseae* inoculated plants possessed lower root-shoot ratio than singly or uninoculated controls at most pH levels.

The formation of root nodules increased in time and with increase in soil pH and decrease with decrease in pH value above or below pH 6. Dually inoculated seedlings developed more nodules than those inoculated only with *Frankia* at most pH levels. The biomass of root nodule induced by *Frankia* was ameliorated by the colonization of root by *Glomus mosseae*. The rate of nitrogenase activity of root nodules differed considerably between *Frankia* inoculated and *Frankia* + *G. mosseae* inoculated ones at all soil pH levels except at pH 4.

Conversely, the status of VA mycorrhiza, in terms of colonization, arbuscule and spore abundance was ameliorated in *Frankia* - *Alnus* - *G. mosseae* symbioses. This synergistic behaviour was altered or repressed with decreasing soil pH.

Total phosphatase activity of soil was higher in rhizosphere of dually inoculated plants than in only *G. mosseae* inoculated one especially at moderate soil pH i.e. 5-

7 but was equal or lesser at soil pH 4.0.

Concentration of N, P, K and Mn in seedlings of Himalayan alder was significantly improved by dual inoculation with *Frankia* and *G. mosseae*. A marked difference in the nutrient contents of dually, singly or noninoculated control plants was evident at most soil pH and especially between pH range of 5-6. The nutrient content was however, strongly affected by soil pH, type/(s) of organism involved and harvest period.

The seedlings of *A. nepalensis* responded positively to *Frankia* and *G. mosseae* inoculation either singly or in combination at low to intermediate dose of organic matter amendments. Dually inoculated plants showed vigorous growth than singly inoculated either with *Frankia* or *G. mosseae* especially at 0 N and 1 N soil organic matter levels. Seedling growth was adversely affected by the higher doses of organic amendments. Non-mycorrhizal controls showed an increasing trend of growth with increasing gradient of organic matter. *Frankia + G. mosseae* inoculated seedlings exhibited low root-shoot ratio than singly inoculated or noninoculated counterparts at low to intermediate levels of organic matter. In terms of stem collar diameter, leaf number

and leaf area, dually inoculated seedlings showed higher values than singly or uninoculated controls.

The nodulation, nodule biomass, nitrogen fixation and rhizosphere *Frankia* abundance was markedly stimulated by *G. mosseae* in alnus -*Frankia* - *G. mosseae* symbiosis. However, this ameliorative effect was depressed exponentially with increasing organic matter level in soil.

Seedlings of Himalayan alder colonized by *Frankia* + *G. mosseae* exhibited more mycotrophic behaviour than those with only *G. mosseae* normally at lower level of organic matter. The abundance of arbuscules, vesicles and endogonaceous spore was remarkably improved in plants inoculated with *Frankia* + *G. mosseae* which diminished sharply with increasing level of soil organic matter.

Total phosphatase activity in the rhizosphere increased initially depending upon doses of organic matter amendments, which however, was assayed only in *Frankia* + *G. mosseae* and *G. mosseae* inoculated plants. More activity was estimated in dually inoculated plant than singly inoculated counterparts.

Frankia + *G. mosseae* inoculated plants contained higher concentration of N, P, K and Mn both in shoot and roots than in singly or noninoculated ones. Generally shoot harboured higher nutrients than roots.

Study on interaction between *Frankia* and *G. mosseae* at different levels of rock phosphate in containerised seedlings of *A. nepalensis* was conducted. Himalayan alder seedlings inoculated with *Frankia* and/or *G. mosseae* responded differently to various rock P levels in soils. *Frankia* + *G. mosseae* and *G. mosseae* inoculated seedlings performed better at intermediate to high (4 N) levels of rock P, while uninoculated controls and *Frankia* inoculated ones performed better only at highest dose. At any rock P level, root/shoot ratio was considerably lower for dually inoculated seedlings as compared to singly or noninoculated controls.

Himalayan alder seedlings inoculated with *Frankia* + *G. mosseae* exhibited 2-3 folds greater nodulation, nodule biomass and nitrogen fixing ability than those with only *Frankia*. However, increasing level of rock P reduced this difference. The number of *Frankia* propagules in rhizosphere soil was increased in the presence of

mycorrhizal fungus in a tripartite symbiosis. However, This effect was not observed in only *Frankia* inoculated seedlings even at highest level of rock P application. A significant difference in latter population was recorded at low to intermediate rock P levels between *Frankia* + *G. mosseae* and only *Frankia* infected seedlings.

The dependency of seedlings of *A. nepalensis* on mycorrhizal fungus, *G. mosseae* was more in *Frankia* + *G. mosseae* than those only with *G. mosseae*. This, however, was least affected by increasing rock P level in soil. The production of arbuscules and endogonaceous spores was distinctly more in dually inoculated seedling than those only with *G. mosseae* symbiont.

Total phosphatase activity in rhizosphere soil was significantly higher in the rhizosphere of tripartite symbioses involving *Alnus* - *Frankia* - *G. mosseae* than bipartite (*Alnus* - *G. mosseae*) ones. The activity was absent in the rhizosphere of *Frankia* inoculated or noninoculated seedlings. It was high in soils with low level of rock P and decreased with increasing level of latter. Further, at 30 days, the activity in rock P amended soil increased but showed low value at later stages.

In terms of nutrient contents, nodulated mycorrhizal (*Frankia* + *G. mosseae*) seedlings harboured distinctly higher level of nutrients (N, P, K and Mn) in shoot and root tissues than *Frankia* nodulated, mycorrhizal or noninoculated ones. Seedlings with two microsymbionts maintained their lead for nutrient contents throughout the experiment irrespective of rock phosphate level in soil.

R E F E R E N C E S :

- Abbott, L.K. and Robson, A.D. 1977. Growth stimulation of subterranean clover with vesicular - arbuscular mycorrhizas. Aust. J. Agric. Res. 28: 639-649.
- Abbott, L.K. and Robson, A.D. 1978. Growth of subterranean clover in relation to the formation of endomycorrhizas by introduced and indigenous fungi in a field soil. New Phytol. 81 : 575 - 585.
- Abbott, L.K. and Robson, A.D. 1982. The role of vesicular arbuscular mycorrhizal fungi in agriculture and selection of fungi for inoculation. Aust. J. Agric. Res. 33: 389-408.
- Abeysekera, R.M., Newcomb, W., Silvester, W.B. and Torrey J.G. 1990. A freeze - fracture electron microscopic study of *Frankia* in *Alnus incana* nodule grown at three oxygen tensions. Can.J. Microbiol. 113:123 - 125
- Akkermans, A.D.L. 1971. Nitrogen fixation and nodulation of *Alnus* and *Hippophae* under natural conditions. Ph.D. Thesis, Univ. Leiden, pp. 85.
- Akkermans, A.D.L. and Houwers, A. 1979. Symbiotic nitrogen fixers available for use in temperate forestry. In: Symbiotic nitrogen fixation in the management of temperate forests. (Eds. Gordon, J.C., Wheeler, D.T. and Perry, D.A.). For. Res. Lab. Oregon State Univ., Corvallis, pp. 23 - 35.
- Akkermans, A.D.L., Huss - Danell, K. and Roelofsen, W. 1981. Enzymes of the tricarboxylic acid cycle and the malate of *Alnus glutinosa*. Physiol. Plant. 53: 289 - 294.
- Akkermans, A.D.L. and Roelofsen, W. 1980. Symbiotic nitrogen fixation by actinomycetes in *Alnus* type root nodules. In: Nitrogen fixation (Eds. Steward, W.D.P. and Gallon, J.R.), Academic Press, New York pp. 279 - 299.
- Akkermans, A.D.L., Roelofsen, W., Blom, J., Huss - Danell, K. and Harkink, R. 1983. Utilization of carbon and nitrogen compounds by *Frankia* in synthetic media and in root nodules of *Alnus glutinosa*, *Hippophae rhamnoides* and *Datisca Cannabina*. Can. J. Bot. 61 : 2793 - 2800.
- Akkermans, C. and Van Dijk, C. 1976. The formation and nitrogen fixing activity of root nodules of *Alnus glutinosa* under field conditions. In : Symbiotic nitrogen fixation in plant. (Ed. Nutman, P.S), Cambridge Univ. Press, Cambridge, England, pp. 511 - 520.
- Akkermans, A.D.L. and Van Dijk, C. 1981. Non- leguminous root nodule symbioses with actinomycetes and *Rhizobium*. In : Nitrogen fixation Vol. 1 : Ecology, (Ed. Broughton, W.J.), Clarendon Press Oxford, pp. 57 - 103.

- Akkermans, A.D.L., Van Straten, J. and Roelofsen, W. 1977. Nitrogenase activity of nodule homogenate of *Alnus glutinosa* : A comparison with the *Rhizobium* - pea system. In : Recent developments in nitrogen fixation (Eds. Newton, W., Postage, J.R. and Rodriguez - Barrueco, C.), Proc. 2nd Int. Symp. Nitrogen fixation, Academic Press. New York, pp. 591 - 603.
- Aldwell, F.E.B. and Hall, I.R. 1986. Monitoring spread of *Glomus mosseae* through soil infested with *Acaulospora laevis* using serological and morphological techniques. Trans. Br. Mycol. Soc. 87 : 131 - 134.
- Allen, M.F. 1982. Influence of vesicular- arbuscular mycorrhizal fungi on water movement through *Bouteloua gracilis* (H.B.K.) log ex. Steud. New Phytol. 91 : 191 - 196.
- Allen, S.E. 1974. Chemical analysis of ecological materials. Blackwell Scientific Publications. New Delhi.
- Ames, R.N., Reid, C.P.P., Porter, L.K. and Cambardella, C. 1983. Hyphal uptake and transport of nitrogen from two ^{15}N labelled sources by *Glomus mosseae*, a vesicular -arbuscular mycorrhizal fungus. New Phytol. 95 : 381-396.
- Amijee, F., Tinker, P.B. and Stribley, D.P. 1989. The development of endomycorrhizal root systems. VII. A detailed study of effect of soil phosphorus on colonization. New Phytol. 111 : 435 - 446.
- An, C.S., Wills, J.W., Riggsby, W.S. and Mullin, B.C. 1983. Deoxyribonucleic acid base composition of 12 *Frankia* isolates. Can. J. Bot. 61 : 2859 - 2862.
- An, C.S., Riggsby, W.S. and Mullin, B.C. 1985. Restriction pattern analysis of genomic DNA of *Frankia* isolates. Plant Soil 87 : 43 - 48.
- Angulo - Carmona, A.F. 1974. La formation des nodules fixateurs d'azote chez *Alnus glutinosa* (L.)Vill. Acta Bot. Neerl. 23:257-303.
- Antonio, J.P.S. 1990. The possible relationship of spore like structures on the mycelium of *Glomus monosporum* to formation of appressoria. Mycology 82 : 268 - 269.
- Appiah, M.R. and Thomas, R.L. 1982. Inositol phosphate and organic phosphorus content and phosphatase activity of some Canadian and Ghanian soils. Can. J. Soil Sci. 62 : 31 - 38.
- Arines, J. and Vilarino, A. 1991. Growth, micronutrient content and vesicular - arbuscular infection of herbaceous plants on lignite mine spoils : A green house pot experiment. Plant Soil 135 : 266-273.
- Asai, T. 1944. Über die Mykorrhizen bildungder Leguminosen Pflanzten. Japan J. Bot. 12 : 359 - 437.

- Asimi, S., Gianinazzi-Pearson, V. and Gianinazzi, S. 1980. Influence of increasing soil phosphorus level on interaction between vesicular mycorrhizae and *Rhizobium* in soybeans. Can. J. Bot. 58 : 2200 - 2205.
- Avio, L. and Giovanetti, M. 1988. vesicular - arbuscular mycorrhizal infection of lucerne roots in a cellulose amended soil. Plant Soil 112 : 99 - 104.
- Azcon - Aguilar, C. and Barea, J.M. 1992. Nodulation, nitrogen fixation, (^{15}N) and N nutrition relationships in mycorrhizal or P amended alfalfa plants. Symbiosis (Rehovot) 12 :33 -41.
- Azcon-Aguilar, C., Barea, J.M. and Olivares, J 1980. Effects of *Rhizobium* on VA mycorrhiza formation. 2nd Internat. Symp. Microbial Ecol., Univ. Warwick, Coventry, U.K. Abs. 18.7.
- Azcon-Aguilar, C., Gianinazzi - Pearson, V., Fardeau, J.C. and Gianinazzi, S. 1986. Effect of VAM fungi and phosphate solubilizing bacteria on growth and nutrition of soybean in a neutral calcareous soil amended with ^{32}P - ^{43}Ca - tricalcium phosphate. Plant Soil 96 : 3 - 16.
- Azcon, R., Gamez - Ortega, M. and Barea. J.M. 1982. Comparative effects of foliar - and soil applied nitrate in vesicular - arbuscular mycorrhizal infection in maize. New Phytol. 92 : 553 - 559.
- Azcon, R., Azcon - Aguilar, C. and Barea, J.M. 1978. Effect of plant hormones present in bacterial culture on the formation and response to VA endomycorrhiza. New Phytol. 80 : 359 - 364.
- Azcon., R., Gomez - Ortega, M. and Barea, J. 1982. Comparative effects of foliar or soil applied nitrate on vesicular - arbuscular mycorrhizal infection in maize. New Phytol. 92 : 553-559.
- Azcon, R. and O'Campo. J.A. 1981. Factors affecting the vesicular -arbuscular infection and mycorrhizal dependency of thirteen wheat cultivars. New Phytol. 87: 667-685.
- Baas, R., Van Dijk, C and Troelstra, M. 1989. Effect of rhizosphere soil, vesicular - arbuscular mycorrhizal fungi and phosphate on *Plantago major* L. ssp. *pleiosperma* Pilger. Plant Soil 113 : 59-67.
- Bagyaraj, D.J. and Manjunath, A. 1980. Response of crop plants to VA mycorrhizal inoculation in an unsterile Indian soil. New Phytol. 85 : 33 - 36.
- Baker, D. 1979. Relationships among pure cultured strains of *Frankia* based on host specificity. Physiol. Plant. 70 : 245 - 248.
- Baker, D. 1982. A cumulative listing of isolated *Frankiae*, the symbiotic nitrogen- fixing actinomycetes. The Actinomycetes 17 : 35-42.

- Baker, D. 1989. Methods for the isolation, culture and characterization of the Frankiaceae : Soil actinomycetes and symbionts of actinorrhizal plants. In : Isolation of Biotechnical organisms from nature. (Ed. Labeda, D.P.) McGraw Hill, New York, pp. 213-236.
- Baker, D. and Miller, N.G. 1980. Ultrastructural evidence for the existence of actinorrhizal symbioses in the late pleistocene. Can. J. Bot. 58 : 1612-1620.
- Baker, D. and Torrey, J.G. 1979. The isolation and cultivation of actinomycetous root nodule endophytes, In : Symbiotic nitrogen fixation in the management of temperate forests. (Eds. Gordon, J.C., Wheeler, C.T. and Perry, D.A.), Forest. Res. Lab., Corvallis, pp. 38 - 56.
- Baker, D. and O'Keefe, D. 1984. A modified sucrose fractionation procedure for isolation of *Frankiae* from actinorrhizal root nodule and soil samples. Plant Soil 78 : 23 - 28.
- Baker, D., Pengelly, W.L. and Torrey, J.G. 1981. Immunological analysis of relationships among isolated *Frankiae* (Actinomycetales). Int. J. Syst. Bacteriol. 31 : 148 - 151.
- Baker, D., Torrey, J.G. and Kidd, G.H. 1979. Isolation by sucrose density fractionation and cultivation *In vitro* of actinomycetes from N₂ - fixing root nodules. Nature (London) 281 : 76 - 78.
- Bakshi, B.K. 1974. Mycorrhiza and its role in forestry. P.L. 480 Project Report, FRI and colleges, Dehradun, India.
- Bathlenfalvay, G.J., Brown, M.S. and Pacovsky, R.S. 1982. Parasitic and mutualistic associations between a mycorrhizal fungus and soybean. I. Development of the host plant. Phytopathology 72 : 889 - 893.
- Barea, J.M. and Azcon - Aguilar, C. 1983. Mycorrhizal and their significance in nodulating nitrogen fixing plants. Adv. Agron. 36 : 1 - 54
- Barea, J.M., El-Atrach, F. and Azcon, R. 1988. Mycorrhizal and P nutrition as affecting plant development, N₂ - fixation, and N transfer by using a ¹⁵N dilution technique. Soil Biol. Biochem. 21 : 581 - 590.
- Barea, J.M., Escudera, J.L. and Azcon - Aguilar, G. 1980. Effects of introduced and indigenous VA mycorrhizal fungi on nodulation, growth and nutrition of *Medicago sativa* in phosphate fixing soil as affected by P fertilizers. Plant Soil 54 : 283 - 289.
- Barea, J.M., Azcon, R. and Azcon - Aguilar, C. 1989. Time course of N₂ - fixation (¹⁵N) in the field clover growing alone or in mixture with with rye grass to improve pasture productivity

- and inoculated with vesicular-arbuscular mycorrhizal fungi. New Phytol. 112: 399 - 404.
- Barea, J.M., Azcon-Aguilar, C. and Azcon, R. 1987. Vesicular-arbuscular mycorrhiza improve both symbiotic nitrogen fixation and N uptake from soil as assessed with ¹⁵N technique under field conditions. New Phytol. 106 : 717-725.
- Baylis, G.T.S. 1967. Experiment on the ecological significance of phycomycetous mycorrhizas. New Phytol. 66: 231 - 243.
- Bayne, H.G., Brown, M.S. and Bethlenfalvay, G.J. 1984. Defoliation ^{and} effects on mycorrhizal colonization, nitrogen fixation and photosynthesis in the *Glycine - Glomus - Rhizobium* symbiosis. Physiol. Plant. 62:576 - 580.
- Becker, W.N. and Gerdemann, J.W. 1977. Colorimetric quantification of vesicular - arbuscular mycorrhizal infection in onion. New Phytol. 78 : 289 - 295.
- Becking, J.M. 1965. *In vitro* cultivation of alder root nodule tissue containing endophyte. Nature (London) 207 : 885-887.
- Becking, J.M. 1970. Plant endophyte symbiosis in non - leguminous plants. Plant Soil 32 : 611 - 654.
- Becking, J.H. 1970 Frankiaceae fam. nov. (Actinonycatales) with a new combination and six new species of the genus *Frankia* Brunchorst 1886. Int. J. Syst. Bacteriol. 20:201-220.
- Becking, J.H. 1977. Dinitrogen fixing association in higher plants other than legumes. In : A treatise on dinitrogen fixation (Eds. Hardy, R.W.F. and Silver, K.) John Wilder and Sons, Inc. New York, pp. 185.
- Bell, M.J., Middleton, K.J. and Thompson, J.P. 1989. Effects of vesicular -arbuscular mycorrhizae on growth, phosphorus and zinc nutrition of peanut (*Arachis hypogea* L.) in an oxisol from subtropical Australia. Plant Soil 117:49-57.
- Benecke, U. 1970., Nitrogen fixation by *Alnus viridis* (Chaix) DC. Plant Soil 33: 30-48.
- Benson, D.R. 1982. Isolation of *Frankia* strains from alder actinorrhizal root nodules. Appl. Environ. Microbiol : 461 - 465.
- Benson, D.R., Arp, D.J. and Burris, R.H. 1980. Hydrogenase in actinorrhizal root nodules and root nodule homogenate. J. Bacteriol. 142 : 138 - 144.
- Benson, D.R. and Hanna, D. 1983. *Frankia* diversity in an alder stand as estimated by sodium dodecylsulphate polyacrylamide gel electrophoresis of whole cell proteins. Can. J. Bot. 61: 2919-2923.

****Bhuwaneshwari, T., Norman, F. and Hohne, G.K. 1980. Methods for the inoculation of *Frankia*. Aust. Agric. 20: 155-157.**

Berg, A. and Doerksen, A. 1975. Natural fertilization of a heavily thinned Douglas - fir stand by understory red alder. Res. Note 56., For. Res. Lab., Oregon State Univ. Corvallis, Oregon, pp. 3.

Berliner, R. and Torrey, J.G. 1989. On tripartite *Frankia*-mycorrhizal association in the myricaceae. Can. J. Bot. 67:1708-1712.

Berry, A.M., Rodney, K.S.K. and Booth, M.C. 1989. Identification of Indole Compounds secreted by *Frankia* HFP Ar 13 in defined culture medium. Plant Soil 118 : 205 - 209.

Berry, A. and Torrey, J.G. 1979. Isolation and characterization *In Vivo* and *In Vitro* of an actinomycetous endophyte from *Alnus rubra* Bong. In : Symbiotic nitrogen fixation in the management of temperate forests.(Eds. Gordon, J.C., Wheeler, C.T. and Perry, D.A.), Forest Res. Lab., Oregon State Univ. Corvallis, pp. 69 - 83.

Bethlenfalvay, G.J., Ulriah, J.M. and, M.S. 1985. Plant response to mycorrhizal fungi : host, endophyte and soil effects. Soil Sci. Soc. Am. J. 49 : 1164 - 1168.

Bethlenfalvay, G.J. and Yoder, J.F. 1981. The *Glycine* - *Glomus-Rhizobium* symbiosis. I. Phosphorus effect on N₂ - fixation and mycorrhizal infection. Physiol. Plant. 52: 141 - 145

Bhattacharjee, M., Mukerji, K.G. and Mishra, S. 1980. Studies on Indian Endogonaceae III. Further records. Acta. Bot. Indica 8 : 99 - 102.

Bhattacharai, I.D. 1983. Studies on the vesicular - arbuscular mycorrhizal ^{fungi} of certain crop plants of Meghalaya. Ph.D. Thesis, Deptt. Bot., NEHU, Shillong.

Biermann, B. and Linderman, R.G. 1981. Quantifying vesicular - arbuscular mycorrhizae : A proposed method towards standardization. New Phytol. 87 : 63 - 67.

Biermann, B., Linderman, R.G. 1983. Use of vesicular - arbuscular mycorrhizal roots, intraradical vesicles and extramatrical vesicles as inoculum. Trans. Br. Mycol. Soc. 95 : 97 - 105.

Bilenski, R.L. 1973. Phosphate pools, phosphate transport and phosphatase availability. Ann. Rev. Plant Physiol. 24 : 225 - 252.

Blom, J. 1982. Carbon and nitrogen source requirements of *Frankia* strains. FEMS Microbiol. Lett. 13 : 51 - 55.

Bloom, R.A., Lechevalier, M.P. and Tate, R.L. 1989. Physiological, chemical, morphological and plant - infectivity characteristics of *Frankia* isolates from *Myrica pennsylvanica* : Correction to DNA restriction patterns. Appl. Environ. Microbiol. 55 : 2161 - 2166.

- Bolan, N.S., Robson, A.D. and Barrow, N.J. 198. Plant and soil factors including mycorrhizal infection causing sigmoidal response of plants to applied phosphorus. *Plant Soil* 73:187-202.
- Bond, G. 1951. The fixation of nitrogen associated with the root nodules of *Myrica gale* L., with special reference to its pH relation and ecological significance. *Ann. Bot. (London)* 15 : 447-459.
- Bond, 1957. Isotopic studies of nitrogen fixation in non- legumes root nodules. *Ann. Bot. (London)* 21 : 513 - 521.
- Bond, G. 1967. Fixation of nitrogen by higher plants other than legumes. *Annu. Rev. Plant Physiol.* 18 : 107 - 126.
- Bond, G. 1976. The results of the IBP survey of root nodule formation in non-leguminous angiosperms. In: "Symbiotic nitrogen fixation in plants." (Ed. Nutman, P.S.) Cambridge Univ. Press. Cambridge, England, pp. 443-474
- Bond, G. and Mackintosh, A.H. 1975. Effect of nitrate-nitrogen on the nodule symbioses of *Coriaria* and *Hippophae*. *Proc. R. Soc. London, Ser. B.* 190: 199-209.
- Bonfante-Fasolo, P. and Grippiolo, R. 1982. Ultrastructural and cytochemical changes in the wall of a VAM fungus during symbiosis. *Can. J. Bot.* 60: 2303-
- Bonmati, M., Ceceanti, B. and Nanniperi, P. 1991. Spatial variability of phosphatase, urease, organic carbon and total nitrogen in soil. *Soil Biol. Biochem.* 23 : 319 - 396.
- Bormann, B.T. and Debell, D.S. 1981. Nitrogen content and other soil properties related to age of red alder stand. *Soil Sci. Soc. Am. J.* 45 : 428 - 432.
- Bosco, M., Fernandez, M.P., Simonet, P., Materassi, R. and Normand, P. 1992. Evidence that some *Frankia* species strains are able to cross boundaries between *Alnus* and *Elaeagnus* host specificity group. *Appl. Environ. Microbiol.* 58 : 1569-1576.
- Boyetchko, S.M. and Tewari, J.P. 1990. Root colonization of different hosts by the vesicular-arbuscular mycorrhizal fungus *Glomus dimorphicum*. *Plant Soil* 129 : 131 - 136.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Ann. Biochem.* 72 : 248 - 254.
- Braunberger, P.G., Miller, M.H. and Peterson, R.L. 1991. Effect of phosphorus nutrition on morphological characteristics of vesicular - arbuscular mycorrhizal colonization of maize. *New Phytol.* 119 : 107 - 114.

- Brown, M.S. and Bethlenfalvay, G.T. 1987. *Glycine* - *Glomus*-*Rhizobium* symbiosis. IV. Interactions between the mycorrhizal and nitrogen - fixing endophytes. *Plant Cell Environ.* 10: 607 - 612.
- Brown, M.S., Thamasurakul, S. and Bethlenfalvay, G.J. 1988. The *Glycine* - *Glomus*-*Bradyrhizobium* symbiosis VIII. Phosphorus use efficiency of CO₂ and N₂ fixation in mycorrhizal soybean. *Physiol. Plant.* 74 : 159 - 163.
- Brunchorst, J. 1886. Über einige wurzelanschwellungen, besonders diejenigen von *Alnus* und den Elaeagnaceae. *Unters. Bot. Inst. Tusign* 2 : 150 - 177.
- Bur'angulova, M.N. and Khaziev, K.M. 1965. The phosphatase activity of soil as affected by chemical fertilizers. *Agrokem. Talast.* 14 : 101 - 110.
- Burggraaf, A.J.P. and Shipton, W.A. 1982. Estimates of *Frankia* growth under various pH and temperature regimes. *Plant Soil* 19: 135 - 147.
- Burggraaf, A.J.P. and Shipton, W.A. 1983. Studies on the growth of *Frankia* in relation to infectivity and nitrogen fixation (acetylene reduction). *Can. J. Bot.* 61: 2774 - 2782.
- Burggraaf, A.J.P., Quispel, A., Tak, T. and Valstar, J. 1981. Methods of isolation and cultivation of *Frankia* species from actinorrhizas. *Plant Soil* 61 : 157 - 168.
- Burggraaf, A.J.P. and Valstar, J. 1984. Heterogeneity within *Frankia* sp. LDAgp1 studied among clones and reisolates. *Plant Soil* 78 : 29 - 43.
- Burns, R.G. 1982. Enzyme activity in soils : Location and a possible note in microbial ecology. *Soil Biol. Biochem* 14 : 423 - 427.
- Buwalda, J.G., Ross, G.J.S., Studies, D.P. and Tinker, P.B. 1982 a. The development of endomycorrhizal root system. III. The mathematical representation of the spread of vesicular - arbuscular mycorrhizal infection in root systems. *New Phytol.* 91 : 669 - 682.
- Buwalda, J.G., Ross, G.J.S., Stribley, D.P. and Tinker, P.B. 1982 b. The development of endomycorrhizal root system. IV. The mathematical analysis of effects of phosphorus on the spread of vesicular - arbuscular mycorrhizal infection in root system. *New Phytol.* 92 : 391 - 399.
- Callaham, D., Del Tredici, P. and Torrey, J.G. 1978. Isolation and cultivation *In vitro* of the actinomycete causing root nodulation in *Comptonia*. *Science* 199:899-902.
- Carling D.E., Riehle, W.G., Brown, M.F. and Johnson, D.R. 1978. Effect of vesicular-arbuscular mycorrhizal fungus on nitrate

reductase and nitrogenase activities in nodulating and non-nodulating soybeans. *Phytopathology* 68:1590-1596.

- Carpenter, C.V., Robertson, L., Gordon, J.C. and Perry, D.A. 1984. The effects of four new *Frankia* isolates on growth and nitrogenase activity in clones of *Alnus rubra* and *A. sinuata*. *Can. J. For. Res.* 14: 701-706
- Casgrove, D.J. 1967. Metabolism of organic phosphatase in soil. In: *Soil biochemistry*. (Eds. McLaren, I.A.D. and Paterson, G.H.) Marcel Dekker, New York, pp. 216-228.
- Casida, L.E. Jr. 1977. Microbial metabolic activity in soil as measured by dehydrogenase determinations. *Appl. Environ. Microbiol.* 34:630-636.
- Casida, L.E. Jr., Klein, D.A. and Santoro, T. 1964. Soil dehydrogenase activity. *Soil Sci.* 98:371-376.
- Chaboud, A. and Lalonde, M. 1983. Lectin binding on surfaces of *Frankia* strains. *Can. J. Bot.* 61:2889-2897.
- Chambers, C.A., Smith, S.E. and Smith, F.A., 1980. Effect of ammonium and nitrate ions on mycorrhizal infection, nodulation and growth of *Trifolium subterraneum*. *New Phytol.* 85:47-62.
- Chattopadhyay, A.K. and Samaddar, K. 1978. Effects of *Helminthosporium oryzae* infection and ophiobolin on the phenol metabolism of host tissue. *Phytopath. Z.* 98 : 193 - 202.
- Choudhary, A.H. and Mirza, M.S. 1987. Isolation and characterization of *Frankia* from nodules of actinorhizal plants of Pakistan. *Physiol. Plant.* 70:255-258.
- Chilvers, M.T. and Daft, M.F.J. 1981. Mycorrhizae of the liliflorae II. mycorrhiza formation and incidence of root hairs in field grown *Narcissus tulipa* L., and *Grocus* L. cultivars. *New Phytol.* 88:247-261.
- Ching, T.M., Monaco, P.A., and Ching, K.K.. 1983. Energy status and cytochromes in isolated endophytic vesicle clusters of red Alder root nodules. *Can. J. For. Res.* 13:921-928.
- Clelland, D.C., Gardner, I.C. and Scott, A. 1983. The occurrence of *Glomus fasciculatus*, a mycorrhizal endophyte, in the nitrogen fixing non-legume *Hippophae rhamnoides*. *Microbios. Letters.* 24:107-113.
- Cluett, H.C. and Boucher, D.H. 1983. Indirect mutualism in the legume-*Rhizobium*-Mycorrhizal fungus interaction. *Oecologia* 59:405-448.
- Cnios, A.S. 1981. *Gigaspora marginata* inoculation of *Nicotiana tabacum* : Response to fertilization. *Can. J. Bot.* 59:101-103
- Cooper, K.M. and Tinker, P.B. 1978. Translocation and transfer of

- nutrients in vesicular-arbuscular Mycorrhizae II. Uptake and translocation of Phosphorus, zinc and sulphur. *New Phytol.* 81:43- 52.
- Cooper, K.M. and Tinker, P.B. 1981. Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. IV. effect of environmental variables on movement of phosphorus. *New Phytol.* 81:327-339.
- Cox, G., Sanders, F.E., Tinker, P.B. and Wild, J.A. 1975. Ultrastructural evidence relating to host endophyte transfer in a vesicular-arbuscular mycorrhiza. In: *Endomycorrhizas* (Eds. Sanders, F.E., Mosse, B. and Tinker, P.B.). Academic Press (London), pp. 297-312.
- Coyne, P.. 1983. Specificity between *Casuarina* species and root nodule organisms. In: *Casuarina - ecology, management and utilization*, (Eds. Midgley, S.J., Turnbull, J.W. and Johnston, R.D.), Commonw. Sci. Ind. Res. Org., Melbourne, Australia, pp. 205-210.
- Crocker, R.L. and Major, J. 1955. Soil development in relation to vegetation and surface age at Glacier Bay, Alaska. *J. Ecol.* 43:427-448.
- Crush, J.R. 1973. Significance of endomycorrhizas in tussock grassland in Otago, New Zealand. *N.Z.J. Bot.* 11:645-660.
- Crush, J.R. 1974. Plant growth response to vesicular-arbuscular mycorrhiza. VII. Growth and nodulation of some herbage legumes. *New Phytol.* 73:743-749.55
- Daft, M.J. and El-Giahmi, A.A. 1976. Studies on nodulation and mycorrhizal peanuts. *Ann. Appl. Biol.* 83:273-276.
- Daft, M.J. and Hacskeylo, E. 1986. Arbuscular-mycorrhizas in the anthracites and bituminous coal wastes of Pennsylvania. *J. Appl. Ecol.* 13:523-531.
- Daft, M.J. and Hogarth, B.G. 1983. Competitive interactions amongst four species of *Glomus* on maize and onion. *Trans. Br. Mycol. Soc.* 80:339-345.
- Daft, M.J. and Nicolson, T.M. 1972. Effect of *Endogone* mycorrhiza in plant growth. IV. Quantitative relationship between the growth of the host and the development of the endophyte in tomato and maize. *New Phytol.* 73:1129-1138.
- Daft, M.J. and Nicolson, T.H. 1974. Arbuscular mycorrhizas in plants colonizing coal wastes in Scotland. *New Phytol.* 73: 1129-1138.
- Dalton, D.A. and Zobel, D.B. 1977. Ecological aspects of nitrogen fixation by *Purshia tridentata*. *Plant Soil* 48 : 57 - 80.
- Daly, G.T. 1965. Nitrogen fixation by nodulated *Alnus sinuosa*. *Can. J. Bot.* 44:1607-1621.

- Danielson, R.M. and Visser, S. 1989. Effect of forest soil acidification on ectomycorrhizal and vesicular-arbuscular mycorrhizal developments. *New Phytol.* 112:151-158.
- Das, P.K. and Mishra, R.R. 1986. Dehydrogenase activity and CO₂ evolution from *Pinus kesiya* ex. Gordon. forest soil. *Geobios.* 13:12-15
- Davis, E.A. and Young, J.L. 1985. Endomycorrhizal colonization of glasshouse-grown wheat as influenced by fertilizer salts when banded or soil mixed. *Can. J. Bot.* 63:1196-1203.
- Dawson, J.O. and Gordon, J.C. 1979. Nitrogen fixation in relation to photosynthesis in *Alnus glutinosa*. *Bot. Gaz. (Chicago)*, suppl. 140:S 70 - S 75.
- Dawson, J.O. and Sun, S.H. 1981. The effect of *Frankia* isolate from *Comptonia peregrina* and *Alnus crispa* on the growth of *Alnus glutinosa*, *A. cordata* and *A. incana* clones. *Can. J. For. Res.* 11:758-762.
- Dawson, J.O. Knowlton, S. and Sun, S.M. 1981. The effect of juglone concentration on the growth of *Frankia in vitro*. *For. Res. Rep. (Univ. Ill. Urbana-Champaign Agric. Exp. Stn. Dep. For.)* no. 81-2.
- Debell, D.S. and Radwan, M.A. 1979. Growth and nitrogen relation of coppiced black cottonwood and red alder in pure and mixed plantings. *Bot. Gaz.* 93. (Chicago) Suppl. 140:S 97 - S 101.
- Debell, D.S. Radwan, M.A. and Kraft, J.M. 1983. Influence of red alder on chemical properties of a clay loam soil in Western Washington, USDA, For. Serv. PNW For. Range Exp. Stn. Res. paper PNW-313, Portland region.
- Delwiche, C.C, Zinke, P.T. and Jonhson, C.M. 1965. Nitrogen fixation by *Ceanothus*. *Plant Physiol.* 40: 1045-1047.
- Demeterio, J.L., Elias, Jr. J.L. and Panlsen, G.M. 1972. Nodulation and nitrogen fixation by soybeans varieties as affected by phosphorus and zinc nutritions. *Agron. J.* 64: 566-568.
- Dewadar, A. and Mansour, S.R. 1992. Infection events in the establishment of *Casuarina-Frankia* Symbioses: using spot inoculation. *Acta Oecol.* 13 : 379-385.
- Diem, H.G. and Dommergues, Y. 1982. The isolation of *Frankia* from nodules of *Casuarina*. *Can. J. Bot.* 61 : 2822- 2825.
- Diem, H.G. Gauthier, D. Dommergues, Y.R. 1982. Isolation of *Frankia* from nodules of *Casuarina equisetifolia*. *Can. J. Microbiol.* 28:526-530.
- Diem, H.G., Gauthier, D. and Dommergues, Y.R. 1983. An effective

- strain of *Frankia* from *Casuarina* species. Can. J. Bot. 61:2815-2821.
- Dillon, S.S. 1992. Evidence for host mycorrhizal preference in native grassland species. Mycol. Res. 96:359-64.
- Dillon, J.T. and Baker, D. 1982. Variation in nitrogen fixing efficiency among pure cultured *Frankia* strains tested on actinorhizal plants as an indication of symbiotic compatibility. New Phytol. 92: 215-219.
- Dixon, R.K. Garrett, H.E. and Cox, G.S. 1988. Carbohydrate relationships of *Citrus jambhiri* inoculated with *Glomus fasciculatum*. J. Amer. Soc. Hort. Sci. 113:239-242.
- Dixon, M. and Webb, E.C. 1966. Enzymes. Longman London.
- Dixon, R.O.D. and Wheeler, C.T. 1983. Biochemical, physiological or environmental aspects of symbiotic nitrogen fixation. In : Biological nitrogen fixation in forest ecosystems: Foundations and Applications, (Eds. Gordon, J.C. and Wheeler, C.T.), Nijhoff/W. Junk, the Hague, pp: 107-171.
- Dixon, R.O.D. and Wheeler, C.T. 1986. Nitrogen fixation in plants. Blackie, Glasgow, pp. 25.
- Dobritsa, S.V. and Stupar, D.S. 1989. Genetic heterogeneity among *Frankia* isolates from root nodules of individual actinorhizal plants. FEMS Microbiol. Lett. 58: 287-292.
- Dodd, J.C., Burton, C.C., Burns, R.G. and Jeffries, P. 1987. Phosphatase activity associated with vesicular-arbuscular mycorrhizal fungi. New Phytol. 107: 163-172.
- Domenach, A.M., Kurdali, F. and Bardin, R. 1989. Estimation of symbiotic dinitrogen fixation in alder forest on the method based on natural ^{15}N abundance. Plant Soil 118: 51-59.
- Douds, D.D. Jr., and Chaney, W.R. 1986. The effects of high nutrient addition upon seasonal patterns of mycorrhizal development, host growth and root phosphorus and carbohydrate content in *Fraxinus pennsylvanica* Marsh. New Phytol. 103 : 91-103.
- Eissenstadt, D.M. and Newman, E.I. 1990. Seedling establishment near large plants: Effects of vesicular-arbuscular mycorrhiza on the intensity of plant competition. Funct. Ecol. 4: 95-99.
- Faure-Raynaud, M., Bonne Foy-Poirier, M.A. and Moiroud, A. 1986. Low pH influence on *Frankia* strain viability. Plant Soil 96 : 341 - 358.
- Faure-Raynaud, M., Bonne Foy-Pairier, M.A. and Moiround, A. 1990. Diversity of *Frankia* strains isolated from actinorhizae of a single *Alnus rubra* cultivated in nursery. Symbiosis 8 : 147 - 160.

- Faure-Raynaud, M., Daniere-Ravellin, C. & Moiroud, A. 1992. Evaluation of strain preference by four *Alnus glutinosa* genotypes on *Frankia* soil population. *Acta Oecologica* 13:513.
- Ferguson, T.P. and Bond, G. 1953. Observation on the formation and function of the nodules of *Alnus glutinosa* (L.) Gaertn. *Ann. Bot. N.S.* 17 : 175-188.
- Fogel, R. 1980. Mycorrhizae and nutrient cycling in natural forest ecosystem. *New Phytol.* 86 : 199 - 212.
- Fogel, R. and Hunt, G. 1979. Fungal and arboreal biomass in the Western Oregon Douglas - fir ecosystem : distribution pattern and turnover. *Can. J. For. Res.* 9:245 - 256.
- Fox, T.R. and Comerford, N.B. 1992. Rhizosphere phosphatase activity and phosphate hydrolyzable organic phosphate in two forested spodosols. *Soil Biol. Biochem.* 24 : 579 - 584.
- Fraga - Beddiar, A and Tacon, F.Le. 1990. Interactions between a VAM fungus and *Frankia* associated with alder (*A. glutinosa* (L.) Gaertn.). *Symbiosis* 9 : 247 - 257.
- Frank, A.B. 1885. Über die auf wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze. *Ber. dtsh. Bot. Ges.* 3 : 128 - 145.
- Frankenberger, W.T. and Dick, W.A. 1983. Relationships between enzyme activities and microbial growth and activity indices in soil. *Soil Sci. Soc. Am. J.* 47 : 945 - 951.
- Frankland, J.C. and Harrison, A.F. 1985. Mycorrhizal infection of *Betula pendula* and *Acer Pseudoplatanus* : relationships with seedling growth and soil factors. *New Phytol.* 101 : 133 - 151.
- Furlan, V. and Fortin, A.J. 1973. Formation of endomycorrhiza by *Endogone calospora* on *Allium Cepa* under three temperature regimes. *Naturaliste Ca.* 100 : 467 - 477.
- Garcia - Garrido, J.M., Capello, M.N., Garcia - Romera, I. and O'Campo, J.A. 1992. Endoglucanase activity in lettuce plants colonized with the vesicular - arbuscular mycorrhizal fungus, *Glomus fasciculatum*. *Soil Biol. Biochem.* 24 : 955 - 959.
- Garcia - Garrido, J.M., Garcia - Romera, I. and O'Campo, J.A. 1992. Cellulase production by vesicular - arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe. *New Phytol.* 121 : 221 - 226.
- Gardner, I.C. 1986. Mycorrhizal status of actinorhizal plants. *MIRCEN. J. Appl. Microbiol. Biotech.* 2 : 147 - 160.
- Gardner, I.C. and Bond, 1957. Observation on root nodules of *Shepherdia*. *Can J. Bot.* 35 : 305 - 314.
- Gardner, I.C., Clelland, D.M. and Scott, A. 1984. Mycorrhizal improvement in non leguminous N₂ - fixing association with

particular reference to *Hippophae rhamnoides* L. Plant Soil 78: 189 - 199.

Garrett, S.D. 1950. Ecology of root - inhabiting fungi. Biol. Rev. 25: 220 - 254.

Gauthier, D., Diem, H.G. and Dommergues, Y. 1981. *In Vitro* nitrogen fixation by two actinomycete strains isolated from *Casuarina* nodules. Appl. Environ. Microbiol. 41 : 306 - 308.

Gauthier, D., Frioni, L., Diem, H.G. and Dommergues, Y. 1984. The *Colletia spinosissima* - *Frankia* symbiosis. Acta Oecol./Oecol. Plant. 5 : 231 - 239.

Gazey, C., Abbott, L.K. and Robson, A.D. 1992. The rate of development of mycorrhizal affects the onset of sporulation and production of external hyphae by two species of *Acaulospora*. Mycol. Res 96 : 643 - 650.

Geary, T.F. 1983. Casuarinas in Florida, U.S.A. and some Carribean Islands. In : Casuarina ecology, management and utilization. (Eds. Midgley, S.J., Turnbull, J.W. and Johnston, R.D.), Commonw. Sci. Ind. Res. Org., Melbourne, Australia, pp. 107 - 109.

Gemma, J.N. and Koske, R.. 1988. Preinfection interaction between roots and mycorrhizal fungus *Gigaspora gigantea*: Chemotropism of germtubes and root growth response. Trans. Br. Mycol. Soc. 91 : 123 - 132.

Gerdemann, J.W. 1975. Vesicular-arbuscular mycorrhizae. In: The development and function of roots (Eds. Torrey, J.G. and Clarkson, D.T.). Academic Press, London , pp. 575

Gerdemann, J.W. and Nicolson, T.H. 1963. Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Trans. Br. Mycol. Soc. 46 : 235 - 244.

Gerdemann, J.W. and Trappe, J.M. 1974. The Endogonaceans in the Pacific Northwest. Mycologia Memoirs 5 : 1 -76.

Gianinazzi - Pearson, V., Branzanti, B. and Gianinazzi, S. 1989. *In Vitro* enhancement of spore germination and early hyphal growth of a vesicular - arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis (Rehovot) 7 : 243 - 255.

Gianinazzi - Pearson, V., Fardeau, J.C., Asimi, S and Gianinazzi, S. 1981. Source of additional phosphorus absorbed from soil by vesicular - arbuscular mycorrhizal soybeans. Physiol. Veg. 19 : 33 - 43.

Gianinazzi, S., Gianinazzi-Pearson, V., and Dexheimer, J. 1979. Enzymatic studies on the vesicular - arbuscular mycorrhizae. III Ultrastructural localization of acid and alkaline phosphatase in onion root influenced by *Glomus mossea*

(Nicol. and Gerd.). New Phytol. 82 : 127 - 132.

- Gianinazzi-Pearson, V. and Gianinazzi, S. 1978. Enzymatic studies on the metabolism of vesicular - arbuscular mycorrhiza. II. soluble alkaline phosphatase specific to mycorrhizal infection in onion roots. *Physiol. Plant Pathol.* 12 : 45 - 53.
- Gibson, A.H. 1976. Limitations to dinitrogen fixation in legumes In: Proceedings of 1st International symposium on nitrogen fixation, Vol. II. (Eds. Newton, W.E. and Nyman, C.J.), Washington State University Press, Pullman, W.A. pp. 400 - 428.
- Giovanetti, M. and Mosse, B. 1980. An evaluation of techniques for measuring VAM infection in roots. *New Phytol.* 64 : 489 - 500.
- Gitte, R., Rai, P. and Patil, R.E. 1978. Chemotaxis of *Rhizobium* pp. towards roots exudates of *Cicer acratinum*. *Plant Soil* 50 : 553 - 556.
- Golebiewska, J. 1956. A method of investigation of microbial population playing a part in the transformation of phosphorus compounds in the soil. *Acta. Microbial. Polon.* 5 : 125 - 127.
- Gordon, J.C. and Dawson, J.O. 1979. Potential use of nitrogen fixing trees and shrubs in commercial forestry. *Bot. Gaz.* 140 (Suppl.) : 88 - 90.
- Gordon, J.C. and Wheeler, C.T. 1978. Whole plant studies on photosynthesis and acetylene reduction in *Alnus glutinosa*. *New Phytol.* 80 : 179 - 186.
- Graham, J.K. and Timmer, L.W. 1985. Rock phosphate as a source of phosphorus for VAM development and growth of *Citrus* in a soil less medium. *J. Am. Soc. Hort. Sci.* 110 : 489 - 492.
- Granhall, U., Ericsson, T. and clarholm, M. 1983. Dinitrogen fixation and nodulation by *Frankia* in *Alnus incana* as affected by inorganic nitrogen in pot experiment with peat. *Can. J. Bot.* 61 : 2956 - 2963.
- Graw, E. 1979. Influence of soil pH on the efficiency of VA mycorrhiza. *New Phytol.* 82 : 687 - 695.
- Gray, L.E. and Gerdemann, J.W. 1973. Uptake of Sulphur - 35 by vesicular - arbuscular mycorrhiza. *Plant Soil* 39 : 687 - 689.
- Greaves, M.P. and Welbley, D.M. 1965. A study of the breakdown of organic phosphate by microorganism from the root region of certain pasture grasses. *J. Appl. Bacteriol.* 28 : 454.
- Green, N.E. Graham, S.O. and Schenck, N.C. 1976, The influence of pH on the germination of vesicular - arbuscular mycorrhizal spores. *Mycologia* 68 : 929 - 934.

- Gunderson, P. 1991. Nitrogen deposition and the forest nitrogen cycle : Role of denitrification. For. Ecol. Manag. 44 : 15 - 28.
- Guofan, L. and Tingxiu, D. 1987. A study of nodulation and nitrogen - fixation of alder on the purplish soil of China. Plant Soil 99 : 285 - 290.
- Hafeez, F., Akkermans, A.D.L. and Choudhary, A.H. 1984. Morphology, physiology and infectivity of two *Frankia* isolates An1 and An2 from root nodules of *Alnus nitida*. Plant Soil 78 : 45 - 59.
- Hahn, D., Dorech, M. Stockebrandt, E. and Akkermans, A.D.L. 1989. Evidence for a close phylogenetic relationship between members of genera *Frankia*, *Geodermatophilus* and *Blastococcus* and emendatio the family *Frankiaceae*. Syst. Appl. Microbiol. 11 : 236 - 242.
- Hahn, D., Starrenburg, M.J.C. and Akkermans, A.D.L. 1988. Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains. Plant Soil 107 : 233 - 243.
- Hall, I.R. and Fish, B.J. 1979. A Key to the Endogonaceae. Trans. Br. Mycol. Soc. 68 : 341 - 356.
- Hall, I.R., Johnstone, P.D. and Dolby, R. 1984. Interactions between endomycorrhizas and soil nitrogen and phosphorus on growth of ryegrass. New Phytol. 97 : 447 - 453.
- Hall, R.B., McNabb, H.S. Jr., Maynard, C.A. and Green, T.L. 1979. Towards development of optimal *Alnus glutinosa* symbioses. Bot. Gaz. (Chicago) Suppl. 140 : S 120 - S 126.
- Hamdi, Y.A. 1974. Vertical movements of rhizobia in soil. Zentralblatt für Bakteriologie. Abt. 11 129 : 373-377
- Harding, T.P.C., and Whitton, B.A., 1977. Environmental factors reducing the toxicity of zinc to *Stigeodinium tenue*. Br. Phycol. J. 12 : 17 - 21.
- Hardy, R.W.F., Holsten, R.D., Jackson, E.K. and Burns, R.C. 1968. The acetylene - ethylene assay for nitrogen fixation : laboratory and field evaluation. Plant Physiol. 43: 1185 - 1207.
- Harley, J.L. 1969. The biology of mycorrhiza, 2nd edition, Leonard Hill, London.
- Harrison, A.F. 1979. Variation of four phosphorus properties in woodland soils. Soil Biol. Biochem. 11 : 393 - 403.
- Harrison, A.F. 1983. Relationship between intensity of phosphatase activity and physico - chemical properties in woodland soils. Soil Biol. Biochem. 15 : 93 - 93.
- Harris, D., Pacovsky, R.S. and Paul, E.A. 1985. Carbon economy of

- soybean - *Rhizobium* - *Glomus* associations. New Phytol.101 : 427 - 440.
- Harrison,A.F. and Pearce,T.1979. Seasonal variation of phosphatase activity in woodland soils.Soil Biol.Biochem.11 : 405 - 410.
- Harrison,J.T. and Seilor,J.R.1988.Acetylene reduction in black alder seedlings as affected by direct and indirect moisture stress using a spilt pot growing system.Environ.Expt.Bot.28: 225 - 230.
- Haselwandter, K.1979.Mycorrhizal status of ericaceous plant in alpine and sub-alpine areas. New Phytol.83 : 427 - 431.
- Hayman,D.S.1970. Endogone spore numbers in soil and vesicular - arbuscular mycorrhiza in wheat as influenced by season and soil treatment.Trans. Br.Mycol.Soc.54 : 53 - 63.
- Hayman,D.S.1975. The occurrence of mycorrhiza in crops as affected by soil fertility. In : Endomycorrhizas, (Eds.Sanders,F.E.,Mosse, B. and Tinker ,P.B) ,Academic Press,London ,pp.495 - 509.
- Hayman,D.S.1978. Mycorrhizal population of sown pastures and native vegetation in Otago, New Zealand. N.Z.J.Agric.Res. 21 : 217: 276.
- Hayman,D.S.,Johonston,A.M. and Ruddlesdin,I.1975.The influence of phosphate and crops species on *Endogone* spores and vesicular - arbuscular mycorrhiza under field conditions.Plant Soil 43 : 489 - 495.
- Hayman,D.S. and Mosse,B.1971. Plant growth responses to vesicular-arbuscular mycorrhiza in wheat as influenced by season and soil treatment.New Phytol .70 : 19 - 27.
- Hayman,D.S. and Mosse,B. 1971. Plant growth responses to vesicular - arbuscular mycorrhiza . I.Growth of endogone inoculated plants in phosphate - deficient soils.New Phytol.70 : 19 - 27.
- Hayman,D.S. and Tavares,M.1985. Plant growth responses to vesicular - arbuscular mycorrhiza XV. Influence of soil pH on the symbiotic efficiency of different endophytes.New Phytol.100 : 367 - 377.
- Hennessey ,T.C.,Vishniac, H.S, Lorenzi, E.M. and Williams,J.C. 1989.Dinitrogen fixation in a water stressed *Alnus* clone is limited by host xerotolerance.Plant Soil 118 : 89 - 96.
- Hepper,C.M.1983. Effects of phosphate on germination and growth of VAM fungi.Trans.Br.Mycol.Soc.80 : 487 - 490.
- Hewitt,E.J. 1958. Some aspects of mineral nutrition in legumes. In : Nutrition of the legumes.(Eds. Hallsworth, E.G.,and Butterworths,A.K.) London,pp.15.

- Hiyoshi, T., Sassakawa, H. and Michihiko, Y. 1989. Isolation of *Frankia* strains from root nodules of *Myrica rubra*. Soil Sci. Plant Nutr. 34 : 107 - 116.
- Hoagland, D.R. and Arnon, D.I. 1950. The water culture method for growing plants without soil. Calif. Agric. Sta. Circ. 347.
- Hoffmann, G. and Elias - Azar, K. 1965. Various soil fertility factors of North Persian soils and their connections with the activity of hydrolytic enzymes. Z. Pflanzener - Nahrung Dungyng und Boden Kunde 108 : 199 - 217.
- Hogberg, P. 1982. Mycorrhizal association in some woodland and forest trees and shrubs in Tanzania. New Phytol. 92 : 407 - 417.
- Hooker, J.E. and Wheeler, C.T. 1987. The effectivity of *Frankia* for nodulation and nitrogen fixation in *Alnus rubra* and *A. glutinosa*. Physiol. Plant. 70 : 333 - 341.
- Horriere, F. 1984. In Vitro Physiological approach of classification of *Frankia* isolates of the *Alnus* group based on urease, protease and glucosidase activities. Plant Soil 78 : 7 - 13.
- Houwers, A. and Akkermans, A.D.L. 1984. Influence of inoculation on the yield of *Alnus glutinosa* in the Netherlands. Plant Soil 61 : 189 - 202.
- Huss - Danell, K. 1978. Nitrogenase activity measurement in intact plants of *Alnus incana*. Physiol. Plant. 43 : 373 - 376.
- Huss - Danell, K. 1980. Nitrogen fixation and biomass production in clones of *Alnus incana*. New Phytol. 85:503 - 511.
- Huss - Danell, K. and Bergman, B. 1990. Nitrogenase in *Frankia* from root nodules of *Alnus incana* (L.) Moench. : Immunolocalization of the Fe and MO- Fe protein during vesicle differentiation. New Phytol. 116 : 443 - 455.
- Huss - Danell, K. and Frej, A.K. 1986. Distribution of *Frankia* in soil from forest and afforested sites in Northern Sweden. Plant Soil 90 : 407 - 418.
- Huss - Danell, K. and Hahlin, A.S. 1988. Nitrogenase activity decay and energy supply in *Frankia* after addition of ammonium to the host plant *Alnus incana*. Physiol. Plant. 74 : 745 - 751.
- Huss - Danell, K., Lundquist, P.O. and Eckland, A. 1989. Growth and acetylene reduction activity by intact plants of *A. incana* under field conditions. Plant Soil 118 : 61 - 73.
- Huss - Danell, K., Roelofsen, W., Akkermans, A.D.L. and Meijer, P. 1982 a. Carbon metabolism of *Frankia* Spp. in root nodules of *Alnus glutinosa* and *Hippophae rhamnoides*. Physiol. Plant.

Huss - Danell, K. and Sellstedt, A. 1985. Nitrogenase activity in response to darkening and defoliation of *Alnus incana* J. Expt. Bot. 36 : 1352 - 1358.

Huss - Danell, K., Sellstedt, A., Flower - Ellis, A., Sjoström, M. 1982 b. Ammonium effect on function and structure of nitrogen - fixing root nodules of *Alnus incana* L. Moench. Planta 156 : 332 - 340.

Huss - Danell, K., Winship, L.J. and Hahlin, A.S. 1987. Loss and recovery of nitrogenase in *Alnus incana* nodules exposed to low Oxygen and low temperature. Physiol. Plant. 70 : 355 - 360.

Ingestad, T. 1980. Growth, nutrition and nitrogen fixation by grey alder at varied rate of nitrogen addition. Physiol. Plant. 50: 353-364.

Jakobsen, I., Abbott, L.K. and Robson, A.D. 1992. External mycelium of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. II. Hyphal transport of ³²P over defined distances. New Phytol. 120: 509-516.

Jayachandran, K., Schwab, A.P., and Hetrick, B.A.D. 1992. Mineralization of organic phosphorus by vesicular mycorrhizal fungi. Soil Biol. Biochem. 24: 897-904.

Jensen, A. and Jakobsen, P. 1980. The occurrence of vesicular-arbuscular mycorrhiza in barley and wheat grown in some Danish Soil with different fertilizers treatments. Plant Soil 55: 403-414.

Jha, D.K. 1990. Ecological studies on vesicular- arbuscular mycorrhizal (VAM) fungi in secondary successional plant community at higher and lower altitudes of Meghalaya. Ph. D. Thesis. North-Eastern Hill Univ. Shillong, India.

Jha, D.K., Sharma, G.D. and Mishra, R.R. 1992. Soil microbial population numbers and enzyme activities in relation to altitudes and forest degradation. Soil Biol. Biochem. 24: 761-767.

Jha, D.K., Sharma, G.D. and Mishra, R.R. 1993. Mineral nutrition in the tripartite interreaction between *Frankia*, *Glomus* and *Alnus* at different soil phosphorus regimes. New Phytol. 123: 307-311.

Jibaji-Hare, S.H., Piche, Y. and Fortin, J.A. 1986. Isolation and structural characterization of soil-borne auxiliary cells of *Gigaspora marginata* Becker & Hall, a Vesicular-arbuscular mycorrhizal fungus. New Phytol. 108:777-784

Johansen, D.A. 1940 Plant microtechnique, 2nd edition, Tata McGrawHill Company Ltd., pp.189-193.

* Jiaben, H., Zheyang, Z., Guanxiong, C. and Huichang, L. 1985. Host range of *Frankia* endophytes. Plant Soil 87: 61-65.

- Johnsen, A., Jakobsen, I. and Jensen, E.S. 1992. Hyphal transport of ^{15}N labelled nitrogen by a vesicular-arbuscular mycorrhizal fungus and its effect on depletion of inorganic soil N. *New Phytol.* 122: 281-288.
- Johnson, C.R., Joiner, J.N. and Crews, C.E. 1980. Effects of nitrogen, potassium and magnesium on growth and leaf nutrients composition of three container grown woody ornamentals inoculated with mycorrhizae. *J. Am. Soc. Hortic. Sci.* 105: 286-288.
- Johnsrud, S.C. 1978. Nitrogen fixation by nodules of *Alnus incana* in a Norwegian forest ecosystem. *Oikos* 30: 475-479.
- Kant, S. and Narayana, H.S. 1978. Effect of water stress on growth, nodulation and nitrogen fixation in *Casuarina equisetifolia*. *Ann. Arid Zone* 17: 216-221.
- Kariya, N. and Toth, R. 1981. Ultrastructure of the mycorrhizal association formed between *Zea diploperennis* and *Glomus fasciculatum*. *Mycologia* 73: 112-117.
- Keilling, J., Camus, A., Avignac, G., Dauchez, P. and Boitel, M. 1960. Contribution to the study of soil biology. *Compt. Rend. Acad. Agric. Fran.* 46: 617-652.
- Khan, A.G. 1971. Occurrence of *Endogone* species in West Pakistan Soils. *Trans. Br. Mycol. Soc.* 56: 217-224.
- Khan, A.G. 1978. Vesicular-arbuscular mycorrhizas in plants colonizing black wastes from bituminous coal mining in the Illawa region of New South Wales. *New Phytol.* 81: 53 - 62.
- Khaziev, F.Kh. and Burangolova, M.N. 1965. Activity of enzymes which dephosphorylate organic phosphorus compounds of soil. *Prikl. Biok. Microbiol.* 1: 373-379.
- Khruscheva, E.P. 1977. Wheat mycorrhizal vesicles and their formation depending on fertilizers. *Mikol. Fitopatol.* 11: 471 - 473.
- Kinden, D.A. and Brown, M.F. 1975. Techniques for scanning electron microscopy of fungal structures within plant cells. *Phytopathology* 65: 74 - 76.
- Knowlton, S. and Dawson, J.O. 1983. Effects of *Pseudomonas cepacia* and cultural factors in the nodulation of *Alnus rubra* by *Frankia*. *Can. J. Bot.* 61: 2877 - 2882.
- Kohls, S.J. and Baker, D.D. 1989. Effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants. *Plant Soil* 118: 171 - 179.
- Koske, R.E. and Gemma, J.N. 1988. A modified procedure for staining roots to detect VA mycorrhizas. *Mycol. Res.* 92: 486-505.

- Kothari, S.K., Marschner, H. and Romheld, V. 1991 Effect of a vesicular-arbuscular mycorrhizal fungus and rhizospheric microorganisms on manganese reduction in the rhizosphere and manganese concentration in maize (*Zea mays* L.) .New Phytol . 117: 649-655.
- Krishna, K.R. and Bagyaraj, D.J. 1983 Changes in the free amino nitrogen and protein fraction of groundnut caused by inoculation with VA Mycorrhiza. Ann. Bot.51: 399-401.
- Krebber, O. 1932. Untersuchungen über die warzelknolchen der erle. Arch. Mikrobiol. 3, 588-608.
- Kruckelmann, H.W. 1973. Die Vesikular- arbuskulare mykorrhiza and ihre Beeinflussung in land wirtschaftlichen Kulhiren. Diss., Braunschweig.
- Kucey, R. M. N. and Paul, E.A. 1982. Carbon flow, photosynthesis and N_2 - fixation in mycorrhizal and nodulated faba beans (*Vicia faba* L.). Soil Biol. Biochem. 14: 407-412.
- Kulinska, D., Camargo., V.L.I. and Drozdowicz, A. 1982. Enzyme activities in cerrado soil in Brazil. Pedobiol. 24: 101-107.
- Kurakov, A.V. 1985. Some aspect of the ecology of VAM Selskokhozyaisstennya Biologiya 10: 101-111.
- Kurdali, F. and Domenach, A.M. 1991 Evaluation of nodulation capacity in *Alnus glutinosa* seedlings grown in soil sampled from various depth. Acta Oecologia 12: 397-401.
- Kurdali, F., Rinando, G., Moiround, A. and Domenach, A.M. 1990. Competition for nodulation and N_2 - fixation between a Sp⁺ and Sp⁻ *Frankia* strain on *Alnus incana*.. Soil Biol. Biochem. 22: 57-64.
- Ladd, J.N. and Butler, J.N.A. 1975. Humus enzyme systems and synthetic organic polymer enzyme analogs. In: Soil biochemistry (Eds. Paul, E.A and McLaren, A.D.). Dekker, New York, pp.143-194.
- Lalonde, M. and Calvert, H.E. 1979. Production of *Frankia* hypha and spore as an infective inoculant for *Alnus* sp. In: Symbiotic N_2 - fixation in the management of temperate forests (Eds. Gordon, J.C. Wheelers, C.T. and Perry, D.A.) Corvallis, Oregon, USA. pp.95-110.
- Lalonde, M. Calvert, H.E. and Pine, S. 1981. Isolation and use of *Frankia* strains in actinorhizal formation. In: Current perspective in nitrogen fixation in the management of temperate forests. (Eds. Gibson, A.H. and Newton, W.E.), Aust. Acad. Sci. Canberra, Australia. pp.296-299.
- Lalonde, M. and Devoe, I.W. 1975 Scanning electron microscopy of *Alnus crispa* Var. Mollis fern. root nodules endophyte. Archiv.

Microbiol. 105:87-94.

- Lalonde, M. and Knowles, R. 1975. Ultrastructure, composition and biogenesis of the encapsulation material surrounding the endophyte in *Alnus crispa* var. *Mollis* root nodules. Can. J. Bot. 53:1951-1971
- Lalonde, M., Simon, L., Bonsquet, J. and Seguin, A. 1988. Advance in the taxonomy of *Frankia*: Recognition of species of *alni* and *elaeagni* and novel subspecies *pommerii* and *vanDijkii*. In: nitrogen fixation: three hundred years after (Eds., Bothe H., Bruijn, J. and Newton, W.E.), Fischer, Stuttgart, pp. 671-680.
- Lambert, D.H., Cole, H. Jr. and Baker, D.E. 1980. Adaptation of vesicular-arbuscular mycorrhizae to edaphic factors. New Phytol. 85:513-520.
- Lechevalier, M.P. 1984. The taxonomy of the genus *Frankia*. Plant Soil 78:1-6.
- Lechevalier, M.P., Baker, D.D. and Horriere, F. 1983. Physiology, chemistry, serology and infectivity of two *Frankia* isolates from *Alnus incana* subsp. *rugosa*. Can. J. Bot. 61: 2826-2833.
- Lechevalier, M.P., Horriere, F. and Lechevalier, H. 1982. The Biology of *Frankia* and related organism. Dev. Ind. Microbiol. 23: 51-61.
- Lechevalier, M.P. and Lechevalier, H.A. 1980. The Chemotaxonomy of actinomycetes. In: Actinomycete taxonomy, (Eds. Dietz, A. and Thayer, D.W.), Soc. Ind. Microbiol. Arlington, VA, pp. 227-291.
- Lechevalier, M.P. and Lechevalier, H.A. 1989. Genus *Frankia* *brunchorst* 1886, 174(a) AL. In: Bergley's manual of systematic bacteriol. Vol. 4. (Ed. William, S.T. Wilkins, A.), Baltimore, pp. 2410-2417.
- Lechevalier, M.P. and Lechevalier, H.A. 1990. Systematics, isolations, and culture of *Frankia*. In: The biology of *Frankia* and actinorhizal plants. (Eds. Schwinger, C.R. and Tjepkema J.D.), Academic press, Inc, San Diego, Calif. pp. 35-60.
- Lechevalier, M.P. and Ruan, J.S. 1984. Physiological and chemical diversity of *Frankia* spp. isolated from the nodules of *Comptonia peregrina* (L) Coult. and *Ceanothus americanus* (L). Plant Soil 78: 15-22.
- Leikola, M. 1976. Minirotation forestry in Finland. In: Man and boreal forests. (Eds. Tamm, C.O.), Ecol. Bull. 21, Stockholm, pp. 127-130.
- * Lenhard, G. 1956. The dehydrogenase activity of soil as a measure of the activity of soil micro-organisms. Z. Pflanzenernahr Dugn Bodenkd. 73: 1-11.

- Li, X.L. Eckhard, G. and Marschner, H. 1991 a. Extension of the phosphorus depletion zone in the VA mycorrhizal white clover in a calcareous soil. *Plant Soil* 136: 41- 48.
- Li, X.L. Marchner, H. and Eckhard, G. 1991 b. Acquisition of P and Cu by VAM hyphae and root to shoot transport in white clover. *Plant Soil* 136, 49- 57.
- Lopez, M.F and Torrey, J.G. 1985. Enzymes of glucose metabolism in *Frankia*. *J. Bacteriol.* 162: 110- 116.
- Louis, St. Laurent. and Lalonde, M. 1986. Isolation and characterization of *Frankia* strains isolated from *Myrica gale*. *Gen. J. Bot.* 65: 1356-1363.
- Lundquist, P.O. and Huss-Danell K. 1990. Nitrogenase activity and nitrogenase protein levels in a *Frankia* - *Alnus incana* symbiosis subjected to darkness. *Physiol. Plant.* 79 : 240 - 244.
- Lundquist, P.O. and Huss-Danell K. 1991. Response of nitrogenase to altered carbon supply in a *Frankia* - *Alnus* Symbiosis. *Physiol. Plant.* 83: 331-338.
- Mac Donald, R.M. 1981. Routine production of axenic vesicular-arbuscular mycorrhizas. *New Phytol.* : 86-93.
- Mac Donald, R.M. and Lewis, M. 1978.. The occurrence of some acid phosphatases and dehydrogenases in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. *New Phytol.* 80: 135-141.
- Mac Kay, J. Simon, L. and Lalonde, M. 1987. Effects of substrate nitrogen on the performance of *In vitro* propagated *Alnus glutinosa* clones inoculated with Sp^+ and Sp^- *Frankia* strains. *Plant Soil* 103:21-31
- Mc Lachlan, K.D. and Norman, B.W. 1961. Phosphores and symbiotic nitrogen fixation in subterranean clover. *J. Aust. Inst. Agric. Sci.* 27: 2444- 245.
- Manjunath, A. and Bagyaraj, D.J. 1981. Components of VA mycorrhizal inoculum and their effect on growth of onion. *New Phytol.* 87: 355- 361.
- Manjunath, A. and Bagyaraj, D.N. 1984. Response of pigeonpea and cowpea to phosphate and dual inoculation with vesicular-arbuscular mycorrhiza and *Rhizobium*. *Trop. Agric.* 61: 48-53.
- Manjunath, A., Hue, N.V. and Habte, M. 1989. Response of *Leucaena leucophala* to vesicular-arbuscular mycorrhizal colonization and rock phosphate fertilization in an oxisol. *Plant Soil* 144: 127- 133.
- Mansour, S.R. and Torrey, J.G. 1991. *Frankia* spores from strain

HFPCg 14 as inoculum for seedling of *Casuarina glauca*. Can. J. Bot. 69: 1251-1256.

Marshall, K.C. 1969. Studies by microelectrophoretic and microscopic techniques of the sorption of illite and montmorillonite to rhizobia. J. Gen. Microbiol. 56 : 301-306.

Marx, C. Dexheimer, J. Gianinazzi- Pearson, V. and Gianinazzi, S. 1992. Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza IV. Ultracytoenzymological evidence (ATPase) for active transfer processes in the host-arbuscule interface. New Phytol. 90: 37- 43.

Mayen, J. 1829. Über das Haverwachsen parasitischer Gebilde aus den wurzeln anderer Pflazen. Flora (Sena) 12: 49- 64.

Mays De Manzi, J. and Cartwright, P.M. 1984. Effect of pH and aluminium toxicity on growth and symbiotic nitrogen development of cowpea (*Vigna unculata* (L) Walp.). Plant Soil 80: 423- 430.

McGee, P.A. 1989. Variation in propagule number. of vesicular-arbuscular mycorrhizal fungi in semi-arid soil. Mycol. Res. 92: 28- 33.

Mc Kell, C.M., Wilson, A.M. and Jones, M.B. 1961. A flotation method for easy separation of roots from soil samples. Agrono. J. 53: 56- 57.

Medina, O.A., Sylvia, D.M. and Kretchmer, Jr. A.E. 1988. Response of siratro to vesicular-arbuscular mycorrhizal fungi II. Efficiency of selected vesicular-arbuscular fungi at different phosphorus levels. Soil Sci. Soc. Am. J. 52 : 416- 419.

Meesters, T.M. 1987. Localization of nitrogenase in vesicles of *Frankia* sp. Cc1 17 by immunogoldlabelling on ultrathin cryosections. Arch. Microbiol. 146; 327- 331.

Meesters, T.M. Van Vliet, M.W. and Akkermans, A.D.L. 1985. Growth, acetylene reduction activity and localization of nitrogenase in relation to vesicle formation in *Frankia* strains Cc1.17 and Cp 12. Arch. Microbiol. 143 : 137- 142.

Menge, J.A. Steirle, D. Bagyarraj, D.J., Johnson, E.L. and Leonard R.T. 1978. Phosphorus concentration in plants responsible for inhibition of mycorrhizal infection. New Phytol. 80 : 575- 578.

Mian, S. and Bond, G. 1978. The onset of nitrogen fixation in young alder plant and its relation to differentiation in the nodular endophyte. New Phytol. 80 : 187- 192.

Midgley, S.J., Turnbull, J.W. and Johnston, R.D. 1983. *Casuarina*- ecology, management and utilization. Commonw. Sci. Ind. Res. Org., Melbourne, Australia.

*** McGonigle, T.P. and Fitter, A.H. 1988. Growth and phosphorus inflow of *Trifolium repens* L. with a range of indigenous vesicular-arbuscular mycorrhizal infection levels under field conditions. New Phytol. 108: 59-65.

- Mikola, P., Vomala, P. and McKaren, E. 1983. Application biological nitrogen fixation in European silviculture. In: Biological nitrogen fixation in forest ecosystems: Foundations and application. (Eds. Gordon, J.C. and Wheeler, C.T.), Nijhoff Junk, The Hague, pp 279- 294.
- Miller, R.E. and Murray, M.D. 1978. The effects of red alder on Douglas- fir growth. U.S. Dep. Agric. For. Serv. Gen. Tech. Rep. PNW- 115, pp. 8.
- Mishra, R.R. and Sharma, G.D. 1979. Distribution of endogonaceae in a subtropical humid forest of Meghalaya. National Academy of Science, INDIA, 61- 65.
- Mishra, R.R., Sharma, G.D. and Kharsyntiew, I.B. 1981. Response of maize to different inoculum densities of vesicular-arbuscular mycorrhizal endophytes. Experimentia 31 : 568- 569.
- *Moller, H. 1890. Beitrage Zur Kenntnis der *Frankia subtilis* Brunthorst. Ber. Dtsch. Bot. Ges. 8: 215- 224.
- Moiroud, A., Faure- Raynaud, M. and Simonet, P. 1984. Influence of temperature on the growth and viability of pure strains of *Frankia* isolated from *Alnus* nodules. Plant Soil 78: 91- 97.
- Mort, A., Normand, P. and Lalonde, M. 1983. 2-O- Methyl D-Mannose, a Key sugar in the taxonomy of *Frankia*. Can. J. Microbiol. 28: 993- 1002.
- Morton, J.B. 1990. Evolutionary relationship among arbuscular mycorrhizal fungi in the endogonaceae. Mycol. 82 : 192 - 207.
- Mosse, B. Fructifications associated with strawberry roots. Nature(London) 171: 974.
- Mosse, B. 1956. Fructifications of Endogone species causing endotrophic mycorrhiza in fruits plants. Ann. Bot. (London) 20: 349- 362.
- Mosse, B. 1957. Growth and chemical composition of mycorrhizal and non- mycorrhizal apple .Nature (London) 179: 922- 924.
- Mosse, B. 1959. The regular germination of resting spores and same observation on the growth requirement of an *Endogone* sp. causing vesicular- arbuscular mycorrhiza. Tran. Brit. Mycol. Soc. 42; 273- 286.
- Mosse, B. 1972. The influence of soil type and endogone strain on the growth of mycorrhizal plant on phosphate- deficient soil. Rev. Ecol. Boil. Sol. 9 : 529
- Mosse, B. 1973. Advances in the study of vesicular- arbuscular mycorrhiza. Ann. Rev. Phytopathol. 11: 171- 196.
- Mosse, B. and Bowen G.D. 1968. The distribution of *Endogone* spores in some Australian and New Zealand soil and in an

- experimental field soil at Rothamsted. Trans. Br. Mycol. Soc. 51: 485- 492.
- Mosse, B., Powell, C.L. and Hayman, D.S. 1976. Plant growth response to vesicular-arbuscular mycorrhiza IX. Interactions between VA mycorrhiza, rock phosphate and symbiotic nitrogen fixation. New Phytol. 76: 331- 342.
- Mowry, H. 1933. Symbiotic nitrogen fixation in the genus *Casuarina*. Soil Sci. 36: 409-425.
- Mugwira, L.M. 1980. Growth and Ca, Mg, K and P uptake by triticale, wheat and rye at four Al levels. J. Plant Nutr. 2: 591- 606.
- Munns, D.N. and Mosse, B. 1980. Mineral nutrition of legume crops. In: Advances in Legume science, (Eds. Summerfield, R.J. and Burtin, A.H.), HMSO, London, pp. 115- 125. 1980.
- Murry, M.A., and Lopez, M.F. 1989. Interaction between hydrogenase, nitrogenase and respiratory activity in a *Frankia* isolate from *Alnus rubra*. Can. J. Microbiol. 35 : 636- 641.
- Nair, M.G., Safir, G.R. and Sequeira, J.O. 1991. Isolation and identification of vesicular-arbuscular mycorrhiza stimulatory compounds from clover (*Trifolium repens*) roots. Appl. Environ. Microbiol. 57: 434- 439.
- Nazaret, S., Cournoyer, B., Normand, P., Simonet, P. 1991. Phylogenetic relationship among *Frankia* generic species determined by use of amplified r - DNA sequence. J. Bacteriol. 173: 4072- 4078
- Neal, J.R. 1973. Influence of selected grasses and herbs on soil phosphatase activity. Can. J. Soil. Sci. 53: 119- 121.
- Nemec, S. and Merdith, F.I. 1981. Amino acid content of leaves in mycorrhizal and non mycorrhizal citrus roots stocks. Ann. Bot. 47: 351- 58.
- Nesme, X., Normand, P., Trembley, F. M and Lalonde, M. 1985. Nodulation speed of *Frankia* sp. on *Alnus glutinosa*, *A. crispa* and *Myrica gale*. Can. J. Bot. 63: 1292-1295.
- Newbould, P. and Rangle, A. 1984. Effect of lime, phosphorus and mycorrhizal fungi on growth, nodulation and N₂ fixation by white clover (*Trifolium repens*) grown in U.K hill soil. Plant Soil 76: 105- 114.
- Newcomb, W., Peterson, R.L., Callahan, D., and Torrey, J.G. 1978. Structure and host-actinomycete interaction in developing host nodules of *Comptonia peregrina*. Can. J. Bot. 56: 502- 531.
- Nielson, J.D. and Eiland, F. 1980. Investigation on the relationships between P- fertility, phosphatase activity and ATP content in soil. Plant Soil 57: 95- 103.

- ***Fairman, A.K., Robson, A.D. and Abbot, L.K. 1980. The effectiveness of vesicular-arbuscular mycorrhizas in increasing growth and phosphorus uptake of subterranean clover from phosphorus sources of different solubilities. *New Phytol.* 84: 327-338.
- Nielsen, J.D. and Jenson, A. 1983. Influence of vesicular-arbuscular mycorrhizal fungi on growth and uptake of various nutrients as well as an ratio of fertilizer P for lucerne (*Medicago sativa*.) *Plant soil* 70: 165-172.
- Nohrstedt, H.O. 1985. Biobological activity in soil from forest stands in central Sweden as related to site properties. *Microbial Ecol.* 11: 259-266.
- Noordwijk, M.V. and Hairiah, K. 1986. Mycorrhizal infection in relation to soil pH and soil P content in rainforest of North Sumatra. *Plant Soil* 96: 299-
- Norridge, N.A. and Benson, D.R. 1986. Isolation and nitrogen fixing activity of *Frankia* sp. strain cp 11 vesicles. *J. Bacteriol.* 166: 301-305.
- Normand, P. and Lalonde, M. 1982. Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. *Can. J. Microbiol.* 28: 1133-1142.
- Normand, P., Simonet, P., Butour, J.L., Rosenberg, C., Moiroud, A. and Lalonde, M. 1983. Plasmids in *Frankia* sp. *J. Bacteriol.* 155: 32-35.
- O'Campo, J.A. and Azcon, R. 1985. Relationships between the concentration of sugars in the roots and VA mycorrhizal infection, *Plant Soil*. 86: 95-100.
- Olivers, A.J., Smith, S.E., Nicholas, D.J.D., Wallace, W. and Smith, F.A. 1983. Activity of nitrate reductase in *Trifolium subterraneum*: Effect of mycorrhizal infection and phosphate nutrition. *New Phytol.* 94: 63-79.
- Olsen, S.R., Cole, C.V., Watanable, F.S. and Deen, L.A. 1954. Estimation of available phosphorus in soil by extraction with sodium bicarbonate. *Circ.* 939. USDA. Sup. of Documents US Govt. Printing Office, Washington, pp. 19.
- Owusu-Bennoah, E. and Wild, A. 1979. Autoradiography of the depletion zone of phosphate around onion plants in the presence of vesicular-arbuscular mycorrhiza. *New Phytol.* - 82: 133-140.
- Pacovsky, R.S., Paul, E.A. and Bethlenfalvay, G.J. 1986. Response of mycorrhizal and P-fertilized soybeans to nodulation by Bradyrhizobium or ammonium nitrate. *Crop. Sci.* 26: 145-150.
- Paccini, D., Ocampo, J.A. and Bedmer, E.J. 1986. Possible influence of *Rhizobium* on VA mycorrhiza metabolic activity in double symbiosis of alfalfa plants (*Medicago sativa* L.) grown in pot experiment. *Biol. Fertil. Soils* 6: 65-67.
- *** Palmgren, K., Saarsalmi, A. and weber, A. 1985. Nitrogen fixation

- and biomass production in some alder clones. Silva Fenn. 19 : 407 -420.
- Pang, P.C. and Kolenko, H. 1986. Phosphomonoesterase activity in forest soils. Soils Biol. Biochem. 18:35-40.
- Parkinson, D., Balasopriya, I. and Winter Holder, K. 1968. Studies in fungi in a pinewood soil. III. Fungal growth and total microbial activity. Rev. Ecol. Biol. Sol. 4: 637-645.
- Parson, W.L., Robertson, L.R. and Carpenter, C.V. 1985. Characterization and infectivity of a spontaneous variant isolated from *Frankia* sp. WEY 0131391. Plant Soil 87:31-42
- Parsons, R., Silvester, W.B., Harris, S., Grueters, W.T.M. and Bullivant, S. 1987. *Frankia* vesicles provided inducible and absolute oxygen protection for nitrogenase. Plant Physiol. 83 : 728 - 731.
- Paula, M.K. and Sequiera, J.O. 1990. Stimulation of hyphal growth of VA mycorrhizal fungus *Gigaspora marginata* by suspensions cultured *Pueraria phaseoloides* and cell products. New Phytol. 115: 69-75.
- Paulino, V.T., Ocampo, J.A. and Bedmar, E.J. 1987. The effect of interaction : *Rhizobium* - VAM in N_2 fixation in tropical fodder legumes growth on an inert medium. Revista de Agriculture Brazil 62: 3 - 15.
- Pedersen, C.T., Safir, G.R., Sequeira, J.O. and Parent, S. 1991. Effect of phenolic compounds on asparagus mycorrhiza. Soil Biol. Biochem. 23 : 491 - 494.
- Perinet, P., Brouillette, J.G., Fortin, J.A. and Lalonde, M. 1985. Nitrogen fixation and biomass production in some alder clones. Silva Fenn. 19 : 407 - 420.
- Perradin, Y., Mottet, M.J. and Lalonde, M. 1983. Influence of phenolics on *In vitro* growth of *Frankia* strains. Can. J. Bot. 61:2807-2814.
- Perry, C.B., Bvess, V.H. and Stulz, J.C. 1991. Ultrastructure of actinorhizal nodules of *Cowania subtintegra* (Rosaceae). Bot. Gaz. 152: 8-12.
- Peterson, N.A., Smadar, W., Hana, B. and Yoram, K. 1991. N_2 fixation activity and nitrate concentration do not affect *Glomus macrocarpum* infection of *medicago sativa* L. Soil. Biol. Biochem. 23: 703-705.
- Phillips, J.M. and Hayman, D.S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55 : 158-161.
- Pizzelle, G. 1984. Seasonal variation of the sexual reproductive growth and nitrogenase activity (C_2H_2) in mature *Alnus*

- *Raman, N. and Elumalai, S. 1991. Studies on mycorrhizal and actinorhizal association in *Casuarina equisetifolia* in coramandal coastal region. *J. Trop. Forestry* 7 : 138-150.
glutinosa. *Plant Soil* 78: 181 - 184.
- Pizelle, G., Bendmar, S.,m & Thiery, G.1992. Nitrate reductase and nitrogenase activities in black alder: relationship and effects of dark/light treatments. *Acta Oecologica* 13 : 510.
- Powell, C.LL. 1975. Plant growth response to vesicular-arbuscular mycorrhiza VIII. Uptake of P by onion and clover infected with different *Endogone* spore types in 32 P labelled soils. *New Phytol.* 75 : 563-566.
- Powell, C.LL. and Daniel, J. 1978. Mycorrhizal fungi stimulate Uptake of soluble and insoluble phosphate fertilizer from a phosphate - deficient soils. *New Phytol.* 80 : 351 - 358.
- Pregent, G. and Camire, C. 1985. Biomass production by alders on four abandoned agricultural soils in Quebec. *Plant Soil* 87 : 185 - 193.
- Quispel, A., Burggraaf, A.J.P., Borsje, K.A. and Tak, 1983. The role of lipid in the growth of *Frankia* isolates. *Can. J. Bot.* 61: 2801 - 2806.
- Quispel, A. and Tak, T. 1978. Studies on the growth of the endophyte of *Alnus glutinosa* (L) Vill. In nutrient solutions. *New Phytol.* 81 : 587 - 600.
- Racette, S. and Torrey, J.G. 1988. The isolation, culture and infectivity of a *Frankia* strain from *Gymnostoma papuanum* (Casuarinaceae). *Plant Soil* 118:165-170.
- **
- Rai, L.C., Gour, J.P. and Kumar, M.D. 1981. Protective effect of certain environment factors on the toxicity of Zinc, Mercury, or Methylmercury to *Chlorella villgaris*. *Environ. Res.* 25: 250-259
- Rajapakse, S. Zuberer, D.A and J.C. Miller JR. 1989. Influence of phosphorus level on a mycorrhizal. Colonization and growth of cowpea cultivars. *Plant Soil* 114: 45-52.
- ***
- Ratnayake, M., Leonard, R.T. and Mengè, J.A. 1978. Root exudation in relation to supply of phosphorus and its possible relevance to mycorrhizal formation. *New Phytol.* 81: 543-552.
- Read, D.J., Koveheri, H.K. and Hodson. J. 1976. Vesicular-arbuscular mycorrhiza in natural vegetation systems. *New Phytol.* 77: 641-653.
- Reddell, P. 1986. Soil and plants factors affecting nodulation and nitrogen fixation in *Casuarina-Frankia* Symbioses. Ph.D. Thesis. Univ. of Western Australia, Perth, Australia.
- Reddell, P. and Bowen, G.D. 1985. Do single nodules of *Casuarinaceae* contain more than one *Frankia* strain. *Plant Soil* 88: 275-280.
- *Ragupathy, S., Mohankumar, V. and Mahadevan, A. 1988. Distribution of VAM in Thanjavur district flora. In: *Mycorrhiza for green Asia* (Eds. Mahadevan, A., Raman, N. and Natarajan, K.) centre for advanced studies in Botany, University of Madras. pp 95-98.

- Reddell, P., Bowen, G.D. and Robson, A.D. 1985. The effects of soil temperature, on plant growth, nodulation and nitrogen fixation in *Casuarina cunninghamiana* Mig. New Phytol. 101:441-450.
- Reddell, P. and Spain, A.V. 1991. Transmission of infective *Frankia* (actinomycetales) propagules in casts of the endogeic earthworm *Pontoscolex Corethrurus*. Soil Biol. Biochem. 32:775-778.
- Rendette, E.F. and Reeves, F.B. 1981. Interactions between vesicular mycorrhizas and *Rhizobium* and their effect on sweet vetch growth. Soil. Sci. 132:410.
- Redhead, J.F. 1975. Endonophic mycorrhizas in Nigeria: Some aspects of the ecology of the endotrophic mycorrhizal association of *Khaya grandifolia*. C.Dc> In: Endomycorrhizas (Eds. Sanders, F.E., Mosse, B. and Tinker, P.B) Academic Press, London pp. 447-459.
- * Reissek, S. 1984. Endophyten der Pflanzen Zelle, Naturn. Abb. 1:31.
- Rhodes, L.H. and Gerdemann, J. W. 1975. Phosphate uptake zones of mycorrhizal and nonmycorrhizal onions. New Phytol. 75: 555-561.
- Rhodes, L.H. Gerdemann, J.W. 1978. Influence of phosphorus nutrition of sulfur uptake by vesicular-arbuscular mycorrhizae. Soil Biol. Biochem. 10:361-364.
- Ride, J.P. and Drysdale, R.B. 1972. A rapid method for the chemical estimation of filamentous fungi in plant tissue. Physiol. Pl. Pathol. 2: 7-15.
- Righetti, T.L. Chard, C.H. and Backhaus, R.A. 1986. Soil and environmental factors related to nodulation in *Cowania* and *Purshia*. Plant and Soil 91: 147-160.
- Robson, A.D. and Loneragan, J.F. 1978. Response of pasture grasses to soil chemical factors other than nitrogen and phosphorus with particular emphasis on the legume symbiosis. In: Plant relations in pastures. (Ed. Wilson. J.R.) CSIRD, Melbourne, pp. 128-142.
- Rodriguez- Barrueco, C. 1966. Fixation of nitrogen in root nodules of *Alnus jorru[un]ensis* HBK. Phytol 23: 103-110.
- Rodriguez- Barrueco, C. 1968. The occurrence of the root nodule endophyte of *Alnus glutinosa* and *Myrica gale* in soil. J.Gen. Microbiol. 52: 189-194.
- Rogers, H.T., Pearson, R.W. and Pierre, W.H. 1942. The source and phosphatase activity of exoenzyme systems of corn and tomato root. Soil Sci. 54: 354-366.
- Rosbrook, P.A. 1990. Effect of inoculum type and placement on

- nodulation and growth of *Casuarina cunninghamiana* seedlings. For. Ecol. Manag. 36:135-147.
- Rose, S. 1980. Mycorrhizal associations of some actinomycete nodulated nitrogen-fixing plants. Can J. Bot. 58: 1449-1454.
- Rose, S. and Trappe, J.M. 1980. Three new endomycorrhizal *Glomus* spp. associated with actinorrhizal shrubs. Mycolaxon. 10:413-420.
- Rose S.L. and Youngberg C.T. 1981. Tripartite association in snowbush (*Ceanothus velutinus*): Effect of VAM on growth, nodulation and nitrogen fixation. Can. J. Bot. 59: 34-39.
- Rosendahl, S., Sen, R. Heppers, C.M. and Aguilar, C.A. 1989. Quantification of three vesicular-arbuscular mycorrhizal fungi (*Glomus* sp.) in root of leek (*Allium porrum*) on the basis of activity of diagnostic enzyme after polyacrylamide gel electrophoresis. Soil Biol Biochem. 21: 519-522.
- Ross, D.J., Spair, T.W., Gilltrap, D.J., Mcneilly, B.A. and Molloy, L.F. 1975. A principal component analysis of some biochemical activities in climosequence of soils. Soil Biol. Biochem.:349-355.
- Ross, J.P., 1971. Effects of phosphatase fertilization on yield of mycorrhizal and nonmycorrhizal soybeans. Phytopathol. 61: 1400-1403.
- Rovira, A.D. 1965. Interaction between plant root and soil microrganisms Annu. Rev. Microbiol. 12: 241-266.
- Russo, R.O. 1989. Evaluating *Alnus* endophytes (*A. acuminata*-*Frankia*-mycorrhizae). Interaction. 1. acetylene reduction in seedlings inoculated with *Frankia* strain Ar 13 and *Glomus intra-radices* under three Phosphorus levels. Plant Soil 118:151-155.
- Ryan, J.J., Stroehlein, L. and Miyanoto, S. 1975. Sulphuric acid application to calcareous soils: Effect on growth and chlorophyll content of common Bermuda grass in the greenhouse. Agron. J. 116:633-637.
- Rytter, L. 1989. Distribution of root and nodules and biomass allocation under young intensively managed grey alder stand on a peat bog. Plant Soil 119: 71-79.
- Saif, S.R. 1987. Growth responses of tropical forage plant species to vesicular-arbuscular mycorrhizae. 1. Growth, minerals uptake and mycorrhizal dependency. Plant Soil 97: 25-35.
- Same, B.I., Robson, A.D. and Abbott, L.K. 1983. Phosphorus, soluble carbohydrate and endomycorrhizal infection. Soil Biol. Biochem. 15 :593-598.

- Sanders, F.E. and Tinker, P.B. 1971. Mechanism of absorption of phosphorus from soil by *Endogone* mycorrhizas. *Nature* 233:278-279.
- Sanders, I.R. and Fitter, A.H. 1992a. The ecology and functioning of vesicular-arbuscular mycorrhizas in coexisting grassland species. I. Seasonal patterns of mycorrhizal occurrence and morphology. *New Phytol.* 120: 517-524.
- Sanders, I.R. and Fitter, A.H. 1992b. The ecology and functioning of vesicular-arbuscular mycorrhizas in coexisting grassland species. II. Nutrient uptake and growth of vesicular-arbuscular mycorrhizal plants in a seminatural grassland. *New Phytol.* 120: 525-533.
- * Schaede, R. 1938. The Actinomyceten-symbiose von *Myrica gale*. *Planta* 29:32-46.
- * Schaede, R. 1962. "Die pflanzlichen symbiosen" 3rd ed., pp. 42-54. Fischer, Stuttgart (originally published in 1943)
- Schenck, N.C. and Smith, G.S. 1982. Additional new and unreported species of mycorrhizal fungi (Endogonaceae) from Florida. *Mycologia* 74 : 77 - 92.
- Schinner, F.A. and Gurschler, A. 1978. Saccharase and dehydrogenase activities as messungen in verschiedepin Boden der oberen Subalpinen stufl. *Universitat Sverlag* 10: 275-288.
- Schubert, A. and Hayman, D.S. 1986. Plant growth response to vesicular-arbuscular mycorrhiza XVI. Effectiveness of different endophytes at differnt levels of soil phosphate. *New Phytol.* 103: 29-90.
- Schubert, A., Marzachi, C., Mazzilitelli, M., Cravero, M.C. and Bonfante -Fasolo, P. 1987. Development of total and viable extraradical mycelium in the vesicular-arbuscular mycorrhizal fungus *Glomus clarum* Nicol. and Schenck. *New Phytol.* 107: 183-190.
- Schwab, S.M. and Reeves, F.B. 1981. The role of endomy corrhizae in vegetation practices in the semiarid waste. III: Vertical distribution of vesicular-arbuscular (VA) mycorrhiza inoculum potential. *Am. J. Bot.* 68: 1293-1297.
- Schwab, S.M., Johnson, E. L. V. and Menge, J.A. 1982. Influence of Simazine on formation of vesicular-arbuscular mycorrhization *Cohenopodium quinona* wild. *Plant Soil* 64: 284-287.
- Schwab, S.M., Menge, J. A. and Leonard, R.T. 1983. Comparison of stages of vesicular-arbuscular mycorrhiza formation in sudangrass (*sorghum vulgare*) grown at two levels of phosphorus nutrition. *Am. J. Bot.* 70: 1225-1232.
- Schwinker, C.R., Berry, A.M. and Disney L.D. 1982. Seasonal pattern

of root nodules growth, endophyte morphology, nitrogen activity and shoot development in *Myrica gale*. Can. J. Bot. 60: 746-757.

Seguin, A. and Lalonde, M. 1989. Detection of pectolytic activity and pel homogenous segmenes in *Frankia*. Plant Soil 118: 221-229.

Seikh, N.A., Saif, S. R. and Khan, A.G. 1975. Ecology of *Endogone*: II relationship of *Endogone* spore population with chemical soil factors. Islamabad J.Sci. 2:6.

Seilor, J.R. and Johnson, J.D. 1984. Growth and actylene reduction of black alder seedlings in response to water stress Can. J. For. Res. 14: 477-480.

Seilor, J. R. and Mc Cormick. L.H. 1982. Effects of soil acidity and phosphorus on the growth and nodule development of black alder. Can. J. For. Res. 12: 576-581.

Sellstedt, A. 1986. Nitrogen and carbon utilization in *Alnus incarrah* fixing N_2 or supplied with NO_3 at the same rate. J.Expt.Bot. 37:786-797.

Sellstedt, A. , Huss-Danell, K and Ahlqvist, A.S. 1986. Nitrogenase fixation and biomass production in symbioses between *Alnus incana* and *Frankia* strain with different hydrogen metabolism. Physiol. Plant. 66:99-107.

Sen, R. and Hepper, C.M. 1986. Characterization of vesicular - arbuscular mycorrhizal fungus(*Glomus Spp.*)by selective enzyme staining folowing Polyaerlamide gel electrophoresis. Soil Biol. Biochem. 18: 29-34.

Shaede, R. 1933. user die symbionten in ten knolleren der Erle and des Sanddornes and die cytologischen verhaltrisse in innen. Planta 19: 389-416.

Sharma, E. 1988. Altitudinal variation in nitrogenase activity of the Himalayan alder naturally regenerating on land slide affected sites. New Phytol. 108 : 522 - 531.

Sharma, E. and Ambasht, R.S. 1984. Seasonal variation in nitrogen fixation by differnt age of root nodules of *Alnus nepalensis* plantations, in the Eastern Himalayas. J.Appl. Ecol.21:265-270.

Sharma, E. and Ambasht, R.S. 1986. Root nodules age -class transition, prodution and decomposition in an age sequence of *Alnus nepalensis* plantation stands in the Eastern Himalayas. J. Appl. Ecol. 23:689-701.

Sharma, E., Ambasht, R.S. and Singh, M.P. 1985. Chemical soil properties under five age series of *Alnus nepalensis* plantation in the Eastern Himalayas. Plant Soil 84:105-113.

- Sharma, S.K. Sharma, G.D. and Mishra, R.R. 1986. Endogonaceae are sub-tropical forest of North-East India. J. Ind. Bot. Soc. 66:266-268.
- Sharma, S.K., Sharma, G.D. and Mishra, R.R. 1986. Status of mycorrhizae in subtropical forest ecosystem of Meghalaya. Acta Bot. Indica 14 : 87 - 92.
- Sheppard, L.J. and Floate, M.J.S. 1984. The effect of soluble Al on root growth and radicle elongation. Plant Soil 80: 301-306.
- Sheppard, L.J., Hooker, J.E., Wheeler, C.t. and. Smith, R.I. 1988. Glasshouse evolution of the growth of *Alnus rubra* and *A. glutinosa* on peat and brown earth soil when inoculated with four sources of *Frankia*. Plant Soil 110: 187-198.
- Shibata, K, and Tahara, M. 1917. studies iiber die wurzelknollchen. Jahrb. Wiss. Bot. 31: 157-182.
- Shipton, W.A. and Burggraaf, A.J.P. 1982. *Frankia* growth and activity as influenced by water potential. Plant Soil 69:293-297.
- Shipton, W.A. and Burggraaf, A.J.P. 1983. Aspect of the cultural behaviour of *Frankia* and possible ecological implications. Can. J. Bot. 61:
- Shnyreva, A.V. and Kulayev, I.S. 1990. Extracellular xylanases of endomycorrhizal fungus *Glomus* Spp. Moscow. Univ. Biol.Sci.Bull. 45: 47-50.
- Shuji, and Takao, K. 1980. Relations between soil microbial and soil properties in grassland. Soil Sci. Plant Nutr. 32:587-598.
- Si-dhu, O.P., Behl, H.M., Gupta, M.L. and Janardhanan, K.K. 1990. occurance of VA mycorrhizae in *Casuarina equisetifolia*. Current Sci. 59: 422.
- Silvester, W.B. 1977, Dinitrogen fixation by plants association excluding legumes. In: A treatise on dinitrogen fixation (Eds. Hardy, R.W.F. and Gibson, A.H.). Wiley, New York PP. 141-189.
- Simon, L., Lalonde, M. and Bruns, T.D. 1992. Specific amplication of 18 S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungus colonizing roots. Appl. Environ. Microbiol. 58:291-295.
- Simonet, P., Capellono, A., Navarro, E., Bardin, R. and Moiroud, A. 1984. an improved method for lysis of *Frankia* with achromopeptidase, allows detection of new plasmids. Can. J. Microbiol. 30: 1292-1295.
- Simonet, P., Le, T.N, Moiroud, A. and Bardin, R. 1989. Diversity of *Frankia* strains isolated from a single alder stand. Plant Soil 118: 13-22.

- Simonet, P., Normand, P., Hirseh, A.M. and Akkermans, A.D.L. 1990. The genetic of the *Frankia* actinorrhizal symbiosis, In : The Moleender biology of symbiotic nitrogen fixation. (Ed. Greesshoff, P.M.) CRC press, Boca Raton, pp.77-109.
- Simonet, P., Thiyle, N., Moiroud, A., and Bardin, R. 1989. Deversity of *Frankia* strains from a single alder stand. Plant Soil 118: 13-22.
- Singh, R.L. 1971 India, a regional geography. New Delhi, India.
- Siquiera, J.O., Hubell, D.H. and Mohmud A.W. 1990 Effect of liming on spore germination, germs tube growth and root colonization on by VAM fungus. Plant Soil 76: 115-124.
- Skipper, H.D. and Smith, G.W. 1979. Influence of soil pH on the soybean- endomycorrhizae symbiosis. Plant Soil 53: 559-563.
- Skujins, J.J. and McLaren, A.D. 1968. Persistence of enzymatic activities in stored and geologically preserved soils. Enzymologia 34: 213-225.
- Smith, S.E. 1982. Inflow of phosphate into mycorrhizal or non-mycorrhizal plants of *Trifolium subterraneum* at different level of soluable phasphate. New Phytol. 90: 293-303.
- Smith, G.S. and Roncadori, R.W. 1986. Respones of three vesicular-arbuscular mycorrhizal fungus at four soil temperatures and their effects on cotton growth. New Phytol. 104: 89-95.
- Smith, F.A. and Smith, S.E. 1990. Solute transport at the interface: Ecologycal implications. Agric.Ecosyst. Environ. 28:
- Smith, S.E. 1982. Inflow of phosphate into mycorrhizal and normycorrhizal plants of *Trifolium subterraneum* at differnt levels of soil phosphate. New Phytol. 90: 293-303.
- Smith, S.E. and Daft, M.J. 1977. The effects of mycorrlizae on the phosphate content, nitrogen fixation and growth of *Medicago sativa*. In : Microbiol Ecology (Eds. Loutit, M.W. and Miles, J.A.R. Sprigner-Verlag Berlin, Heidelberg, pp.314-319.
- Smith, S.E., St. John, B.J. Smith, F.A. and Nicholas, D.J.D. 1985. Activity of glumate synthetase and gluamate dehydrogenase in *Trifolium subterraneum* L. and *Allium cepa* L. New Phytol. 99: 211-227.
- Smith, S.E., Teester, M. and Walker, N.A. 1986. The development of mycorrhizal root system in *Trifolium subterraneum* L.: Growth of mycorrhizal roots and uniformity of spatial distribution of mycorrhizal infection units in young plants. New Phytol. 103: 117-131.
- Smith, S.N and Pugh, G.J.F. 1979. Evalution of dehydrgpgease as a suitable indicator of soil microflora activity. Enzyme Microb. Technol. 1: 279-281.

- Smith, S.E. and Walker, N.A. 1981. A quantitative study of mycorrhizal infection in *Trifolium* : Separate determination of the roots of infection and mycelial growth. *New Phytol.* 89:225-240.
- Smolander, A. 1990. *Frankia* populations in soil under different tree species with special emphasis on soil under *Betula pendula*. *Plant Soil* 121:1-10.
- Smolander, A., Nurmiäho-Lassila and Sundman, V. 1986. Effect of clay mineral (montmorillonite) on the nodulation of *Alnus* and nitrogenase activity of *Frankia* in pure culture. *Symbiosis* 6:37-52.
- Smolander, A. and Sundman, V. 1987. *Frankia* in acid soils of forest devoid of actinorhizal plants. *Phytol. Plant.* 70: 297-303.
- Smolander, A., Van Dijk, C. and Sundman, V. 1988. Survival of *Frankia* strains introduced into soil. *Plant Soil* 106:65-72.
- Spanu, P., Bonfante-Fasolo, P. and Boler, T. 1990. Chitinase activity and VA mycorrhizal development. *Agric. Ecosyst. Environ.* 29:
- Spiers, G.A. and McGill, W.B. 1979. Effects of phosphorus addition and energy supply on acid phosphatase production and activity in soil. *Soil Biol. Biochem.* 11:3-8.
- Sprent, J.I., Scott, R. and Perry, K.M. 1978. The nitrogen economy of *Myrica gale* in the field. *J. Ecol.* 66: 657-668.
- Stahl, D. and Christensen, M. 1982. Mycorrhizal fungus associated with *Bouteloua* and *Agropyron* in Wyoming sagebrush-grasslands. *Mycologia* 74: 877-885.
- Sterks, N.M. 1971. Mycorrhizae and nitrogen cycling in the tropics. In : *Mycorrhizae*. (Ed. Hacskeylo, F.F.), US Dep. Agric. For. Serv. Misc. Pub. 1189.
- Stevenson, I.L. Dehydrogenase activity in soil. *Can J. Microbiol.* 5: 229-235.
- Stevenson, I.L. 1956. Some observations on the microbiol activity in remoistened air dried soils. *Plant Soil* 8: 170-182.
- Stewart, W.D.P. and Bond, G. 1961. The effect of ammonium nitrogen on fixation of elemental nitrogen in *Alnus* and *Myrica*. *Plant Soil* 14: 347-359.
- Stewart, W.D.P., Fitzgerald, G.P. and Burris, R.H. 1967. In situ studies on N_2 -fixation using the acetylene reduction technique. *Proc. Nat. Acad Sci. USA*, 56: 2071-2073.
- St. John, T.V. 1980. A survey of mycorrhizal infection in an Amazonian rainforest. *Acta Amazonica* 10:527-534.

- ** Torrey, J.G. and Tjepkema, J.D. 1979. Symbiotic nitrogen fixation in actinomycete nodulated plants. Bot. Gaz. 140(suppl.); S 126-S129.**
- Scutellospora calospora* (Nicol.& Gerd.) Walker & Sander on subterranean clover. New Phytol. 118: 463-469.
- Thompson, L.M. and Troeh, F.R. 1985. Soils and soil fertility. Tata McGraw-Hill Publishing company, New Delhi-110020.
- Tinker, P.B. 1975. Effects of vesicular-arbuscular mycorrhizas on higher plants. Symp. Expt. Biol. 29: 325-349.
- Tinker, P.B. 1982. Mycorrhizas: The present position. In: wither soil research. Trans. 12th Int. Congr. Soil Sci., New Delhi.
- Tisa, L.S. and Ensign, J.C. 1987. The calcium requirement for functional vesicle development and nitrogen fixation by *Frankia* strain EAN 1pec and Cp11. Arch. Microbiol. 149: 24-29.
- Tisa, L.S., McBride, M. and Ensign, J.C. 1983. Studies of growth and morphology of *Frankia* strains EAN 1 pec, EU11c, Cp11 and ACN1g. Can. J. Bot. 61:2768-2773.
- Tisserant, B., Gianinazzi-Pearson, V., Gianinazzi, S. and Gollotte, A. 1993. In Planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient mycorrhizal infection. Mycol. Res. 97: 245-250.
- Tjepkema, J.D. and Murry, M.A. 1989. Respiration and nitrogenase activity in nodules of *Casuarina cunninghamiana* and cultures of *Frankia* Sp. HFP 020203: Effects of temperature and partial pressure of oxygen. Plant Soil 118: 251-260.
- Torrey, J.G. 1978. Nitrogen fixation by actinomycete nodulated angiosperms. Bioscience 28 : 586-592.
- Torrey, J.G. 1987. Endophyte sporulation in root nodules of actinorhizal plants. Physiol. Plant. 70: 279-288.
- ** Trappe, J.M. 1982. Synoptic keys to the genera and species of zygomycetous mycorrhizal fungi. Phytopathol. 72:1102-1108.**
- Trappe, J.M. and Fogel, R.D. 1977. Ecosystematic functions of mycorrhizae. In: The below ground ecosystem, a synthesis of plant associated processes. Range Sci. Dep. Serv. No. 26, Colorado State Univ., Fort Collins, pp. 205-214.
- Trevors, J.T. 1984. Effect of substrate concentration, inorganic nitrogen, O₂ concentration, temperature and pH on dehydrogenase activity in soil. Plant Soil 77: 285-293.
- Tripps, L.N., Bezdicsek, D.F. and Heilman, P.E. 1979. Seasonal and diurnal patterns and rates of nitrogen fixation by young red alder. For. Sci. 25: 371-380.
- Turner, J., Kelly, J. and Newman, L.A. 1978. Soil nutrient-vegetation relationship in the Eden area. N.S.W. 11 vegetation- Soil associations. Aust. For. 41: 223- 231.

- Vandenbosch, K.A. and Torrey, J.G. 1965. Host-endophyte interactions in effective and ineffective nodule induced by the endophyte of *Myrica gale*. Can. J. Bot. 61: 2899-2909.
- Van Dijk, C. 1978. Spore formation and endophyte diversity in the root nodule of *Alnus glutinosa* (L.) Vill. New Phytol. 81 : 601 - 615.
- Van Dijk, C. 1979. Endophyte distribution in soil. In : Symbiotic nitrogen fixation in the management of temperate forests (Eds. Gordon, J.C., Wheeler, C.T. and Perry, D.A.), Oregon St. Univ. Press, pp. 84 - 94.
- Van Dijk, C. 1984. Ecological aspects of spore formation in the *Frankia-Alnus* symbiosis. Ph.D. thesis, State Univ. The Netherlands.
- Van Dijk, C. and Merkus, E. 1976. A microscopical study of the development of a spore-like stage in the life cycle of the root - nodule endophyte of *Alnus glutinosa* and *Alnus incana* in Finland. New Phytol. 77 : 73 - 91.
- Van Niegroet, H. and Cole, D.W. 1984. The impact of nitrification on soil acidification and cation leaching in a red alder forest. J. Environ. Qual. 13 : 585.
- Van Nuffelsen, M. and Schenck, N.C. 1984. spore germination, penetration and root colonization of six species of vesicular - arbuscular mycorrhizal fungi on soybean. Can. J. Bot. 62 : 624 - 628.
- Van Straten, J., Akkermans, A.D.L. and Roelofsen, W. 1977. Nitrogenase activity of endophyte suspensions derived from root nodules of *Alnus*, *Hippophae*, *Shepherdia* and *Myrica* spp. Nature (London) 266 : 257 - 258.
- Vekemans, X., Godden, B. and Pennick, M.J. 1989. Factor analysis of the relationships between several physico - chemical and micro²biological characteristics of some Belgian agricultural soil. Soil Biol. Bio²chem. 21 : 53 - 58.
- Verma, N.K. 1982. Studies on the ecology of vesicular-arbuscular mycorrhiza of *Eupatorium riparium* Regel, *E. adenophorum* spreng and *Ospeckia grinda* Wall. ex. D.Don, the common weeds of N.E. region of India. Ph.D. thesis, N.E.H.University, Shillong.
- Vernaud L., Chaboud, A., Prin, Y. and Rougier, R. 1985. Preinfection events in the establishment of *Alnus* - *Frankia* symbiosis : Developments of a spot inoculation technique. Plant Soil 87 : 67 - 78.
- Vikman, P.A. and Huss Danell, K. 1987. Capacity for hexose respiration in symbiotic *Frankia* from *Alnus incana*. Physiol. Plant. 70 : 349 - 354.
- Visser, S., Danielson, R.M. and Parkinson D. 1991. Field performance of *Elaeagnus commutata* and *Shepherdia canadensis* inoculated with VA mycorrhizal fungi. Can. J. Bot. 69 : 1321 - 1328.

- **** Xiao, L.L., Marschner, H. and Eckhard, G. 1991. Acquisition of P and Cu by VAM hyphae and root to shoot transport in white clover. *Plant soil* 136: 49-57.
- 218.
- Whittingham, J. and Read, D.J. 1982. Vesicular - arbuscular mycorrhiza in natural vegetation systems. III. Nutrient transfer between plants with mycorrhizal interconnections. *New Phytol.* 90 : 277 - 284.
- Williams, S.E. 1979. Vesicular - arbuscular mycorrhizae associated with actinomycete nodulated shrub *Cercocarpus montanus* Raf. and *Purshia tridentata* (Pursh.) Dc. *Bot. Gaz. (Suppl.)* 140 : S 115 - S 119.
- Williams, S.E. and Aldon, E.F. 1976. Endomycorrhizal (vesicular - arbuscular) associations of some acid zone shrubs. *South-West Nat.* 20 : 437 - 444.
- Wilson, J.M., Trinick, M.J. and Parker, C.A. 1983. The identification of vesicular mycorrhizal fungi using immunofluorescence. *Soil Biol. Biochem.* 15 : 439 - 445.
- Winship, L.J. and Tjepkema, J.D. 1985. Nitrogen fixation and respiration by root nodules of *Alnus rubra* Bong : Effect of temperature and oxygen concentration. *Plant Soil* 87 : 91 - 107.
- *Woronin, M. 1866. Über die bei der Schwarzerle (*Alnus glutinosa*) and bei der gewöhnlichen gar tenlipine (*Lupinus nutabilis*) auftretenden wurzelanschwellungen. *Mem.Acad. Imp.Sci. St. Peterburg Ser.71* : 1 - 13.
- Worrall, V. and Roughley, R.J. 1991. Vertical movements of *Rhizobium leguminosarum* bv. *trifolii* as influenced by soil water potential and water flow. *Soil Biol. Biochem.* 23 : 485 - 486.
- Wright, S.F., Morton, J.B. and Scrorobuk, J.E. 1987. Identification of a vesicular - arbuscular mycorrhizal fungi by using monoclonal antibodies in an enzyme linked immunosorbent assay. *Appl. Environ. Microbiol.* 53 : 2222 - 2225.
- ****
- Young, J.L., Ho, I. and Trappe, J.M. 1972. Endomycorrhizal invasion on free amino acids contents of corn roots. *Agron. abs.* 102.
- Zaag, P.V., Fox, R.L.K., Pena, R.S.D. and Yost, R.S. 1979. P nutrition of Cassava including mycorrhizal effects on P, K, S, Zn, and Ca uptake. *Field Crops Res.* 2 : 253 - 263.
- Zar, J.H. 1974. *Biostatistical Analysis*. Prentice Hall, Inc. London.
- Zavitkovski, J. and Newton, M. 1971. Litterfall and litter accumulation in red older stands in western Oregon. *Plant Soil* 35 : 257 - 268.

- Zhang, Z., Lopez, M.F. and Torrey, J.G. 1984. A Comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. Plant Soil 78 : 79 - 90.
- Zhao, B. 1988. Effect of soil pH on the production of external hyphae by three VAM fungi. In: Asia conference on mycorrhizae. (Eds. Mahadevan, A., Raman, N. and Natarajan, K.), Centre for Advances Studies in Botany, University of Madras, pp. 189 - 191.
- Ziegler, H. and Huser, R. 1963. Fixation of atmospheric nitrogen by root nodules of *Comptonia peregrina*. Nature (London) 244 : 508 -509.
- Zhizhivkaya, S. 1982. Molybdenum and nitrogen fixation. Mos. Sci. J. 13 : 15 - 17.

* Original not seen

