Study of Genetic Diversity of *Frankia alni* Strains Isolated from *Alnus nepalensis* Root Nodules found in Meghalaya

By

Ganesh G.

Thesis submitted in fulfilment of the Degree of Doctor of Philosophy in Botany

NORTH-EASTERN HILL UNIVERSITY
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CERTIFICATE

We certify that the thesis entitled, ` Study of genetic diversity of *Frankia alni* strains isolated from *Alnus nepalensis* root nodules found in Meghalaya', submitted by Mr. Ganesh G. for award of Ph.D. degree of the North Eastern Hill University, embodies the record of the 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. The work has not been submitted for any degree of this or any other University.

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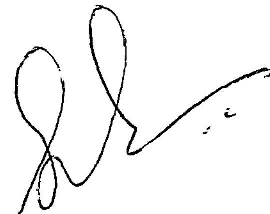
STATEMENT

I submit this thesis for the degree of Doctor of Philosophy (in Botany) of North-Eastern Hill University. I declare that this thesis records details of experiments carried out by me (in the School of Life Sciences) and is of my own composition and has not been previously accepted in part or whole for a higher degree of this or any other University.



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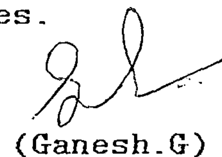

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CHAPTER I

INTRODUCTION

Early man derived his requirements from nature like any other animal. He was a hunter of animals and gatherer of plants. With the advent of modern civilization man became moulder of environment, disturbing the living crust of the earth and changing it from self sufficient life supporting system to a mere system of resources for themselves. Life could originate and flourish on earth only because the environment of this planet provided conducive conditions for it.

The perturbances in the living crust due to "civilization" cause loss of productive land resources, a loss that a developing country like India can ill afford. It is estimated that at least one third of the geographical area of the country is degraded and about one half of the forest area has poor or no forest cover (Ghosh , 1990). The loss of top soil due to deforestation is a more serious matter since it threatens to undermine the very means of our support, the biggest threat being from wind and water action. We cannot replace the lost soil since it takes centuries for the natural process to occur.

Loss of vegetation is due to a variety of reasons, one of which is shifting cultivation. It is believed that about 250 million people, thinly scattered over 300 million hectare of forest land of tropics, still follow an ancient form of agriculture which involves slashing and burning of vegetation, followed by the cultivation of crops (Goodland, 1980). This system of agriculture, characterized by rotation of fields rather than crops, is variously called in different regions eg. *Podu* in Orrisa, *Deppa* in Bastar, *Dahia* in Madhya Pradesh and *Jhum* in North East.

In *Jhum* cultivation the entire vegetation, including large and small trees are felled during the dry season. Large boles and branches are removed from the site and used as firewood. The slash is allowed to dry on the ground during the winter months which are rainless and burnt *in situ* whereas larger logs may

be heaped up and burnt a few times. After the first shower in April/ May weeding is done followed by the sowing of a mixture of crops by dibbling the seeds using a digging stick. Eight to thirteen crop species are grown together on a single field by the Garos. The practice followed by the Khasi tribe is a modified version of the typical type in that normally only lower branches of the sparsely distributed pine trees are cut instead of the whole tree, and seeds dibbled directly into the soil-ash complex (Mishra, 1981). Fertilizers are not used and rapid depletion of the fertility of the fields, which are often too steep to hold soil, water and nutrients, compels the farmer to shift to new site. These practices, and others created due to mine spoils etc., offer a hostile environment for plant growth and revegetation at some sites is often very difficult (Carpenter and Hensley, 1979).

In most forest soils, sufficient nitrogen is not present in a form that can be taken up and metabolized by plants to support optimal tree growth. Thus nitrate, urea or ammonia are the nutrients most frequently added to forest soils to increase their productivity (Gordon and Dawson, 1970). Industrial production of fertilizers is an energy demanding process and moreover the cost of nitrogen fertilizers has increased sharply. It should be noted here that farming, especially high tech one, consumes a sizable quantity of fertilizers.

Nature provides a way out for the nitrogen defeciencies under

natural conditions. A few free living bacteria like *Clostridium pasteurianum, Klebsiella pneumoniae* etc. fix nitrogen present in the atmosphere. Many cyanobacteria produce a characteristic cell called as heterocyst, which has hyaline contents and a thick, refrective wall. The firm identification that heterocysts as the site of nitrogen fixation and of the nitrogenase activity was given by Stewart *et al.* (1969). Nitrogenase enzyme comprises of a molybdenum and iron containing protein (dinitrogenase-1) and an iron containing protein (dinitrogenase reductase-1). Alternative nitrogenases have also been noticed and for further reading one is referred to the review article of Bishop *et al.* (1988) and Pau (1989). The fixed nitrogen is directly utilized by higher plants and could play a substantial part in the earth's nitrogen cycle (Torrey, 1978). In fact in *Gunnera-Nostoc* symbiosis the host *Gunnera* may rely completely on the cyanobacterial partner for the supply of combined nitrogen (Silvester and Smith, 1969). Besides cyanobacteria, bacteria of the genus *Rhizobium* fix atmospheric nitrogen in symbiotic association with roots of leguminous plants. The resulting, symbiosis occurs in a specialized organ, the nodule. The nature of nodules was not understood until 1886 when Hellriegel was able to demonstrate the ability of the legumes to take up atmospheric nitrogen. This facility is actually provided by assimilating bacteria in the nodules. In another type of symbiosis, diverse woody dicotyledenous plants have established symbiosis with an actinomycete called *Frankia*. *Frankia* sp. nodulated plants have

been assigned to 25 genera distributed among eight families of dicot woody species and include over 170 species. The list continues to grow. The compatibility between host and actinomycete is evidenced by the formation of root and stem nodules. Examples of such a type of symbiosis include genera *Comptonia, Elaeagnus, Casuarina, Myrica, Hippophae, Alnus* etc.

Alnus, commonly known as alder, belongs to the family Betulaceae comprising of six genera (*Betula, Alnus, Corylus, Ostrya, Carpinus and Ostrypsis*) (Lawrence, 1967). Of these six genera only *Alnus* forms symbiotic association with *Frankia.* The root nodules are mainly found 1-3 cm below the topsoil and correspond to "*Alnus* type" nodule (Zhongze and Torrey, 1985). The nodule type is specified by the host. Similar to other actinorhizal plants, the symbiont resides in the cortical cells of root nodules. Within the cortical cells the symbiont generally differentiates into hyphae and vesicles. In some cases sporangia too are seen and this is reported to be a genetic trait of the endophyte (Lumini *et al.*, 1992).

Nitrogen fixing rate of *Myrica gale* was recorded to be comparable to annual legumes under similar laboratory conditions (Bond, 1951). N_2 fixation rates as high as 260 kg/ha/y have been reported for *Casuarina equisetifolia* (Dommergues *et al.*, 1984). At this juncture one should note that nitrogen fixation is a highly energy requiring process. As much as 18.8 g of glucose is needed to fix 1 g of nitrogen (Gutschick, 1978). Therefore, the

plant sacrifices metabolic energy which otherwise could be utilized for potential yield (Bormann and Gordon, 1984). Nevertheless, the great contribution of *Frankia* - actinorhizal plants in nitrogen cycle in forests is quite obvious.

Extensive investigations on all aspects of *Frankia* - actinorhizal plant symbiosis have been taken up in the last fifteen years. Unfortunately very little work has been done on *Frankia* isolates from India. Not only this, although a great volume of information has been gathered the world over on morphological, physiological and molecular characterization of isolates, no comprehensive work has been taken up with a view to collect germplasm and screen it for better strains. If one intends to collect germplasm and screen it for genetic divesity, one should minimize, as far as possible the environmental component of total phenotypic variation. Moreover, some proven traits, that have limited or identified environmental dependence should be studied.

The fact that the genus *Alnus* is thought to have originated in the Indo-China region (Furlow, 1979) prompted us to hypothesize that there could be genetic diversity for alder compatible frankiae in the region. Consequently the present study was taken up with the aim of collecting and screening for alder compatible *Frankia* (called *Frankia alni* by Lalonde *et al.*, 1988) germplasm from Meghalaya.

The investigation carried out could be grouped into two distinct classes -

1. Collection, isolation and confirmation of identity of the endophyte from *A. nepalensis* (Don.) root nodules. Specially in view of the fact that no report of isolation of *Frankia* from this species was available.

2. Screening the isolates for genetic diversity, for which certain morphological, physiological, molecular and genetic parameters listed below were chosen :

A. Morphological parameters :

i. Presence or absence of vesicles under different conditions

ii. Hyphal diameter

iii. Vesicle diameter

iv. Presence or absence of sporangia

B. Physiological parameters :

i. Growth response in different culture media

ii. Preferences for carbon sources

iii. Responses to various nitrogen sources

iv. Nitrogenase activity under varying culture conditions

C. Molecular parameter :

i. Presence or absence of plasmids

D. Genetic parameters :

i. Search for antibiotic resistance genes as markers on plasmids

ii. Possible location of *nif* genes on plasmids

CHAPTER II

REVIEW OF LITERATURE

2.1 Shadows of applied aspects

Drastically disturbed soils offer a hostile environment for plant growth and revegetation is often very difficult. Some species appear to be more tolerant to these conditions than others. *Alnus glutinosa, Elaeagnus umbellata, Robinia fertilis,* and *Rubus pseudoacacia* show great promise in such soils. These woody plants fix atmospheric nitrogen by *Frankia* root nodule symbiosis and offer a system for continued supply of nitrogen to the soil and plant community (Carpenter and Hensley, 1979).

Actinomycetous nodulated plants are likely to be of major importance in wood yield improvement efforts because of their ability to produce high biomass in harsh environmental conditions. Young natural stands of red alder in the Pacific North west of the United States have been estimated to yield 15-25 tons of above ground dry biomass/hectare/y without man's assistance (Gordon and Dawson, 1970). In addition to increased wood yields, using alder as a rotation crop may also suppress two important fungal diseases of conifers (*Phellinus weirii* and *Fomes annosus*). They are also valued as food and cover for wildlife (Klemmedson, 1979).

This group is genetically diverse, indicating that the relationship between host and endophyte is more flexible than in legumes. The actinorhizal plants are native to a wide variety of geographic locations and forest habitats, making utilization of forest culture possible (Gordon and Dawson, 1970).

2.2 Variations in nodule morphology

Frankia induced actinorhizal nodules can be categorized into two morphologically distinct types. Both types are derived from localized proliferation of modified lateral roots. In the "*Alnus* type nodule" each modified lateral root is rounded and knobby while in the "*Myrica* type nodule" each nodule lobe forms a terminal elongated nodule root which tends to grow vertically upward (Moiroud and Gianinazzi-Pearson, 1984). *Casuarina* root nodules characteristically are of *Myrica* type. However, *C.*

cunninghamiana growing in the La Reunion Island, was reported to have aerial nodules (Prin *et al.*, 1992).

Zhongze and Torrey (1985) were the first to report that both morphological types of root nodules could be produced by a single *Frankia* strain on different host species. This would clearly mean that the type of nodule produced is a function of host and not that of the microsymbiont. In addition to host type, nodulation is affected by some physical factors such as soil clay particles (Smolander *et al.*, 1988) and soil depth (Kurdali and Domench, 1991).

Sometimes nodule like structures on actinorhizal plants are induced by microorganisms other than *Frankia*. For example, myconodules resembling young actinorhizae were induced by *Penicillium nodositatum* on alder (Capellano *et al.*, 1987; Valla *et al.*, 1989). Similarly, infections by *Agrobacterium rhizogenes* strain 8196 gave rise to nodule like root structures on cotyledons of *Elaeagnus angustifolia* (Savka *et al.*, 1992). Consequently, care must be taken to avoid errors arising out of such infections while conducting nodulation studies.

2.3 Morphology of *Frankia in situ*

Two distinct types of *Frankia* can be recognised on the basis of whether they produce sporangia within the nodule (spore positive, Sp^+), or not (spore negative, Sp^-). Later type is more frequent except in Finland (Weber *et al.*, 1988).

Experimental evidence suggests that sporulation *in vivo* is a genetic trait of the microsymbiont, but the degree of sporulation may be affected by the environment and host genotype (Schwinizer, 1990). It has been found that Sp^+ types are generally slower growing, less efficient and more difficult to isolate (Burggraaf *et al.*, 1981; Normand and Lalonde, 1982). Several cycles of subculturing may often lead to loss of sporangia producing ability of Sp^+ strains (Lechevalier *et al.*, 1982). Lumini *et al.*(1992) have suggested that spore production *in vivo* may be influenced by the degree of incompatibility between the host and the microsymbiont. This suggestion is based on the observation that in heterologous combinations sometimes Sp^- strains may also produce sporangia.

Morphologically *Frankia* is branched, having septate hyphae and produces septate vesicles. *Alnus* type vesicles are spherical and *Comptonia* type are club shaped (Lalonde, 1979). In *Casuarina* compatible frankiae, while vesicles are present in *ex planta* condition, they are not found inside the nodules (Tyson and Silver, 1979). Hyphae and vesicles are encapsulated in a polysaccharide material and surrounded by the host plasma membrane envelope (Lalonde, 1979).

2.4 The infection process

As soon as growing roots of most of the actinorhizal plants come any where near frankiae, or vice-versa, certain changes in the morphology of roots occur. These changes are normally

manifestated in the form of root hair deformations. Lalonde (1977) considered these deformations as plant response to the actinomycete. He did not see any link between the deformations and the mechanism of penetration of the actinomycete. Such an association was first pointed out by Callaham *et al.* (1979). Frankiae enter through the deformed root hair by digesting away the cell wall and quickly colonize the cortical region of the developing roots (Dewedar and Mansoor, 1992), transforming the infected lateral roots into nodules (Fletcher, 1955). In Elaeagnaceae the mode of entry of the endophyte is by direct penetration of the epidermis followed by apoplastic colonization of the root cortex (Miller and Baker, 1985).

The Frankial hypha is the infective agent throughout the infection process, passing first through the root hair wall at a deeply folded region of the deformed hair. The hyphae may branch at the infection site but no specialized adhesive structures such as pili/capsular material are noted (Berry and McCully, 1985). Limited growth often occurs within the middle lamella of the host cell wall (Benson and Eveligh, 1979). During invasion, the host cells become vacuolated, golgi bodies and rough endoplasmic reticulum proliferate and mitochondria increase in number (Benson and Eveleigh, 1979; Newcomb *et al.*, 1979). As *Frankia* passes through a succession of recently divided cortical cells, the cells expand greatly (Berry and McCully, 1985) and in some cases the cortical cells secrete a darkly staining material into the intercellular spaces through which the filamentous bacterium

grows (Miller and Baker, 1985).

The nodule arises in the pericycle (Fletcher, 1955) and in both *Myrica gale* and *Comptonia* prenodule formation is quite similar (Torrey and Callaham, 1979). Most nodules of *M. gale* originate from one to two primary nodule lobes. After complex routes the endophyte comes to lie in the cortical cells of the host and proliferates (Dewedar and Mansoor, 1992). After mycelial proliferation in the host cells, vesicles are formed radially as the hyphal ends become swollen (Benson and Eveleigh, 1979). On the other hand, the cortical cells invaded by fungus degenerate (Capellano *et al.,* 1987).

The encapsulation material surrounding the endophyte of *A. crispa* is composed mainly of granular and fibrillar non-sulfated and de-esterified polygalacturonic acid (Lalonde and Knowles, 1975).

2.5 Host infectivity groups

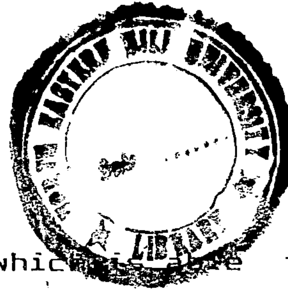
Three main cross inoculation groups have been proposed to classify host infectivity of *Frankia* :

a) strains which nodulate *Alnus* and genera from family Myricaceae;

b) strains which nodulate Elaeagnaceae; and

c) strains which nodulate *Casuarina* (Jiabin *et al.,* 1985).

Baker (1987) redefined the host infectivity groups and



suggested creation of a fourth group whic[illegible]e to nodulate both *Myrica* and *Elaeagnus*. Bosco *et al.* (1992) have shown that some *Elaeagnus* compatible strains may nodulate *Alnus*. Some other workers too have reported cross infectivity (Hall *et al.*, 1979; Redell and Bowen, 1985; Dobrista *et al.*, 1990; Du *et al.*, 1990).

2.6 Status of *Frankia* taxonomy

The criteria used in classifying an actinomycete as a member of the genus *Frankia* include - a) Morphology, b) Chemistry, c) Serology, d) Demonstration of infectivity for host plant, e) DNA homology, f) Determination of 16S rDNA sequence, and g) DNA amplification using *Frankia* genus specific probes (Becking, 1974; Lechevalier, 1984; Baker, 1987; Fernandez *et al.*, 1989 ; Mirza *et al.*, 1991 ; Nazaret *et al.*, 1991; Simonet *et al.*, 1991).

2.7 Isolation of *Frankia*

Isolation of *Frankia* is difficult and different workers have adopted different approaches for the purpose. First reported attempt to isolate the endophyte was made by Peklo (1910). Bottomley (1911) felt that the actinorhizal associations were similar to symbiosis in Leguminosae and thus sought isolation of single eubacteria, and failed for obvious reasons. In fact, all initial attempts failed to establish beyond doubt the authenticity of the isolated endophyte. Hence, it was thought for a long time that *Frankia* was an obligate symbiont.

At last, Callaham *et al.* (1978) succeeded in cultivating the

endophyte *in vitro*. Following this report many isolations of the symbiont from different hosts have succeeded (Baker *et al.*, 1979 a; Berry and Torrey, 1979; Zhongze and Torrey, 1985; Parson *et al.*, 1985).

Prior to plating/dispersing frankial clusters in the medium, surface sterilization is desirable and is done with agents like mercuric chloride (Callaham *et al.*, 1978), sodium hypochlorite (Baker *et al.*, 1979 a) and osmium tetraoxide (Weber *et al.*, 1988) etc.

Isolation techniques employed for *Frankia* so far, can roughly be classified into six types and are as follows:

a) Serial dilutions : It is a widely practiced microbial technique but has been of little help in frankial isolations (Baker and Torrey, 1979).

b) Enzyme maceration : This technique was used by Callaham *et al.*, (1978) for the release of endophyte clusters from root nodules of *Comptonia*.

c) Sucrose density fractionation : *Frankia* sp. were isolated from root nodules of *E. umbellata* and *A. viridis* using this technique, which essentially consists of preparing gradients with a 3 ml cushion of 60% (w/v) sucrose, 3 ml layer of 45% (w/v) sucrose and 6 ml layer of 30% (w/v) sucrose (Baker *et al.*, 1979 a). This technique was also used by Parson *et al.* (1985). The fractionation procedure was modified by Baker and O'Keefe (1984) wherein they

briefly incubated crushed nodule suspensions in 0.7% phenol before application to sucrose density gradient. Phenol incubation decreased the number of contaminating eubacteria and fungi but more importantly increased the number of *Frankia* developing on the isolation plates.

d) Sephadex fractionation : This protocol was proposed for the elimination of phenolic compounds in the nodule suspensions that may inhibit the growth of the actinomycetous endophyte during isolation (Baker *et al.*, 1979 b).

e) Membrane filtration : Benson (1982) and Weber *et al.* (1988) released the endophyte clusters in a mortar containing basal salt solution. The homogenate was filtered through nylon screens of 50 and 20 µm. The cell aggregates on 20 µm screen were washed and diluted with basal salt solution before plating.

f) Microdissection : This is the most widely practiced technique because of its simplicity and majority of strains have been obtained by this method. A wide range of protocols fall under this catagory ranging from crushing of root nodules to careful peeling (Diem and Dommergues, 1983; Boonkerd *et al.*, 1985; Zhongze and Torrey, 1985; Weber *et al.* 1988).

Thus one can conclude that serial dilution techniques have little value in isolating actinomycete from actinorhizal nodules. Techniques based on microdissection of root nodules have been used with more success. Reassuringly, different methods can be

used successfully to achieve isolation of *Frankia* from root nodules and one can expect an increasing number of successful isolations now that one has basic information on the nature of the organism; its growth characteristics in culture and some insight into its nutritional requirements.

2.8 Morphology of *Frankia* in culture

Some frankiae have loosely filamentous thalli with relatively little branching and hyphae that are generally 1-1.5 µm in diameter (Torrey, 1985; Zhongze and Torrey, 1985), while others have finer hyphae (0.5 µm) with highly branched and compact dense thalli (Lechevalier and Lechevalier, 1989). Baker *et al.*,(1979 a) found that *Elaeagnus* isolates could grow on agar surface but true aerial mycelia were not formed. On solid media, *Alnus* and *Elaeagnus* isolates grow in radial colonies composed of branching septate filaments.

Sporangia are generally 20-60 µm in length. Sporangia of loosely filamentous types are larger than compact colonies (Lechevalier and Lechavalier, 1989). CpI1, an isolate from *C. peregrina,* produced enlarged intrahyphal and terminal sporangia of considerable size (upto 60 µm) (Tjepkema *et al.,* 1980). Sporangial shapes are extremely diverse (pear to columnar) and vary even from strain to strain (Horriere, 1984). They are attached to submerged filaments by a thickened sporangiophore (Baker *et al.,* 1979 a) and formed by the growth of longitudinally and transversely oriented septa which

compartmentalize the wide hyphae (Newcomb *et al.*, 1979). Sporulation is reported to be carbon source dependent (Tisa *et al.*, 1983; Zhongze and Torrey, 1985). Cj1-82, in addition to typical sporangia, exhibits intercalary elongated sporangia like structures that could be disrupted into spore like units (Diem *et al.*, 1983).

Spores within the sporangia are roughly 1 µm in diameter and spherical to oblong in shape (Tjepkema *et al.*, 1980). All spores examined to date are smooth surfaced with mature sporangiospores having thick walls and outer membrane like layer (Lechavalier and Lechavalier, 1989). These are the real resting stages of *Frankia* and are released spontaneously. The germination of spores have been reported at variable rates with 1-3 germ tubes for most strains (Lechevalier and Lechevalier, 1989).

Vesicles, which probably are the sites of nitrogen fixation (Baker *et al.*, 1979 a), are spherical-club shaped structures (Akkermans *et al.*, 1983). Under Normarski interference phase optics the vesicles show a thickened wall (Tjepkema *et al.*, 1980). Ineffective, nonvesiculate strains are also found (Lechevalier and Lechevalier, 1989).

Vesicles are formed on most media by certain strains, while others form them only on special media (Lechevalier and Lechevalier, 1989). This may be due to requirement of suitable nutrients, since sporangia and vesicle formation *in vitro* have been found to be dependent on nutritional conditions. Generally

vesicle formation is induced by transfer of culture to nitrogen free medium (Lechevalier and Lechevalier, 1989). However, EAN1pec forms vesicles even in medium containing ammonia, while ACN1^{AG} does not form vesicles in any medium when incubated at temperatures greater than 33° C (Tisa *et al.*, 1983). CpI1 isolate forms vesicles only in absence of fixed nitrogen.

Some workers (Burgraff and Shipton, 1983) have reported structures named 'super clusters' of vesicles. These structures are ball shaped and approximately 0.5-1 mm in diameter consisting of an inner part densely packed with vesicles and surrounded by a dense layer of hyphae.

In *Casuarina*, a unique type of structure results from the enlargement of vegetative hyphae and the segmentation of these hyphae into individual cells by means of a transverse septa. This new type of structure called reproductive torulose hyphae (RTH) appears as a long chain of short cells connected to one another with length reaching 30-50 µm and width of 1.5-4 µm. RTH do not break open easily and morphogenesis of these cells is reminscent of that of spores in some nocardioform actinomycetes. Outgrowth generally occurs from individual RTH cells while still connected in the chain (Diem and Dommergues, 1985).

Hyphae exhibit a finely filamentous nucleoid region, containing numerous mesosomal structures, usually associated with cross wall formation and some particulate inclusions. Amylase digestion studies have shown these inclusions to be glycogen.

These are less prominent in established hyphae (Benson and Eveleigh, 1979).

The surface lamination of the spores is composed of two to three undulating layers, each being 3-4 nm thick. The exact number of these layers varies even among spores of the same sporangia (Berg and Lechevalier, 1985). In addition to laminations the mature spores contain electron dense inclusions resembling eukaryotic nucleoli, vesicular structures and nucleoid region (Newcomb *et al.*, 1979).

Frankia vesicles are shown to possess a specialized layer called vesicle envelope consisting of a multilaminate structure of 12-15 or more closely packed and continuous thin layers surrounding the vesicle and extending along the stem to the basal septum where the vesicle is attached to the bacterial filament. The envelope has special properties that facilitate vesicle function (Torrey, 1985). The lamination resembles spores, the only difference being that layers are discontinuous (Berg and Lechevalier, 1985).

2.9 Growth studies

Growth experiments of *Frankia* strains show that at least four phases can be distinguished - lag phase (0-4 d), log phase (4-12 d), decleration phase (12-24 d) and a starvation phase with ATP contents starting to decrease after day eigthteen. During the lag phase a large increase in infective and viable units has been

observed, followed by a drastic decrease at the onset of log phase (Burggraff and Shipton, 1983). Collins *et al.* (1985) observed a multiphasic mode of growth, characterized by discontinuities in the rate of biomass accretion in stirred aerated batch cultures of *Frankia*.

The optimal temperature for growth of *Frankia* isolates from *Casuarina, Hippophae* and *Myrica* is around 30° C with upper limits close to 40° C and lower limits near 15° C. Water potential below -2 to -6 bar decreases growth while pH values between 6-8 favour growth (Shipton and Burggraff, 1983).

Several *Frankia* strains/isolates have been tested for their growth in media containing glucose, succinic acid, propionic acid etc. as sole carbon sources. Propionic acid was found to be a good carbon source by Burggraff and Shipton (1983) while Weber *et al.* (1988) advocated use of propionate as a carbon source in general. Acetate is decarboxylated by most strains and utilization of acetate as carbon source seems to be the best of physiological tests for differentiation of isolates (Weber *et al.,* 1988). Vitamins like biotin, calcium pantothenate and riboflavin, stimulate growth of some *Frankia* isolates, though the stimulatory effects are negligible (Shipton and Burggraff, 1983).

Members of host compatibility group II utilize a variety of sugars and sugar alcohols in contrast to the host compatibility group I which are unable to utilize them (Tisa *et al.,* 1983). Akkermans *et al.* (1983) showed that *Frankia* strain AvcI1

utilizes only Tween and fatty acids as carbon sources.

Horriere (1984) observed differences in urease, protease and β-glucosidase activities to be invariant. Lechevalier *et al.*, (1983) found that strains of *Frankia* differ in relation to oxygen uptake and utilization of carbohydrates, organic acids and lipids.

The *in vitro* growth of *Frankia* is inhibited by phenolics (Perradin *et al.*, 1983), juglone (Vogel and Dawson, 1985) and sodium chloride (Burleigh and Dawson, 1991 a), while surfactants increase static culture yields (Collins *et al.*, 1985) and aliphatic amino acids increase sporulation (Burleigh and Dawson, 1991 b).

Physiologically, frankiae may be classed into at least two groups. The first group (A) is largely a heterogenous collection of morphologically and chemically diverse strains. They are relatively aerobic, can be maintained on slants, have pigmented cells and show rapid growth in presence of 0.5% carbohydrate in a casein - hydrolysate medium. Group A strains for the most part are serologically and genetically diverse (Lechavalier and Lechevalier, 1989).

Group B strains are physiologically less active. They lack protease activity and do not take up and utilize carbohydrates at 0.5% concentration. However, fatty acids of various types and organic acids are utilized. Carbon metabolizing enzymes that

have been found include some from the Embden - Meyerhof - Parnas and Pentose phosphate pathways as also the tricarboxylic acid and glyoxylate cycles (Lechevalier and Ruan, 1984; Lechevalier and Lechevalier, 1989).

2.10 Maintenance of frankiae

Many media are formulated for the isolation and maintenance of *Frankia*. In general complex media are better than defined ones. Microaerophilic strains, as those of type B, do not grow at the surface of liquid medium and can not be maintained on slants. Long term preservation by lypophilization/storage in sterile soil is found to be satisfactory. Alternatively, stocks in growth medium may be frozen and kept at -20° C, however some strains do not survive after this treatment (Lechevalier and Lechevalier, 1989).

2.11 Nitrogenase activity

Nodules are the sites of nitrogen fixation and provide sufficient fixed nitrogen to sustain healthy growth of plants in nitrogen free medium. Fixation is followed by export of the products to rest of the plant.

When the rate of acetylene reduction assay (ARA) by several actinorhizal plants is measured as a function of time after the addition of acetylene, there is an initial peak rate followed by a decline and partial recovery of activity (Tjepkema and Schwitzner, 1992).

Nitrogenase activity found in actinorhizal nodules, as evidenced by the ARA, varies considerably from site to site and species to species (Binkley, 1981). More than one strain of endophyte may be found at a given site and sometime even within one nodule (Dobrista and Stupar, 1989). Obviously, the variation in nitrogense activity is a function of the interaction of the strain of the endophyte and the host.

The activity of nitrogenase decreases dramatically on cold treatment (-1 to 4° C) (Hensley and Carpenter, 1979; Vogel and Dawson, 1991), incompatible strains (Dillon and Baker, 1982), applications like IAA and handling of nodules (Wheeler *et al.*, 1978), darkness (Landquist and Huss-Danell, 1991) and is strongly affected by oxygen concentration (Hafeez *et al.*, 1984), ammonium addition (Huss-Danell *et al.*, 1982) etc.

Reduction of dinitrogen to ammonia by nitrogenase is normally accompanied by the production of hydrogen gas. However, no hydrogen is evolved by healthy, nitrogen fixing nodules due to the activity of an uptake hydrogenase (Winship and Martin, 1985). *Frankia* is seen to possess superoxide dismutase and catalase activity (Steele and Stowers, 1986).

Phenolic compounds present in large amounts in nodules lead to a pronounced loss in activity during their homogenisation (Akkermans *et al.*, 1977). However, reasonable levels of ARA by nodule homogenates can be measured if 0.3M sucrose and 0.1M dithionate are present during anaerobic homogenization.

2.12 Plasmid studies

Plasmids have been recognized as an important part of some bacterial cells, coding for certain functions which are generally not essential for the subsistence of the host. These include functions like antibiotic resistance, conjugation, symbiosis etc. They have been widely used in cloning systems (see Maniatis *et al.*,1982) and could be used as strain markers (Normand *et al.*, 1983).

Dobrista (1982) isolated plasmids directly from root nodules of *A. glutinosa* by which she classified 14 size classes of plasmids ranging from 1.3 to 85 kb. The main objection to this work, however, concerns the possibility of molecules from host plant cells.

However, majority of experiments conducted are with cultured strains/isolates of *Frankia* with the critical step for plasmid detection being the lysis of frankial cells. Normand *et al.* (1983) used lysozyme, proteases, neuraminidase, crude preprations of cellulase, helicase and mutanase but none of them effectively lysed a significant proportion of cells and a very small amount of DNA was recovered.

A pretreatment of strain ARgN22d by Normand *et al.* (1983) with lysing enzymes from basidiomycetes or with lysozyme and EDTA followed by *in situ* lysis according to Eckhardt (1978) permitted visualization of fuzzy satellite band. Better results were

obtained with a combination of enzymatic attack by lysozyme in presence of EDTA followed by chemical treatment with 10% (w/v) SDS at 90°C.

The best results, however, were obtained with the use of an enzyme, achromopeptidase enabling detection of plasmids ranging from 7 - 190 kb with an additional advantage of requiring only about 1/10 - 1/40 of cell biomass necessary for chemical lysis (Simonet *et al.*, 1984).

After screening 200 strains of collection in Quebec and Lyon, Simonet *et al.* (1985) obtained 11 plasmidic bands which could be grouped in 4 plasmid size classes (7-9, 18-20, 30-35, 50-55 kb). ARgN22d from *A. rugosa* was seen to harbour six plasmids (7.9, 16.2, 20.2, 23.3, 27, 32.2 kb), CpI1 isolated from *C. peregrina* yielded plasmids of smaller sizes and TX41 and TX38 isolated from *A. crispa* contained a 50 kb plasmid.

Normand and Lalonde (1986) observed a marked difference in plasmid stability between strains belonging to *Frankia* group A and B. Strains in group B (CpI1) could be subcultured 50 or more times with few or no changes in copy numbers or restriction patterns. In contrast, in strains of group A (EUN1f, ScN1Oa) the plasmids appeared and dissappeared. This could be attributed to the presence of active exonucleases (Normand *et al.*, 1983).

ArI3 isolated from *A. rubra* and CpI1 showed indistinguish- able 'fingerprints'. Furthermore, an 18 kb plasmid found in the

same two strains, also showed homologous restriction enzyme patterns (Simonet *et al.,* 1985). This may mean that similar plasmids may be found in two apparently unrelated strains. Horizontal mobility of plasmids thus can not be ruled out in *Frankia*, although the same has not been conclusively demonstrated so far.

In *Rhizobium* spp. *nif* and other symbiotic genes have been very often shown to be located on plasmids (Robson *et al.,* 1983), whereas in *Frankia* sp. only some of the *nif* genes are located on plasmids (Simonet *et al.,* 1986), and these plasmids are unlikely to be involved in symbiosis since 35 *Frankia* sp. strains in which no plasmid DNA was detected had similar symbiotic properties (Normand *et al.,* 1983).

A number of small and cryptic plasmids from strain CpI1, ArI3, ArgN22d and EUn1f have been used in hybridization studies with negative results (Normand and Lalonde, 1986) but in future studies where serology, antibiotic resistance and phage sensitivity do not allow differentiation amongst closely related strains, use of plasmids as strain markers might become one of the best tools to identify *Frankia* strains (Simonet *et al.,*1985).

CHAPTER III

MATERIAL AND METHODS

3.1 Isolation and confirmation of identity of the endophyte

3.1.1 Collection of nodules

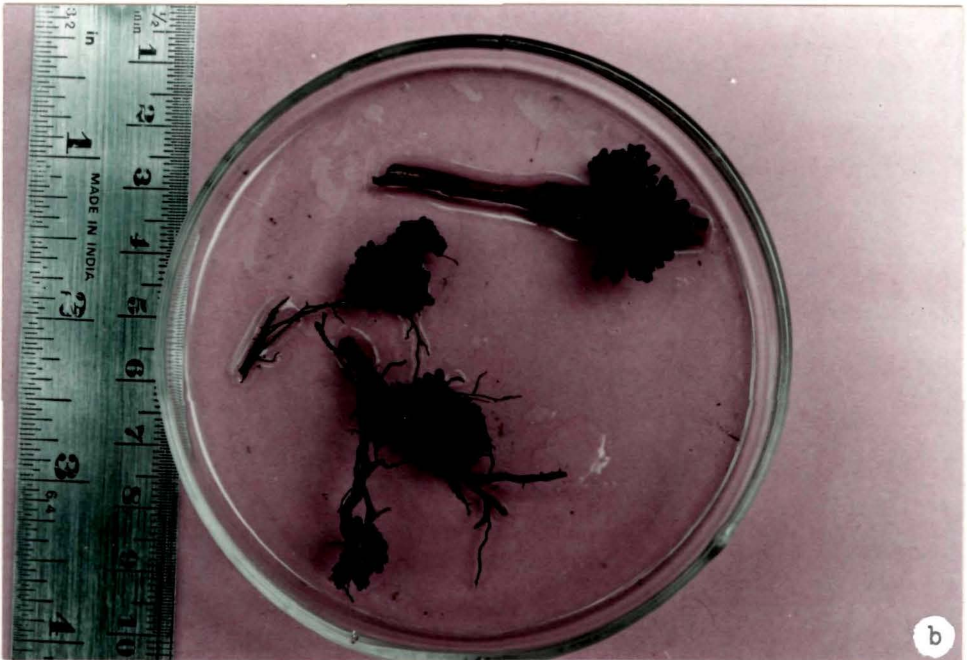
Established trees of *Alnus nepalensis* at four sites in Khasi hills were selected (Fig 3.1 a). Of these four sites Smit was a natural forest with a mixture of actinorhizal plants including *Myrica* and *Elaeagnus* . The forest stands in Upper Shillong consisted of *A. nepalensis* growing luxuriantly with *Pinus kesiya* . The trees in Mawlai and Alugodam were planted.

Fig 3.1 Stand of *Alnus nepalensis* and nodules produced by it

a) Trees of *A. nepalensis*

b) Nodule lobes of *A. nepalensis*

a


MADE IN INDIA
b

Vegetation around the marked tree was cleared off. The soil was dug to reach the nodulated roots. To avoid any confusion arising out of the presence of other nodulated roots, often leguminous in nature, nodulated roots were traced to the tree of their origin. Care was taken to collect only young/developing nodules. Nodule morphology, colour and the depth of its occurrence were recorded.

The nodules (Fig 3.1 b) thus collected were placed in moistened polyethylene bags/cloth and brought to the laboratory on the same day. They were washed under running tap water to remove soil and other foreign particles. This was followed by washing with water to which 2-3 drops of detergent were added for an effective clensing of the nodules. They were then used for isolation of *Frankia*. Some nodules were stored at $-2^{o}C$ for future use.

3.1.2 Isolation of endophyte

Nodules collected from three of the four sites chosen were used for isolation of the endophyte. Nodules from Mawlai were not used because of their poor quality. Root nodules collected and processed as described in Section 3.1.1, were detached into single lobe, rinsed again with running water and were surface sterilized with a variety of surface sterilants (Table 3.1). After surface sterilization and 3-5 washes in sterile distilled water (to free nodules from traces of surface sterilant) any one of the five isolation techniques described below was followed per

Table 3.1

Effectiveness of surface sterilants

Surface sterilant used	Concentration (%)	Time (min)	Degree of effectiveness
Ethanol	70	3	<30
Hydrogen peroxide	15	3	<50
Hydrogen peroxide	30	3	<50
Mercuric chloride	0.01	2	>95
Mercuric chloride	0.1	1	>95

nodule lobe -

a) The root nodule slices were crushed onto the sides of culture tubes containing liquid growth medium for *Frankia*.

b) Epidermal portions of the root nodules were sliced and nodules crushed on the walls of culture tubes.

c) In another modified technique, the surface sterilized root nodules were placed in two drops of sterile PVP (Appendix 3.1) on a sterile microslide and sliced with the help of sterile scalpel. Each slice was incubated in seperate vial containing growth medium.

d) Nodules were initially incubated at 28$\pm$3 ^{o}C for 7 d in an undefined medium containing 1% yeast extract and 1% tryptone. Some vials did not show any fungal and/or bacterial growth, indicating thereby the absence of any contaminating spores at their surface. These nodules were dissected to release frankial clusters.

e) The fifth technique used was serial dilution.

Four growth media, namely Qmod (Lalonde and Calvert, 1979), DPM (Baker and O'Keefe, 1984), F (Simonet *et al.*, 1985) and OS-1 (Dobrista and Stupar, 1989) were used for isolation of the organism (for compositions of media see Appendix 3.2 - 3.5). The pH of all the media were adjusted to 7.0. Both solid and liquid versions were tried. For isolation on solid medium, autoclaved (121° C for 20 min) media were dispersed into petriplates. Crushed nodules as above were spread evenly on the surface of the

solidified medium and another layer of the medium was layered over this to trap the endophyte between the two layers. The plates were incubated at 28 ± 3° C without illumination.

For liquid cultures, half filled tubes of 30, 15 or 5 ml capacity were used. Tubes containing either of the above media and inoculated with processed nodules as above were incubated without agitation. Other conditions of incubation were same as described for solid media.

The vials and plates were observed periodically and those showing contamination were discarded. Others, which showed suspected frankiae were saved and examined for the presence of *Frankia*.

3.1.3 Nodulation tests

The initial step for inoculation studies was standardizing germination of seeds of *A. nepalensis*. Efforts were made to germinate the seeds on filter paper, distilled water, water agar (0.3% agar) and sand. All substrates except the one lastly mentioned were sterilized at 121 ^{o}C/20 min. Sand, after washing with acid and rinsing with sufficient water, was sterilized for 2h at the same temperature. Sterilized sand was put to use 15-20 d after sterilization to eliminate toxicity. The substrates were watered with 1/2 strength Hoagland's solution (Hoagland and Arnon, 1950). Seeds were surface sterilized with 30% hydrogen peroxide for 2 min, rinsed with sterile distilled water and

placed in the substrates. Each substrate was divided into two sets, of which one was incubated at 28°C without illumination and the other incubated under natural conditions (approx. temperature 17-23°C, photoperiod approx. 14h daylight). Germination of seeds was also tried using light filters of various colours.

Fifteen days old seedlings were transferred to sterile, agar/pouches/sand (Fig 3.2 a-c) in batches of 15 or more seedlings and inoculated. The resulting sets could be classified into:

a) Seedlings inoculated with 0.1 ml crushed nodule suspension of *A. nepalensis* (positive control).

b) Seedlings inoculated with 0.1 ml of 15 d old subcultured isolates in DPM (Table 3.2).

c) Strains obtained from Laboratoire de Ecologie Microbienne du Sols (France) as inoculum for *A. nepalensis*. The age and quantity of inoculum being the same as mentioned in (b) and

d) Negative control consisting of uninoculated seedlings.

Seedlings were fed nitrogen free Hoagland's solution and grown under natural conditions of light (approx. 14 h daylight) and temperature (approx. 17-23 $^{\circ}$C). Observations were made under the microscope starting 2h after inoculations and root hair deformations, pre-nodule and nodule formations observed.

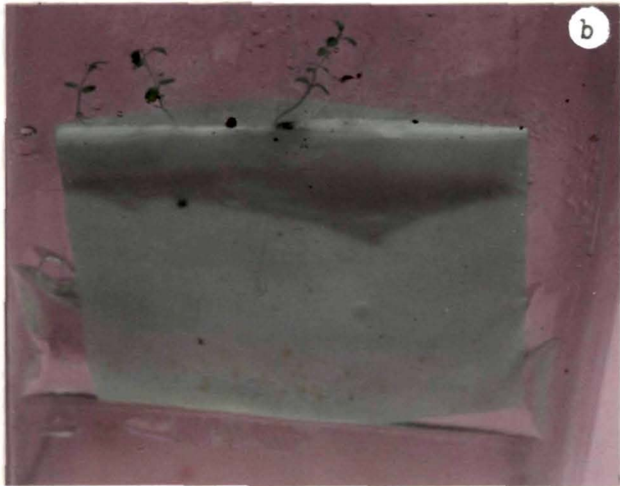
Fig 3.2 Inoculation studies of *Alnus nepalensis* with frankial cultures

a) Agar acting as substrate for seedlings

b) *A. nepalensis* seedlings growing on pouches

c) Inoculated seedlings on sand

2 3 4 5 6
MADE IN INDIA
1 2
a


b

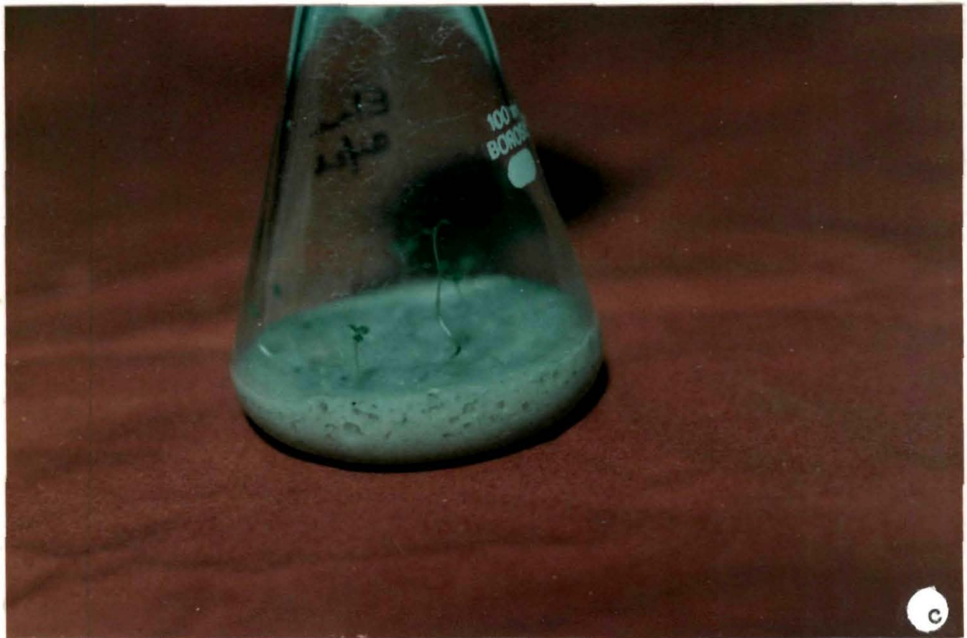

c

Table 3.2

List of *Frankia* isolates and strains used in this study

Isolate/ strain	Host plant	Geographical location obtained from
AnpUS4	*Alnus nepalensis*	Upper Shillong, India
AnpUS8	*Alnus nepalensis*	Upper Shillong, India
AnpST11	*Alnus nepalensis*	Smit, India
AnpAG14	*Alnus nepalensis*	Alugodam, India
ArgP5	*Alnus rugosa*	Lyon collection, France *
ACN1AG	*Alnus crispa*	Lyon collection, France
ArI3	*Alnus rubra*	Lyon collection, France

* Universite Claude-Bernard, Lyon 1, Laboratoire d'Ecologie Microbienne des Sols, Villurbanne, France

3.1.3.1 Cross sections of nodules induced by cultures of *Frankia* in seedlings

Seedlings showing nodule presence were fixed in FAA (Appendix 3.6), dehydrated in alcohol series and their paraffin blocks were made. These blocks were then sectioned using "Leitz" microtome to yield 5-10 µm thick sections. They were then dewaxed, stained with 5% toluidine blue and cortical regions of the sections observed for frankial clusters. Screening was also done to check the presence of hyphae, sporangia and vesicles.

Field collected root nodules were also processed using a similar protocol described above and the *in vivo* characteristics noted.

3.1.4 Morphological observations of isolates

3.1.4.1 Light microscopy

The isolates obtained were mounted with a drop of glycerol on optically flat microslides, stained with 5% toluidine blue and observations made under light microscope to note their morphological features.

3.1.4.2 Scanning Electron Microscopy

In order to obtain an image of higher resolution the isolates/strains were subjected to SEM studies. The following protocol was developed based on Hayat (1979) :

a) Isolates/strains were fixed in 3% gluteraldehyde for 2 h.

b) Washed two to three times in PBS buffer (Appendix 3.7).

c) Mounted on glass stubs smeared with adhesive.

d) Treated with osmium tetraoxide (0.05%).

e) Dehydrated in acetone series (30 min/gradation).

f) Immersed in dry acetone for 2-3 h.

g) Dried to critical point using "Samdri" critical point drier.

h) Gold sputtered using "JOEL" ion sputter JFC 1100 and observed under scanning electron microscope ("JOEL"-JSM-35 CF).

The field collected root nodules which were microtomed and dewaxed were transferred to acetone and finally into dry acetone. Critical point drying, gold sputtering and observations details were the same as mentioned above.

3.2 Screening for genetic diversity

3.2.1 Media preferences

0.1 ml of frankial inoculum was forced through sterile syringes of five ml having needle diameter of approx. 0.1 mm . The colonies thus fragmented were dispersed seperately into 30 ml vials each containing 15 ml of sterilized DPM, F, OS-1 or Qmod medium. Culture tubes with freshly inoculated endophytes were incubated at 28±3 ^{o}C, without illumination and agitation. Growth performances of the isolates and strains in different media were assessed by taking fresh weight increase as the criteria on 30th

and 45th d after subculture. Weighing was done on a sensitive digital "Sartorius" single pan electronic balance. Results obtained in triplicates were unitized according to the equation given below:

$$\text{Gain in weight/unit mass/day} = \frac{\text{mass on 45 d} - \text{mass on 30 d}}{\text{mass on 30 d} \times 15}$$

In addition to fresh weight determination, growth was followed using light microscope. These consisted of assessing the increase in colony diameter, sporangium and vesicle production and counts.The preparatory protocol for this study was similar to the one described in section 3.1.4.1. Screening of the colonies was done after micrometer calibration.

To assess the optimal pH, the pH of DPM was adjusted to 4, 5, 6, 7 and 8 and the power of H ion read both before and after autoclaving (121 ^{o}C/20 min). Preparatory conditions and inoculum volumes were the same as mentioned above. The conditions of incubation and growth assessment too were according to the procedure standardized.

3.2.2 Utilization of carbon sources

To study the performance of the isolates and strains with different carbon sources, sodium propionate, the only carbon source in DPM was substituted by the same amount of sodium pyruvate, sodium acetate or sucrose. The assesment of Tween 80 as a carbon source was by addition of 1.2 ml of it to the medium.

The preparatory protocols, inoculum volume, conditions of incubation and growth measurements were the same as described in section 3.2.1.

3.2.3 Nitrogenase activity

Assays for nitrogenase activity made use of gas chromatographic measurement of acetylene reduction (Burris, 1974). Excised nodules were washed, surface sterilized using 30% hydrogen peroxide (30 s) and placed in sterile stoppered vials (9 ml capacity) containing a moistened piece of sterile filter paper. 1 ml of air was replaced by 1 ml of acetylene. The vials were incubated at 20-22 ^{o}C for 1 h and gas sampled by injection into gas chromatograph. After the assay, fresh weight of nodules was taken. Ethylene production from acetylene was measured with a "Tracor 540" gas chromatograph fitted with "Porapak - T" column. The chromatograph used hydrogen as fuel and nitrogen served as carrier gas.

Assays for pure cultures of isolates, nodulated seedlings, inoculum in sterilized soil, addition of nitrogen sources and metals had a few deviations from the procedure described above and are as follows:

a) Pure cultures of isolates/strains: Three ml of isolates/strains 12, 40 and 109 d after subculture were used for the study. The incubation temperature was 28 $\pm$ 3 ^{o}C and vials shaken intermitantly during the incubation period.

b) Nodulated seedlings: Seedlings six weeks after inoculation and possesing 1-2 nodules were placed in vials of above mentioned capacity for the assay.

c) Isolates/strains in sterilized soil: Soil from Smit was sterilized ($121^{o}C$, 2h) and left for 15 d after autoclaving. This soil was then packed to 1/3 volume in capped vials and 3 ml of inoculum in sterile distilled water was added. After sealing, the vials were incubated at 28 ± 3 ^{o}C for 30 d without illumination. In addition to the assessment of nitrogenase activities in soil, the soil nitrogen status too was estimated according to Allen *et al.* (1974).

d) Effects of nitrogen sources were observed by the addition of 5 mg/l of ammonium chloride, potassium nitrate, urea and EDTA separately to DPM. The assessment of nitrogenase activity which was done 12 days after subculture followed protocols similar to that of pure cultures of isolates/strains. Growth was followed under the microscope and vesicle counts taken.

e) 0.5 mg/l of molybdenum, vanadium or iron salts were added individually to DPM. Care was taken to use starved cultures for the experiment. The protocol followed for study of the effects of these metals on nitrogenase activity was similar to the one used for pure cultures of *Frankia*. Activities were also recorded with cultures grown in DPM lacking the three metals mentioned above.

(note: all the experiments (a-e) had positive and negative controls. While ARA on nodules had a minimum of 30-40 replicates, the remaining ones had 8-15 replicates).

3.2.4 Detection of plasmids

The procedure of Eckhardt (1978) and modified by Simonet *et al.* (1984) was followed. However, some further modifications were made as under:

a) *Frankia* isolates/strains were harvested after different time intervals of growth in DPM and F.

b) 1.2 ml of cultures in eppendorf tubes were centrifuged for 20 min at 1200 g and the pellet obtained was washed thrice in TBE buffer (Appendix 3.8).

c) Repelleted cells were suspended in 0.1M NaOH.

d) Twenty microlitre of 1.2% SDS was added and various combinations and concentrations of lytic agents were tried (see Section 4.2.4).

e) One hour incubation at 37°C was found to be most suitable and followed throughout the experiment.

f) Cell suspension was laid on 0.7% agarose in TBE and

g) Wells of the gel were layered with 5% ficoll and 1% SDS.

Fifteen minutes later the wells were sealed with 0.7% agarose. *Escherichia coli* (pRK 290) and *Rhizobium meliloti* (102F34) were used as size standards. Electrophoresis was carried out at voltages of 25 V for 30 min and 120 V for 3-4 h (6 V/cm after 30 min). The gels were stained in ethidium

bromide for 40 min, destained in water/buffer and observed under UV source ("Pharmacia" transilluminator).

3.2.5 Resistances to antibiotics as markers

Isolates/strains were grown in 15 ml of DPM in the presence of 2, 5 and 10 µg/ml of ampicillin/tetracycline or chloramphenicol. The mode of prepration of these antibiotics has been presented in Appendix 3.9. The details of incubation and growth measurements were the same as described in section 3.2.1.

3.2.6 Possible presence of *nif* genes on plasmids

Comparative study of plasmid harbouring isolates and those without any plasmids was used as a criterion for possible location of *nif* genes on plasmids. Further possible investigations were not done for reasons given later in section 4.2.6.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation and confirmation of identity of the endophyte

4.1.1 Collection of nodules

The abundance of nodules varied between different sites during non rainy months. The nodules collected from Smit were seen in clumps of nodule lobes (above 2 mm in diameter) with a number of developing root nodules (see Fig. 3.1 b). At other

sites nodule clumps were less than 2 mm in diameter and there were fewer developing nodules seen. However, numerous developing nodules were observed after rainy months in alders at all the sites. The almost round the year development of nodules attaining greater diameter at Smit may be due to more moisture retention by the high soil humus present there.

Nodules were found in the soil at depths ranging between 1-9 cm. Similar depths were reported by Kurdali and Domenach (1991) under lab conditions and could be related to oxygen factor, *Frankia* population and nutrient supply. Other than roots (including developing ones), the crown area too was seen to bear nodules. However, aerial nodulation, which occurs in *Casuarina,* was not observed in *Alnus nepalensis* and some actinorhizal plants (*Elaeagnus* spp. and *Myrica* spp.) around Shillong.

Young developing nodules were whitish-yellow to yellow in colour. Nodules in all stages of development were seen in a single cluster and were "*Alnus* type" as described by Zhongze and Torrey (1985). The nodules slowly turned brownish on storage and showed reduced nitrogenase activity as assayed by acetylene reduction. Although the present work indicated a decline in nitrogenase activity of stored nodules, the viability of the endophyte possibly was not affected. *In vitro* isolation of endophytes from preserved actinorhizal nodules has also been reported by other workers (Lechevalier and Lechevalier, 1989). Reduction in nitrogenase activity may result from a reduction in

carbon supply because of removal of nodule from the plant.

4.1.2 Isolation of endophyte

Of the surface sterilants used (see Table 3.1) ethanol (70%) was the least and mercuric chloride (0.1%) the most effective. Addition of cyclohexamide (0.1%) to the growth medium suppressed the growth of fungal contaminants but could not eliminate them completely.

Increase in biomass of *Frankia* was generally obtained after a period of 3-4 weeks. However, in an occassional vial, it took even 4 months. This is in accordance with published reports of frankial isolation where 4-8 weeks are generally reported as the normal time for the visible growth of the organism. It may be due to the inability of some *Frankia* clusters to grow in contact with the growth medium (Diem and Dommergues, 1988), slow doubling rate (Lechevalier and Lechevalier, 1989) and probably some unique growth requirements.

Crushing of the nodules was seen to give good results and all isolates were obtained by this method. This may be due to the requirement of lipids present in root nodules for the initial *in vitro* growth of *Frankia* (Quispel *et al.*, 1983). *Frankia* like colonies could be obtained in F, OS-1 and DPM. However, only confirmed isolates obtained in DPM initially were included in the present study. This was so because morphological screening at initial phase relied heavily on presence or absence of vesicles

which were more striking in DPM than in other media. No *Frankia* isolate could be obtained on solid media. This is despite the precaution taken not to use very hot agar to top the crushed nodule spreads. One possibility could be the thickness of top agar which was not controlled. Instead of creating *microaerophilic conditions, near anaerobic condition might* have been created which prevented the growth of *Frankia.* The fact that the isolates obtained in liquid cultures grew in double layered agar with colonies taking 2-4 months to be visibly seen makes this possibility a difficult one. However, it is worth mentioning here that the phenolics released on crushing nodules would not be able to diffuse so much in solid media and may interfere with the growth of *Frankia*. While when isolates obtained in liquid cultures are grown in double layered plates, no phenolics are present to inhibit their growth.

The cultures obtained were whitish to brownish in colour. Pinhead sized colonies could be seen. Colour of the colonies slowly changed to pále whitish on subculturing. Subculturing also seemed to reduce growth rate. Their microaerophilic nature was indicated by the settling down of the colonies at the bottom of the culture tube, where they could grow best at $28 \pm 3^{o}C$ without agitation and illumination.

4.1.3 Morphological observations of isolates

4.1.3.1 Light microscopy

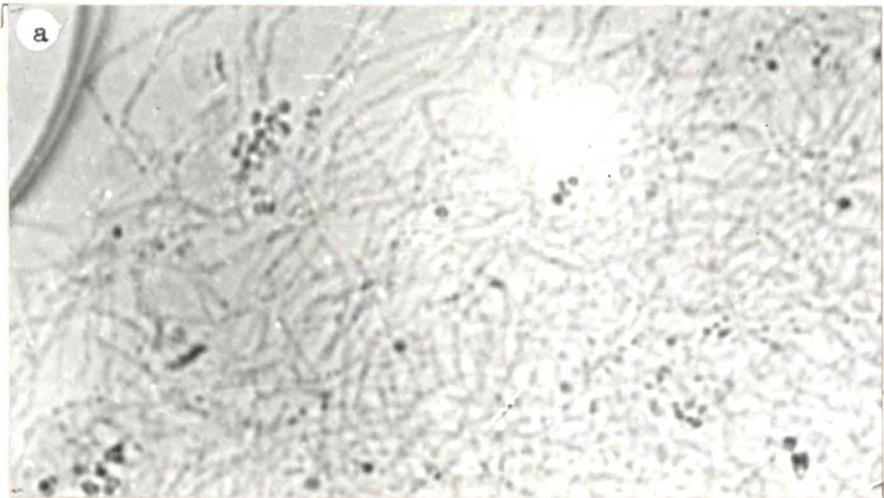
All the isolates of *Frankia* were filamentous, possessing long slender hyphae and bearing terminal and intercalary sporangia (Fig 4.1 a). Sporangia were of two types a) elongated and b) rounded with stalk in many cases being discernible. Vesicles in various stages of development were seen and were spherical in shape. Super vesicles reported by some workers (Burgraff and Shipton, 1983) were not found. The centres of the colonies were devoid of young hyphae and hyphae therein were rather thick. These could be mature hyphae. This also indicates the nature of the symbiont in that the cells are added at the periphery of the colony rather than breaking apart from it.

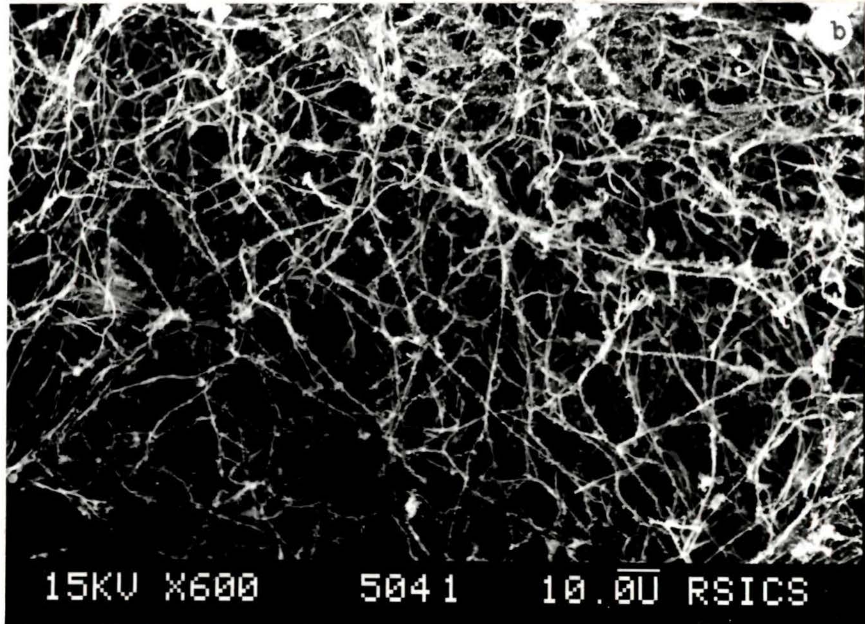
4.1.3.2 Scanning electron microscopy

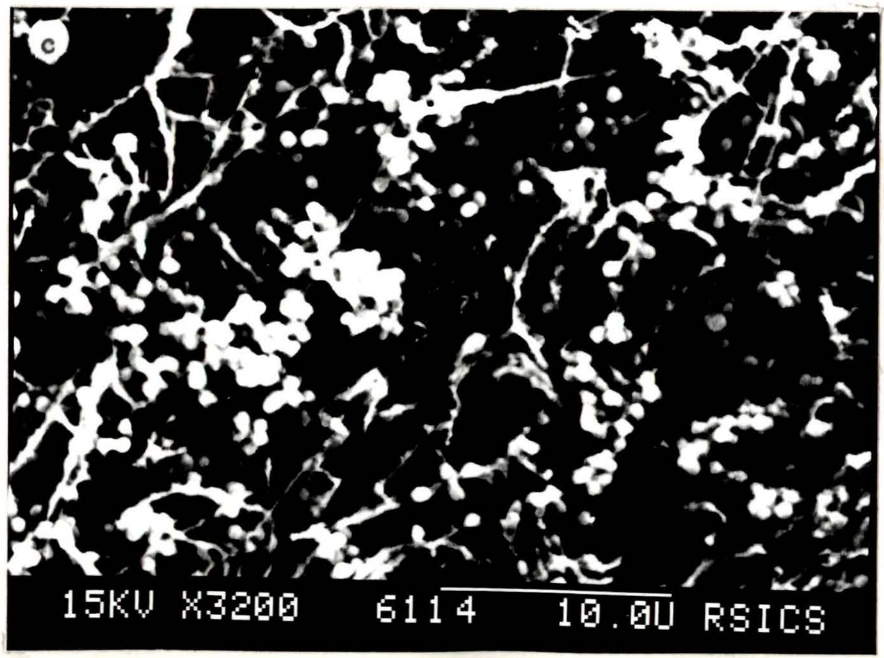
Fig 4.1 (b-h) shows SEM micrographs of various isolates and strains. Measurements revealed a range of 0.30 - 1.20 µm for hyphae and 0.60 - 4.00 µm for vesicles. Details of these measurements are presented in Table 4.1. The observed diameter of hyphae and vesicles of most of the isolates are on the higher side in comparision to ArI3 and ACN1^{AG}. However, hyphal and vesicular diameters of isolates were similar to ArgP5. The differences of these diameters among the isolates could be related to their diversity. Diameter range of vesicles was less in *in vitro* conditions than *in vivo.* This variation could be due to a better environment provided by the host. Similar morphology of reference strains and isolates indicated that the isolates

Fig 4.1 Morphological features of different *Frankia* isolates/strains

a)AnpUS4 b)AnpUS8 c)AnpST11
d)AnpUS4 e)AnpAG14 f)ACN1^{AG}
g)AnpUS8 h)AnpAG14


a


b
15KV X600 5041 10.0U RSICS


c
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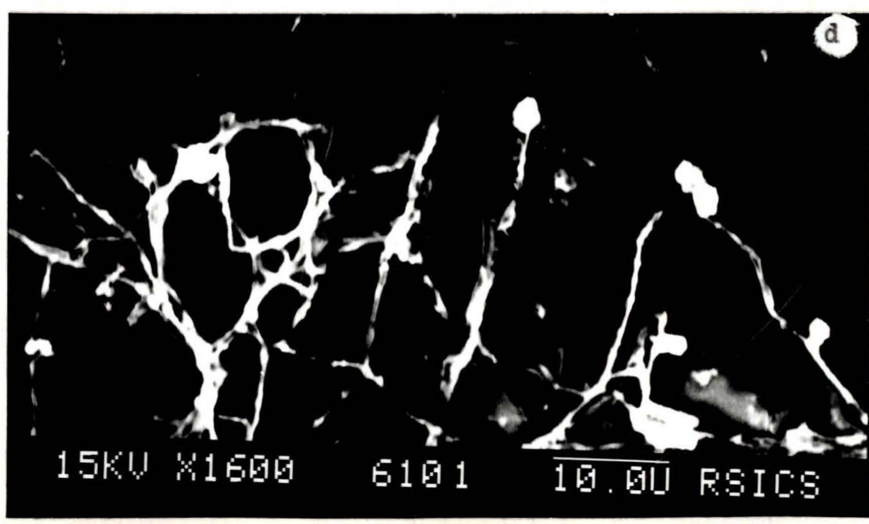
d
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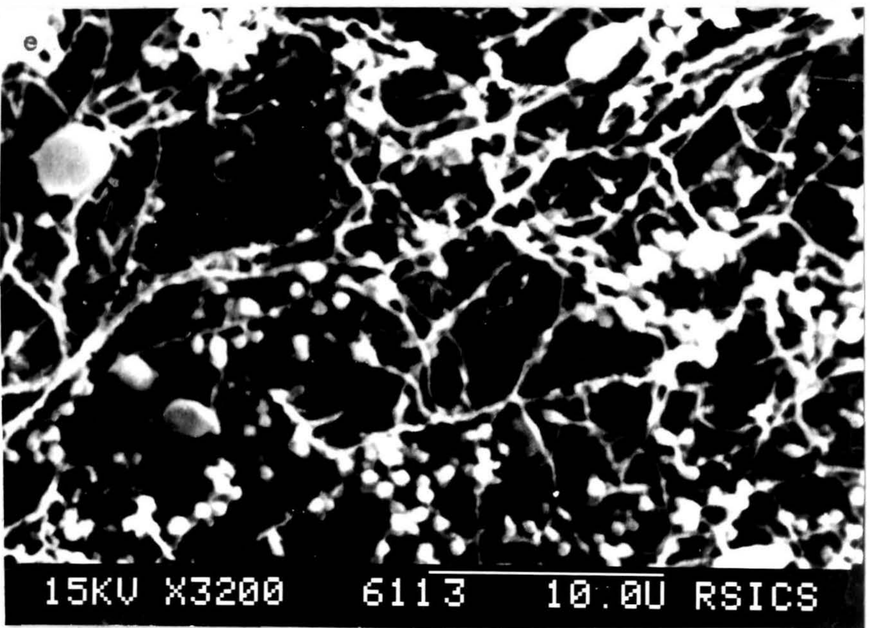
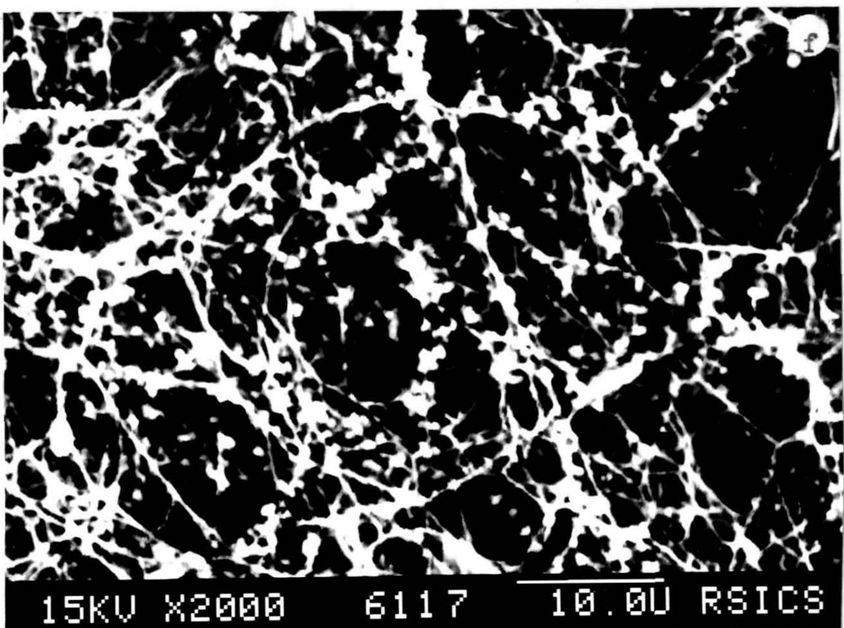

e
15KV X3200 6113 10.0U RSICS


f
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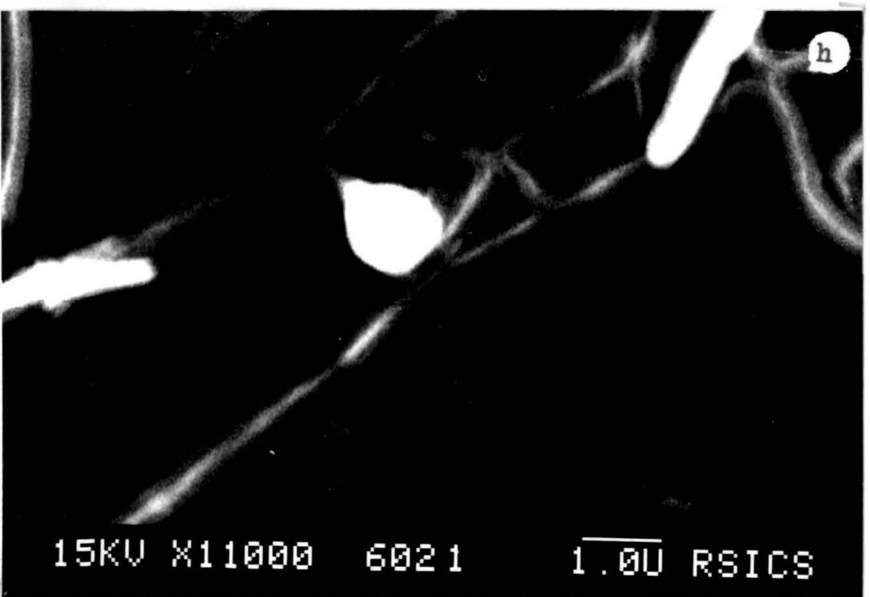

h
15KV X11000 6021 1.0U RSICS

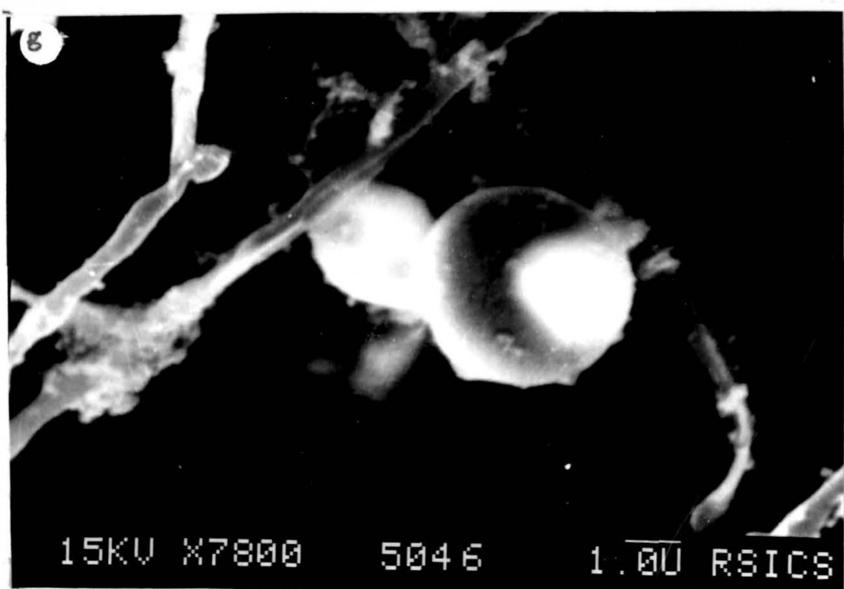

g
15KV X7800 5046 1.0U RSICS

Table 4.1

Table of means of diameter for hyphae and vesicles

Frankia isolate/ strains	Diameters in µm Hyphal	Vesicle
AnpUS4	0.86 (0.11)	2.76 (0.29)
AnpUS8	0.73 (0.11)	4.00 (0.58)
AnpST11	0.63 (0.20)	0.60 (0)
AnpAG14	0.70 (0.16)	1.90 (0.29)
ArgP5	1.20 (0.25)	1.25 (0)
ACN1[AG]	0.30 (0)	0.60 (0.25)
ArI3	0.40 (0.05)	1.47 (0.33)

numbers in parentheses denote SE

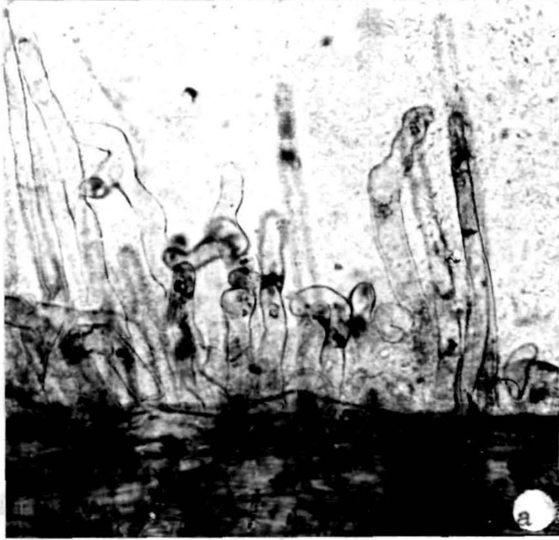
were frankiae.

4.1.4 Nodulation tests

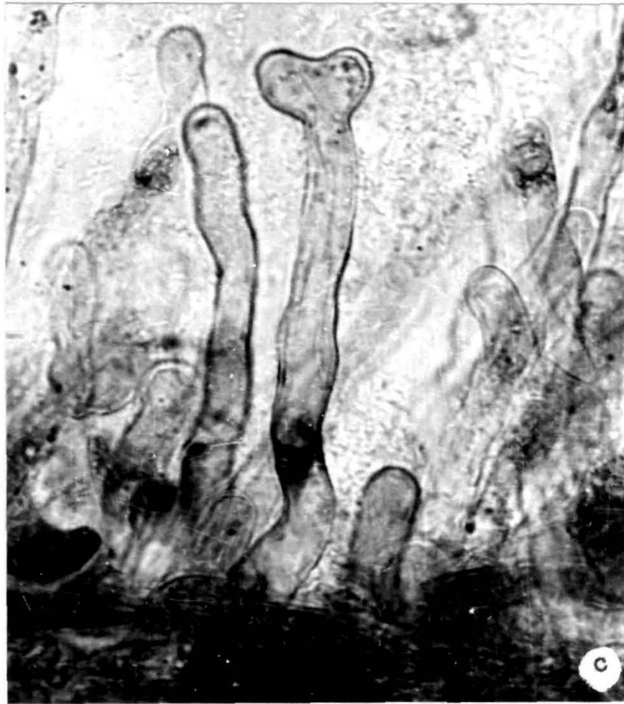
The seeds of alder germinated best in water agar, at 28°C under dark conditions. Colour filters used were seen to be of little help in enhancing seed germination.

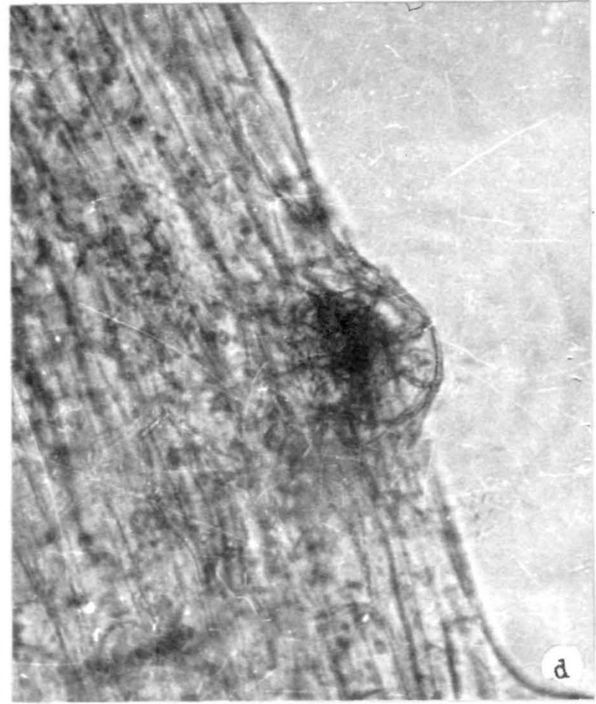
Fifteen day old seedlings were chosen after standardization. Deformational indications were observed as early as four to four and a half hours after inoculations. Complete root hair deformations were observed within 10 d with all isolates, strains and positive control (Fig 4.2 a-c). The deformational changes were noticed between the elongating root hair zone and matured zone. Probably the branched and hooked nature of the deformed root hair could play a role in entrapping frankial units and thus helping the symbiont in entering the plant willing to harbour it. A modified Fahraeus slide technique (Fahraeus, 1957) was tried for monitoring deformations. It did not succeed due to the fragile nature of the seedlings. Infact the seedlings died within 3-5 d after transfer. In case of positive control it was observed that young developing nodules gave mass root hair deformations with the older ones producing less deformations. This could be due to the age of frankial clusters in the nodules, some receptors as noticed in legumes (Mamblin and Kent, 1973) or due to phenolics. Among the three substrates used, pouches were easier to handle and better results were obtained with them. Sand rated next with seedlings grown in agar having less number of

Fig 4.2 Plant response to the symbiont
a&b) Root hair deformations
c) An enlarged view of a single deformed root hair
d) A prenodule


a

b


c

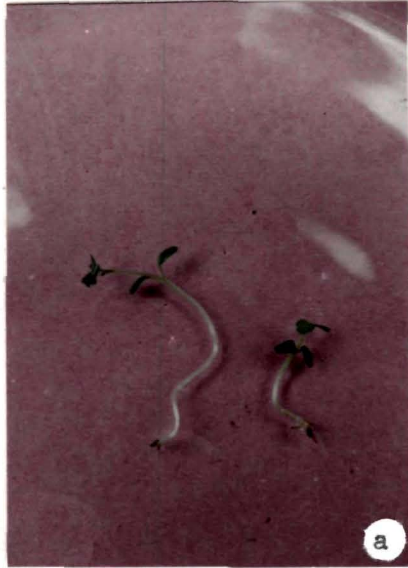

d

deformed root hair. This behaviour in agar could be related to the motility of the organism between layers of agar. Among many things, deformations were seen to be influenced by seed batch, age of seedlings, age of inoculum and nutrients available to the seedlings.

In some cases the seedling roots turned brown after inoculation. Although wall dissolution was not observed, in some deformed hair nuclei were shifted to a side. The deformed root hair slowly bulged.

After four weeks, pre-nodules were seen (see Fig 4.2 d) . These were one to two per seedling . Nodules were visible six weeks after inoculation (Fig 4.3 a-c; Table 4.2). They were brownish in colour, below one mm in diameter and effective in fixing atmospheric nitrogen. It could be hypothesized that due to the size of the seedlings, not more than 1-2 nodules were formed per seedling. This is because it would mean an additional sink for carbon supply. On the other hand negative controls showed stunted growth in comparison to inoculated seedlings and at no stage of the experiment were nodules formed in them. These results confirm the ability of isolates to induce nitrogen fixing nodules on the host. Although certain fungi have been found to induce nodules on alder, they do not fix atmospheric nitrogen (Capellano *et al.*, 1987).

Fig 4.3 Seedlings of *Alnus nepalensis* showing young root nodules (a–c)


a

b

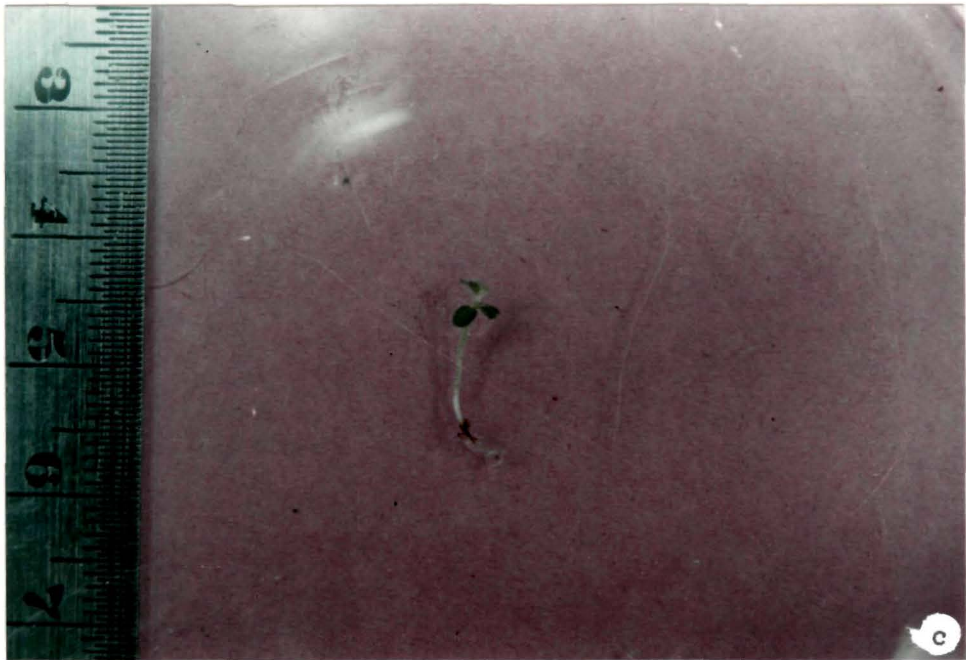

c

Table 4.2

Nodulation studies of *Alnus nepalensis* seedlings inoculated with different isolates of *Frankia*

Inoculum	Plant response (in weeks)					
	1	2	3	4	5	6
AnpUS4	rhd*		p-n**		-	nod***
AnpUS8		rhd		p-n	-	nod
AnpST11	rhd			p-n	-	nod
AnpAG14	rhd		p-n		-	nod
ArgP5		rhd	p-n		-	nod
ACN1AG	rhd		p-n		-	nod
ArI3	rhd		p-n		-	nod

rhd* root hair deformations
p-n** pre-nodule
nod*** nodule

crushed nodule inoculum (positive control) rhd (1 week), p-n (3 weeks) and nod (6 weeks)
negative control - no nodules formed

4.1.5 Cross sections of nodules induced by cultures of *Frankia* in seedlings

The microtomed sections of nodulated seedlings showed the symbiont residing in the cortical cells of nodules (Fig 4.4 a). Sporangia were found to be absent in field collected nodules (Fig 4.4 b-c). No sporangia were seen in sections of nodules induced by cultured isolates either.

4.1.6 Nitrogenase activity

When acetylene reduction assay was done for cultured isolates in nitrogen free medium, it was found that all exhibited nitrogenase activity which was significantly more ($P > 0.05$) than the control (Fig 4.5). Since nitrogen fixing actinomycetes are classified as *Frankia* (Stowers, 1987), the isolates that have typical actinomycetous morphology and fix nitrogen should be *Frankia*.

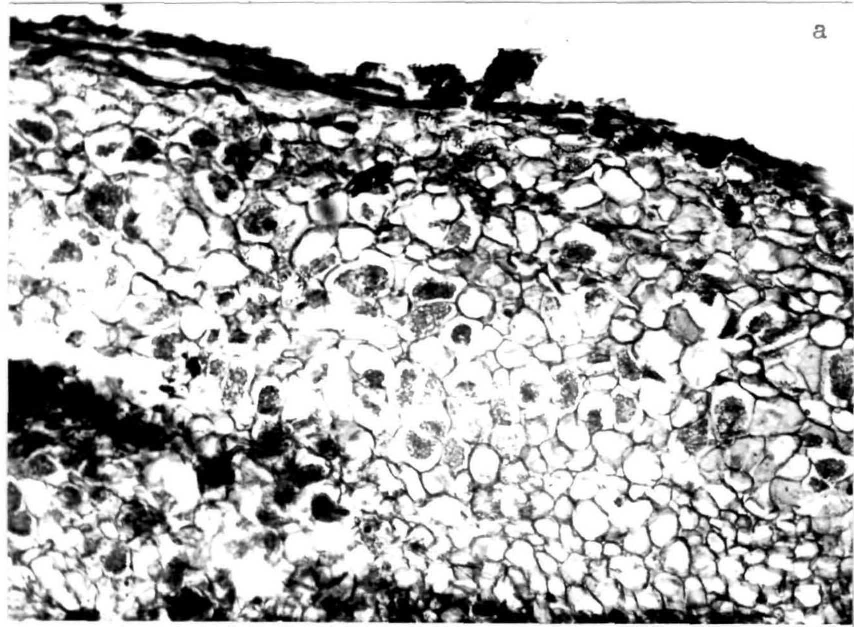
Thus, it was seen that morphologically the isolates resembled the reference strains when cultivated under similar conditions. They induced effective nodules in alder and the sections of induced nodules confirmed the presence of the endophyte in the cortical region. Isolates further showed significant nitrogenase activity in free cultures. Therefore, these results together confirmed the identity of the isolates.

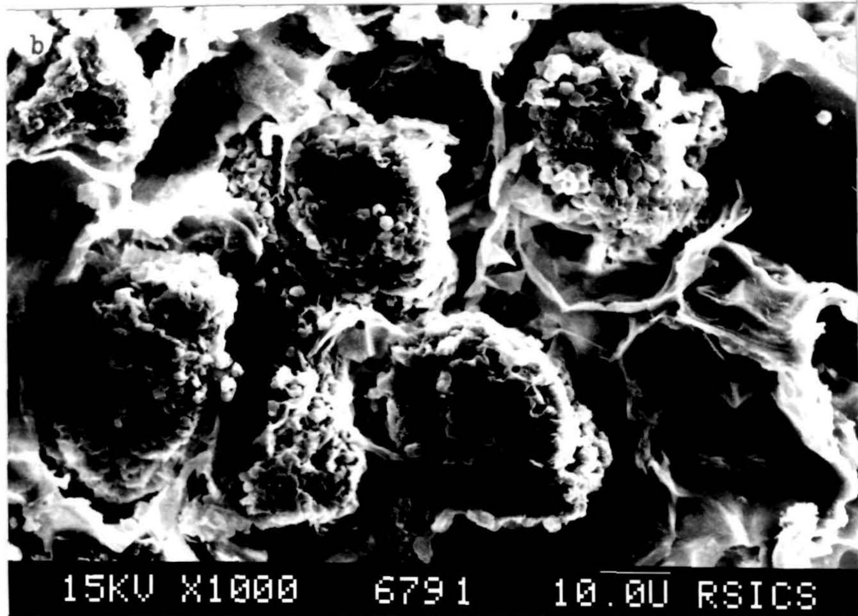
For further confirmation, one of the isolates was sent to Laboratoire de Microbiologie des Sols, Universite Claude Bernard,

Fig 4.4 Nodule sections of *Alnus nepalensis*

a) Inoculated seedling showing some cortical cells filled with symbiont (AnpUS8)

b&c) Micrographs of field collected nodules showing vesicles


a


b
15KV X1000 6791 10.0U RSICS

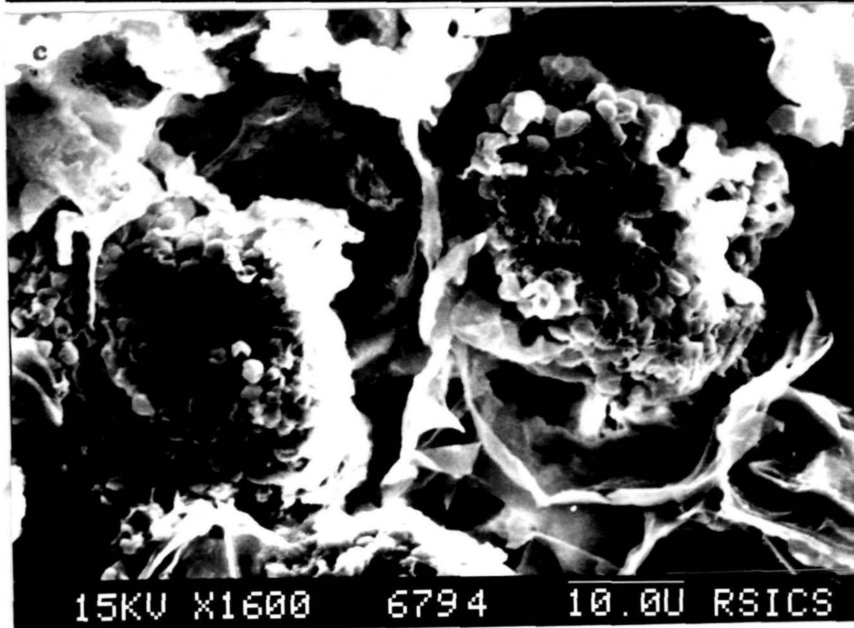
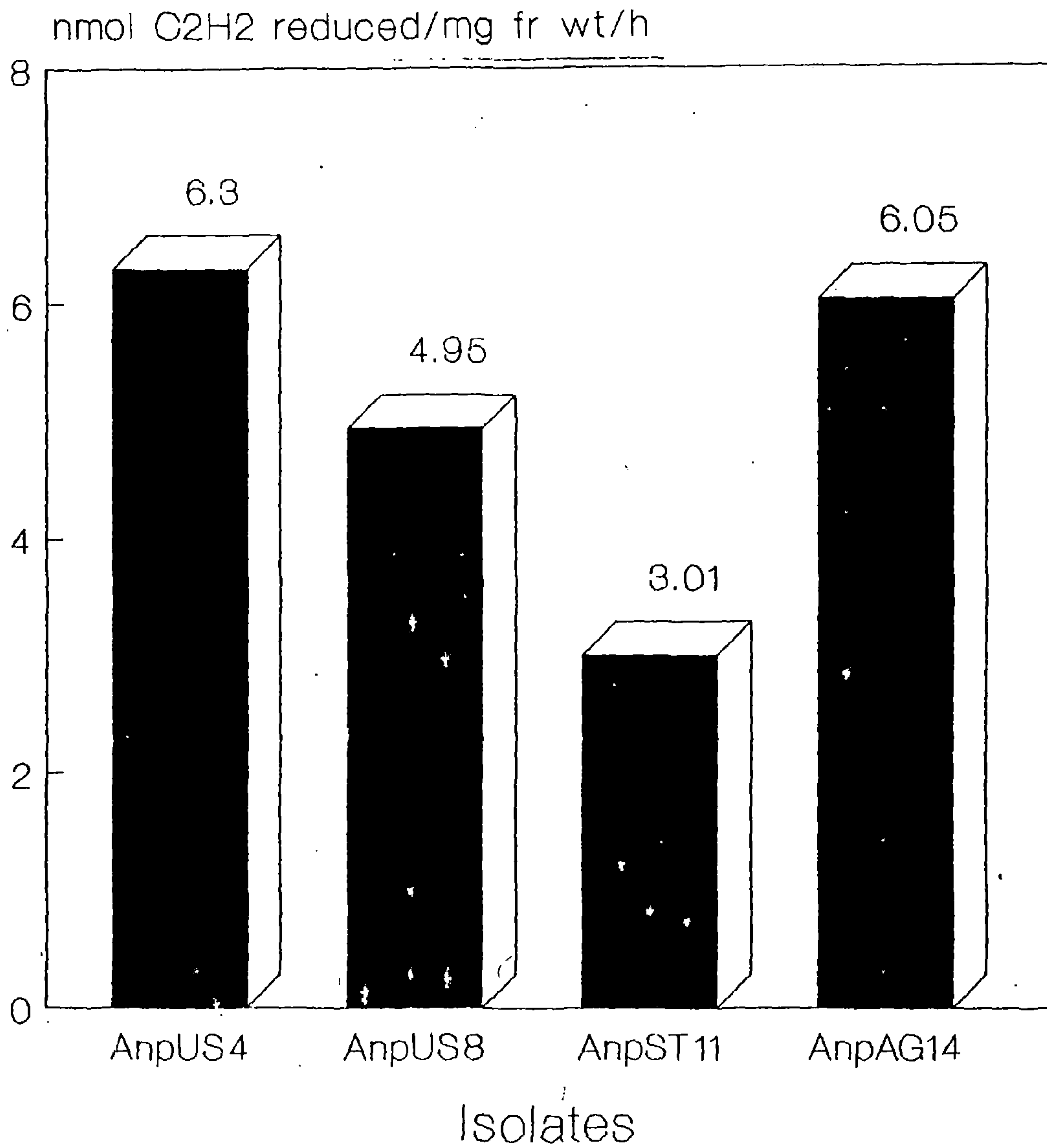

c
15KV X1600 6794 10.0U RSICS

Fig 4.5 Nitrogenase activity for *Frankia* isolates



experiment with 12 d subcultures in DPM

France, where DNA amplification of the isolate was achieved using Frankiaceae and *Frankia* specific DNA probes of the 16S rRNA gene. Positive amplification was also achieved using *nif*H, *nif*D and *nif*K gene probes.

4.2 Screening for genetic diversity

Screening for genetic diversity would involve study of some measurable phenotypic parameters with proven genetic bases. Not only this, one's has to consider the feasibility of studying such attributes under ones work facilities and environment. These considerations prompted use of morphology as revealed by scanning electron microscopy, presence or absence of sporangia in symbiotic condition, media responses, carbon utilization, nitrogenase activity of isolates, presence or absence of plasmids and search for suitable markers for plasmids, if any, as parameters for studying the variability present in the isolates obtained from a limited geographic area around Shillong. Needless to say that all these parameters have proven genetic bases and if environmental component is controlled upto the best possible extent, the variability revealed by these parameters would indicate presence of genetic variability.

4.2.1 Morphological differences

Hyphal and vesicle measurements were done in DPM. The arguments for choosing DPM as the experimental medium is presented in section 4.2.3 . As observed in Table 4.1, the hyphal

diameter ranges of isolates were 0.63-0.86 µm and vesicular diameter ranged between 0.60-4.00 µm. The isolates from Upper Shillong (AnpUS4) showed the largest diameters of hyphae and vesicles. On the other hand AnpST11 had the lowest hyphal and vesicular diameters. The other two isolates had intermediate sizes of hyphae and vesicles. The measurements of AnpUS4 and AnpUS8 isolated from nodules of different plants (approx. 2 km) show differences. This could be due to variability even among closely located isolates. Therefore, variations existed among isolates (isolates from host plants located at close distances and also the ones separated apart).

4.2.1.2 Screening for sporangia *in vivo*

Sporangia have been reported from various actinorhizal nodules (Weber *et al.*, 1988). This has also been documented to be a genetic trait of the symbiont (Lumini *et al.*, 1992). However, absence of sporangia was recorded both in nodules of seedlings inoculated with isolates and the field collected ones (Table 4.3). The presence of Sp^- strains in root nodules possibly points towards a better symbiont since Sp^+ strains are generally inefficient in inducing nodules. It is important to remember that during isolations Sp^+ strains require many unique components for their growth (Lechevalier and Lechevalier, 1989). Although the present study did not detect Sp^+ strains, their presence in the soil can not be ruled out. The obtention of uniformly Sp^- isolates could be due to the isolation technique and media used

Table 4.3

Colony characteristics of *Frankia* species *in vivo**

Location	Hyphae	Sporangia	Mean for vesicles/cell
Alugodam	+	-	23 (1.2)
Mawlai	+	-	10 (1.2)
Smit	+	-	23 (1.1)
Upper Shillong	+	-	21 (1.7)

* host plant *Alnus nepalensis*
+ denotes presence and - absence
numbers in parentheses denote SE

which may be biased towards Sp^- strains.

4.2.1.3 Media preferences

In all the four media tested, colonies grew at the bottom of the culture tubes, were yellowish white in colour and were approximately 0.1 mm in diameter. They did not produce any pigments and their filamentous nature was not visibly seen. A tendency of the colonies to flock together was noticed.

The 15 d subcultured colonies consisted of loosely arranged hyphae with hyphal length ranging from 0.1 - 0.4 mm. Very few colonies had sporangia. It might be presumed that the few colonies which possesed sporangia were from mother colony. Moreover resting stages like sporangia are generally not formed during active growth.

Vesicle counts of 15 d subcultures indicated differences amongst isolates (Table 4.4). All the isolates showed highest vesicle counts in DPM. This could be attributed to lack of available nitrogen in DPM. It is an established fact that vesicles are the sites of nitrogenase activity (Tjepkema *et al.*, 1980). This coincided with the high rate of nitrogenase activity too (maximum values were obtained with 12 d subcultures in DPM). Maximum number of vesicles per colony was found in AnpAG14 in DPM. However, other media too induced vesicle formation in isolates and strains. AnpUS4 showed similar counts in DPM and F media (15 vesicles/colony) while AnpUS8 showed the

Table 4.4

Colony characteristics of 15 d old cultures of different isolates of *Frankia*

Cultures of *Frankia*	Qmod[1] h	s	v	DPM[2] h	s	v	F[3] h	s	v	OS-1[4] h	s	v
AnpUS4	+	-	9	+	-	15	+	-	15	+	3	6
AnpUS8	+	2	12	+	-	12	+	3	6	+	-	5
AnpST11	+	-	8	+	-	10	+	-	8	+	1	4
AnpAG14	+	-	8	+	-	17	+	-	8	+	-	13
ArgP5	+	-	8	+	-	18	+	-	5	+	-	10
ACN1[AG]	+	8	9	+	3	14	+	-	11	+	-	8
ArI3	+	-	4	+	-	7	+	-	6	+	-	14

1 Lalonde and Calvert, 1979 2 Baker and O'Keefe, 1984
3 Simonet *et al.*, 1985 4 Dobrista and Stupar, 1989

h = hyphae, s = sporangia, v = vesicles; + presence and - absence

same in Qmod and DPM (12 vesicles/colony) pointing to possible differences in these two isolates. This among many factors could be related to uptake of nitrogen source in a given medium, size of vesicles and activity period of vesicles. However, analysis of variance indicated no significant differences among isolates.

Thirty and forty five day old colonies had a central region with young hyphae growing out from the edges of the colonies (Table 4.5). It was at these regions that majority of young vesicles were seen, but sporangia of all stages of development were seen to be distributed rather evenly. The presence of young vesicles at the edges might signify the activity of hyphal filaments and the rapid carbon inflow.

Except AnpST11 all the cultures showed medium hyphal network in 'Qmod' medium. It is possible that since 'Qmod' is a complex medium, frankiae take time to adapt to the constituents. AnpST11 showed medium nature network in F. All the colonies had achieved a dense network in DPM. This indicated that DPM supported good growth of isolates. There were more vesicles on an average in forty days old subcultures as compared to the fifteen days old ones. However, many of them might have been inactive.

Medium 'F' induced sporangial numbers above fifty in all the isolates. The formation of sporangia depends on nutrient conditions (Diem *et al.,* 1988). However, AnpST11 produced sporangia (more than fifty) in all the media. This indicated its standing apart from other isolates and improper adaptation to *in*

Table 4.5

Morphological features of 40 day old cultures of different isolates/strains of *Frankia*

Symbionts	Qmod[1]			DPM[2]			F[3]			OS-1[4]		
	h	s	v	h	s	v	h	s	v	h	s	v
AnpUS4	m	a	nm	d	b	nm	d	b	nm	d	a	nm
AnpUS8	m	b	nm	d	a	nm	d	b	nm	m	a	nm
AnpST11	d	b	nm	d	b	nm	m	b	nm	d	b	nm
AnpAG14	m	a	nm	d	a	nm	d	b	nm	d	b	nm
ArgP5	d	a	nm	m	b	nm	m	b	nm	d	b	nm
ACN1AG	d	a	nm	d	a	nm	d	b	nm	m	b	nm
ArI3	d	a	nm	m	b	nm	d	b	nm	d	b	nm

1-4 as in Table 4.4

h = hyphae, s = sporangia, v = vesicles, m = hyphal network of medium nature, nm = number of vesicles higher than 15 d subcultures, d = dense hyphal network, a = sporangial count below 50 and b = sporangial count above 50

vitro conditions. The other isolates, namely AnpUS4 produced below fifty sporangia in Qmod and OS-1, AnpUS8 in DPM and OS-1 and AnpAG14 in Qmod and DPM. Naturally these differences in sporangial numbers are due to variability between the isolates.

The sporangial distribution might reflect the preparation for the stationary phase. However, the shape and size of sporangia or vesicles did not vary from that observed before (see section 4.1.3).

The addition of 0.01% sodium chloride to DPM was seen to enhance sporulation in isolates and strains. Infact 15 d old subcultures showed presence of sporangia. This might reflect the unfavourable conditions induced. However, none of the isolates and strains grew in DPM containing 0.1% phenol. Although one is unable to comment on the survival of these frankiae, Baker and O'Keefe (1984), while isolating *Frankia*, had incubated it for a brief period in 0.7% phenol.

Another feature noticed was the decreasing number of sporangia with maintenance in lab cultures. A comparison made showed a sporangial decline of 75% after 2.5 y of subculture in DPM. A behaviour of similar type has been previously reported for ArI3 on $L+S_2$ medium (Horriere *et al.*, 1983). It is possible that the passage from host to culture medium induced sporangia formation. The endophyte which was well protected probably encountered temporary unfavourable conditions/shock of transfer/protection mechanism and after repeated maintenance,

morphologically and physiologically adapted itself to the conditions of nutrients and growth. The slow doubling time should also be taken into account to explain this.

For growth studies in different media the colonies were broken by passage through a syringe. This would enable the broken hyphal tips grow and also release spores from the sporangia. Relying on previous reports, however, much hopes should not be pinned on spores for their germination.

Selected media could be classified into two categories a) minimal and b) organic. Minimal media are composed of simple inorganic salts and a single carbon compound as energy source. Organic media contain a sugar and either yeast extract or hydrolysate of casein as components. The components of organic media and their degrees of complexity vary. Qmod, to cite an example, is a very complex medium having α -lecithin as lipid supplement. This goes well with the report of Lechevalier and Lechevalier (1984) that lipid supplements increase utilization of particular carbohydrates. However, the role of individual components is unclear till date.

Results of media preferences have been presented in Table 4.6. While ArI3 and ArgP5 had similar preferences ACN1^{AG} grew best in OS-1. All the four local isolates grew well in DPM. In addition to DPM, AnpUS4 liked OS-1, AnpUS8 F and AnpST11 Qmod. Gain in weight/unit mass/day for AnpST11 in Qmod was remarkable, while it grew better than others even in DPM. It is likely that

Table 4.6

Performance of *Frankia* isolates and strains in various media

Isolate/strain	Gain in weight/unit mass/day*			
	Qmod[1]	DPM[2]	F[3]	OS-1[4]
AnpUS4	0.022	0.048	0.032	0.045
AnpUS8	0.029	0.058	0.056	0.024
AnpST11	0.221	0.110	0.018	0.041
AnpAG14	0.019	0.091	0.039	0.037
ArgP5	0.066	0.036	0.027	0.061
ACN1AG	0.054	0.066	0.036	0.112
ArI3	0.055	0.034	0.034	0.049

* Mean values presented were obtained from 3 replicates/medium/organism

1-4 as in Table 4.4

AnpST11's remarkable growth in Qmod might be due to complex interactions between medium constituents and isolates. Tween is reported to be helpful in such cases (Lechevalier and Raun, 1984).

For regular maintenance, the original composition of DPM was altered after various trials (Appendix 4.0). 0.10 g/l yeast extract, 0.1 g/l tryptone and 0.01 g/l molybdic acid were the new additions to DPM. However, it should be noted that the medium ceased to be defined in nature. Yeast extract and tryptone were chosen to provide minor constituents which would help in stimulation of growth. Molybdic acid was added for a faster turnover of amino acids (dinitrogen --- nitrogenase --- amino compounds).

The subculturing must be done every 30-60 d, because of rapid decline in growth 45 d after subculture. Furthermore, after attainment of stationary phase hyphal lysis generally begins (Diem and Dommergues, 1988).

DPM seemed to be a good choice for experimental studies. Its composition is of defined nature and moreover good growth of isolates and strains was obtained on it.

4.2.2 Utilization of carbon sources

The carbon sources chosen represented diverse chemical nature. Propionate was chosen because it is a constituent of DPM and pyruvate because it can be generally substituted for

propionate. Acetate was selected due to its physiological importance (Weber *et al.*, 1988), Tween 80 as a fatty acid and sucrose for being a reducing sugar.

The colonies exhibited three types of structures described in section 4.1.3. All the carbon sources except sucrose gave rise to vesicles before 15 d and sporangia after this period of incubation. The hyphal morphology, type of colony, growth and shape of vesicle or sporangia structures did not show any peculiar deviations.

From this and study of media preferences it could be said that after hyphal proliferation, the endophyte differentiated into vesicles and at a later stage (3 wk) started producing sporangia.

Maximum growth was shown by AnpST11 in acetate (Table 4.7). Propionate supported good growth of isolates. However, in propionate AnpST11 showed the least growth. AnpST11 seemed to be very different in uptake due to excellent growth in acetate but less growth vigour in other carbon sources. A pattern emerged with carbon utilization of isolates. All the isolates except AnpST11 showed best growth in propionate followed by pyruvate. From reports of utilization of carbon it is seen that majority of *Frankia* strains held in cultures do well in propionate. This is also reflected by the chemical composition of many media formulated.

Table 4.7

Carbon utilization by *Frankia* isolates and strains

Frankiae	carbon sources				
	Acetate	Propionate	Pyruvate	Sucrose	Tween 80
AnpUS4	0.015	0.048	0.020	-	0.01
AnpUS8	0.012	0.058	0.020	-	0.016
AnpST11	0.2	0.011	0.021	-	0.005
AnpAG14	0.01	0.091	0.023	-	0.01
ArgP5	0.018	0.036	0.02	-	0.018
ACN1AG	0.01	0.066	0.03	-	0.01
ArI3	0.005	0.034	0.02	-	0.02

increases presented as gain in wt/unit mass/day
each value presented is a mean of 3 replicates
\- absence of growth response

Akkermans *et al.* (1983) reported lower yields with CO_2 devoid cultures on propionate. However, CO_2 was present in the present study. It has been shown that cells grown in Tween 80 or acetate as carbon source contain glyoxylate cycle enzymes (Akkermans *et al.*, 1983). It is quite possible that a situation of a similar type occurred in isolates due to their uptake. Though the present study and an earlier report (Akkermans *et al.*, 1983) demonstrated Tween 80 as sole carbon source, it is also likely that this compound functions as a surface active compound. However, a dependence on fatty acids for growth was not observed. The strains of Quispel *et al.* (1983) had shown a dependence on certain unique fatty acids. Sucrose did not support the growth of any isolate/strain indicating its inability to serve as a sole carbon source.

4.2.3 Nitrogenase activity

4.2.3.1 Soil nitrogen status

Nitrogen status of the soils is presented in Table 4.8. The higher available nirogen content in Upper Shillong was mainly due to its NH_4^+ form with NO_3^- nitrogen being comparable at all the sites. Almost same level of available nitrogen at Smit and Alugodam was probably due to the proximity of the sites. In spite of the low available nitrogen, the actinorhizae at Mawlai showed low levels of nitrogenase activity. This indicated the need to inoculate seedlings with crushed root nodule inoculum, showing high activity, prior to planting at this site.

Table 4.8

Soil nitrogen at the sites

Sites	Available nitrogen[a]		Total nitrogen[a]
	NH_4 -N	NO_3 -N	
Alugodam	6.65 ± 0.01	1.46 ± 0.017	441 ± 0
Mawlai	1.30 ± 0	1.63 ± 0	2226 ± 0
Smit	5.10 ± 0.01	1.64 ± 0	2811 ± 0
Upper Shillong	13.69 ± 2	2.02 ± 0	4326 ± 0

a µg/g
± SE

The total nitrogen too showed the same trend as available nitrogen with Upper Shillong denoting a higher value. The low value at Alugodam might be due to the sandy nature of the site.

4.2.3.2 Nitrogenase activity of field collected nodules

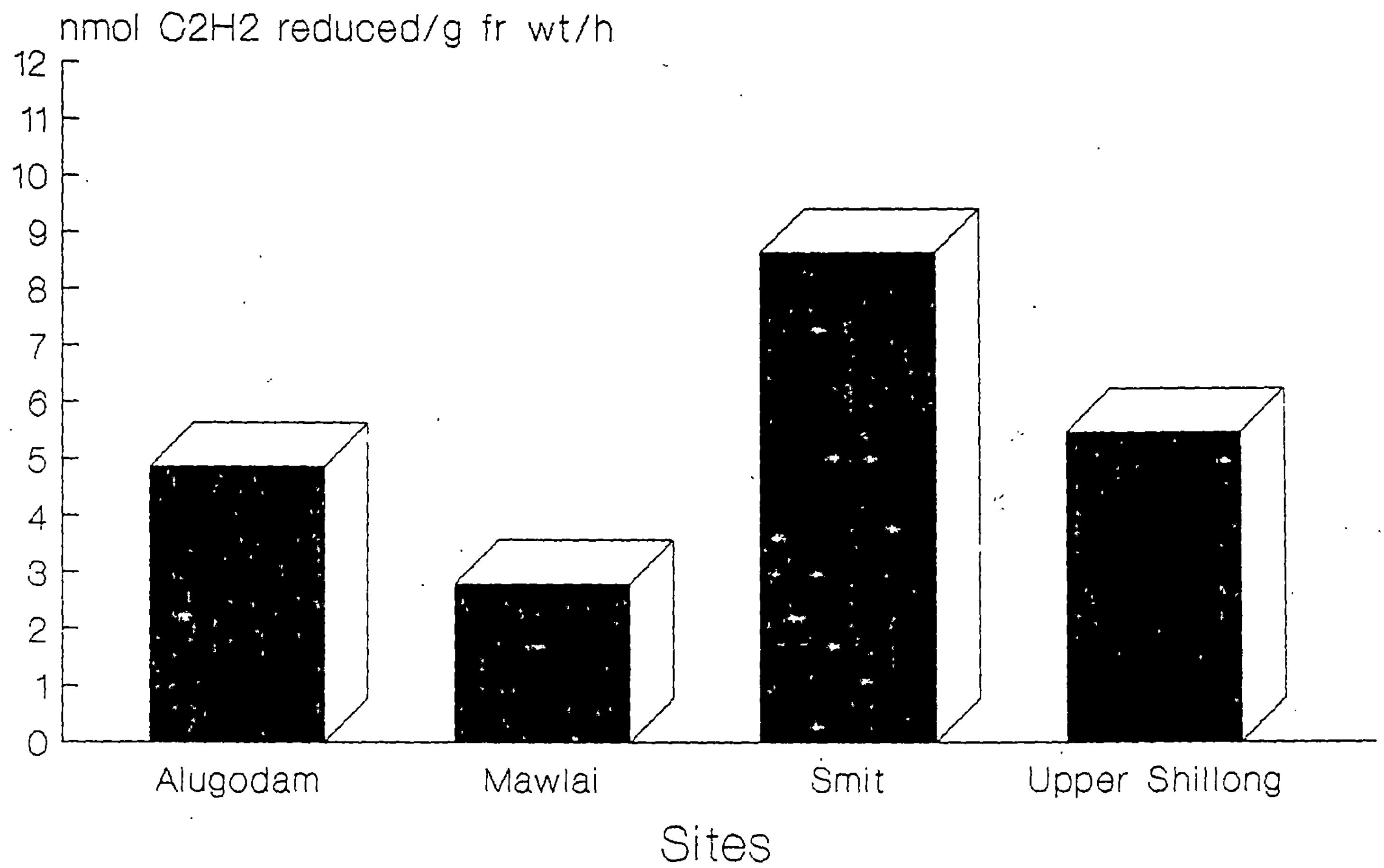
Fig 4.6 depicts the nitrogenase activities of root nodules of various sites, the assays of which were conducted with young and visibly healthy root nodules. Old nodules tended to have more dead and woody tissues. As seen from the Fig. the nodules produced by *A. nepalensis* in Smit had the highest activity which was followed by nodules at Upper Shillong. The enhanced activity of the enzyme from the nitrogen fixer at these sites could be due to efficient symbiont at these sites or host selecting *Frankia* with a higher efficiency due to competition between different strains.

The activity of dinitrogen fixing enzyme nearly ceased at $40^{o}C$ (0.24 n mol C_2H_2 reduced/g fwt/ h) and $4^{o}C$ (0.18 n mol C_2H_2 reduced /g fwt/h).

4.2.3.3 Nitrogenase activity of cultures of isolates

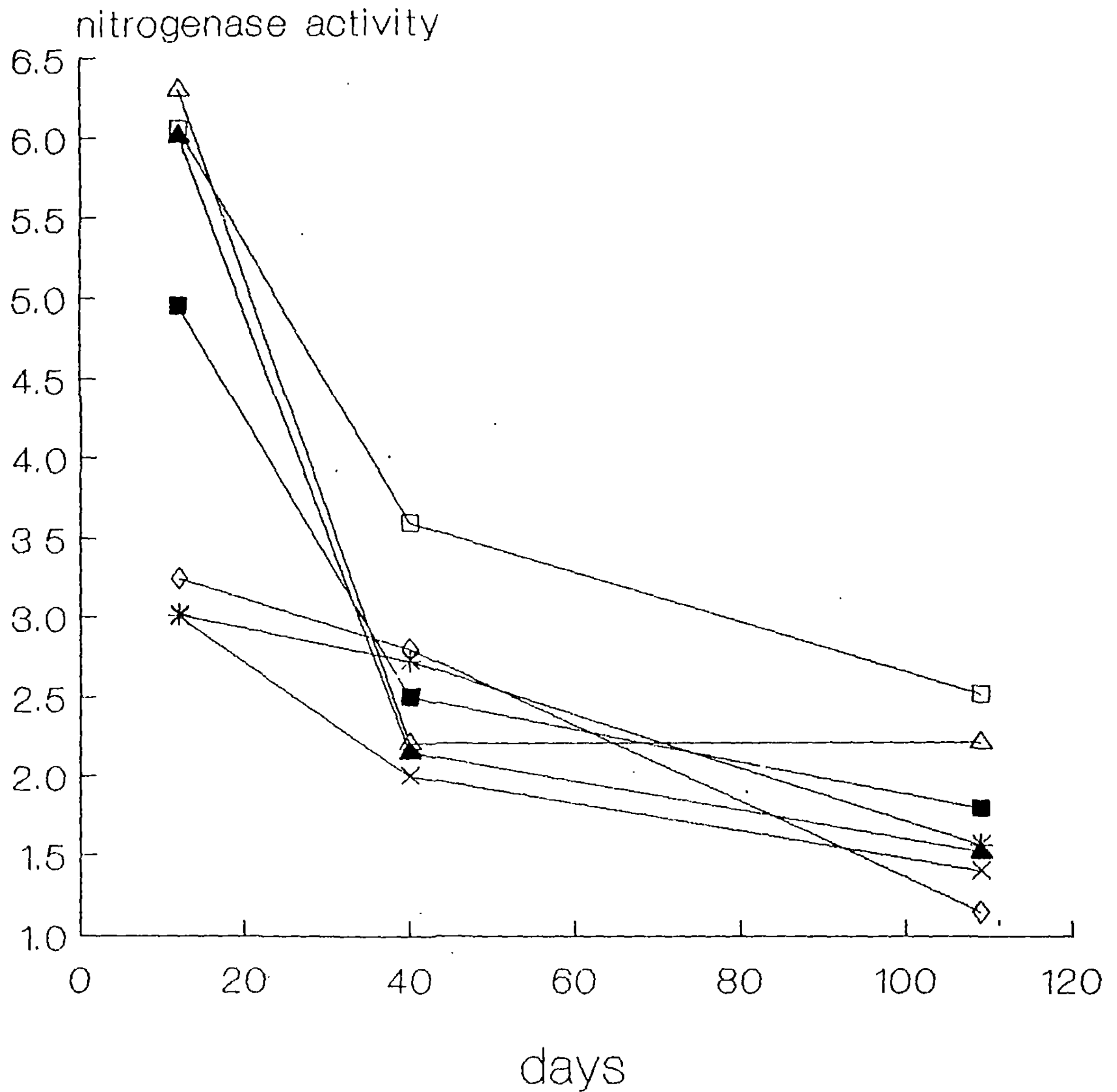
The results of nitrogenase activity of cultures are presented in Fig 4.7. Maximum nitrogenase activity was noticed in twelve day old cultures of *Frankia* isolates. It is possible that this much time is required for the growth of hyphae and formation of vesicles. The activity of twelve day old cultures could be due to active growth after subculture and less number of cells in

Fig 4.6 Comparision of nitrogenase activity of field collected nodules



experiment conducted in September
SE values not above 0.9

Fig 4.7 Nitrogenase activity of *Frankia* cultures against time



AnpUS4 AnpUS8 AnpST11 AnpAG14
ArgP5 ACN1AG Arl3

Nitrogenase activity in nmols
C2H2 reduced/mg fr wt/h.
Medium used DPM.

SE values for 12 d not above 0.7
SE values for 40 & 109 d not above 0.3

mature/static stage.

AnpUS4 exhibited the highest activity while AnpST11 the lowest. A fall in nitrogenase activity was noticed in forty and hundred and nine day old subcultures. The reduction noticed could be due to depletion of nutrients in the growth medium and also due to the lowered activity of cells. However, the fall of nitrogenase activity was less prominent in AnpST11. AnpST11 also showed the lowest values among isolates hundred and nine day old after subculture. The activity decrease in AnpUS4 was marked with AnpAG14 showing the highest activity among forty and hundred and nine day old subcultures. In general the efficiencies of isolates were higher than that of the strains (ArI3 being the only exception where ARA values were nearly equivalent to that of isolates). Even after hundred and nine days of subculture the isolates showed higher activities than the reference strains. AnpST11, $ACN1^{AG}$ and ArgP5 did not have the drastic fall noticed for the isolates. It is possible that these strains had a comparatively efficient uptake of the constituents of the growth medium.

In general the ARA values of isolates on forty and hundred and nine days were clustered. Thus the efficiency of isolates could best be differentiated twelve days after subculture. The rate of decline of nitrogenase activity was very rapid since no marked differences were observed for the activities after forty and hundred and nine days of subculture. The only exception being

AnpAG14 which showed a more steady decline.

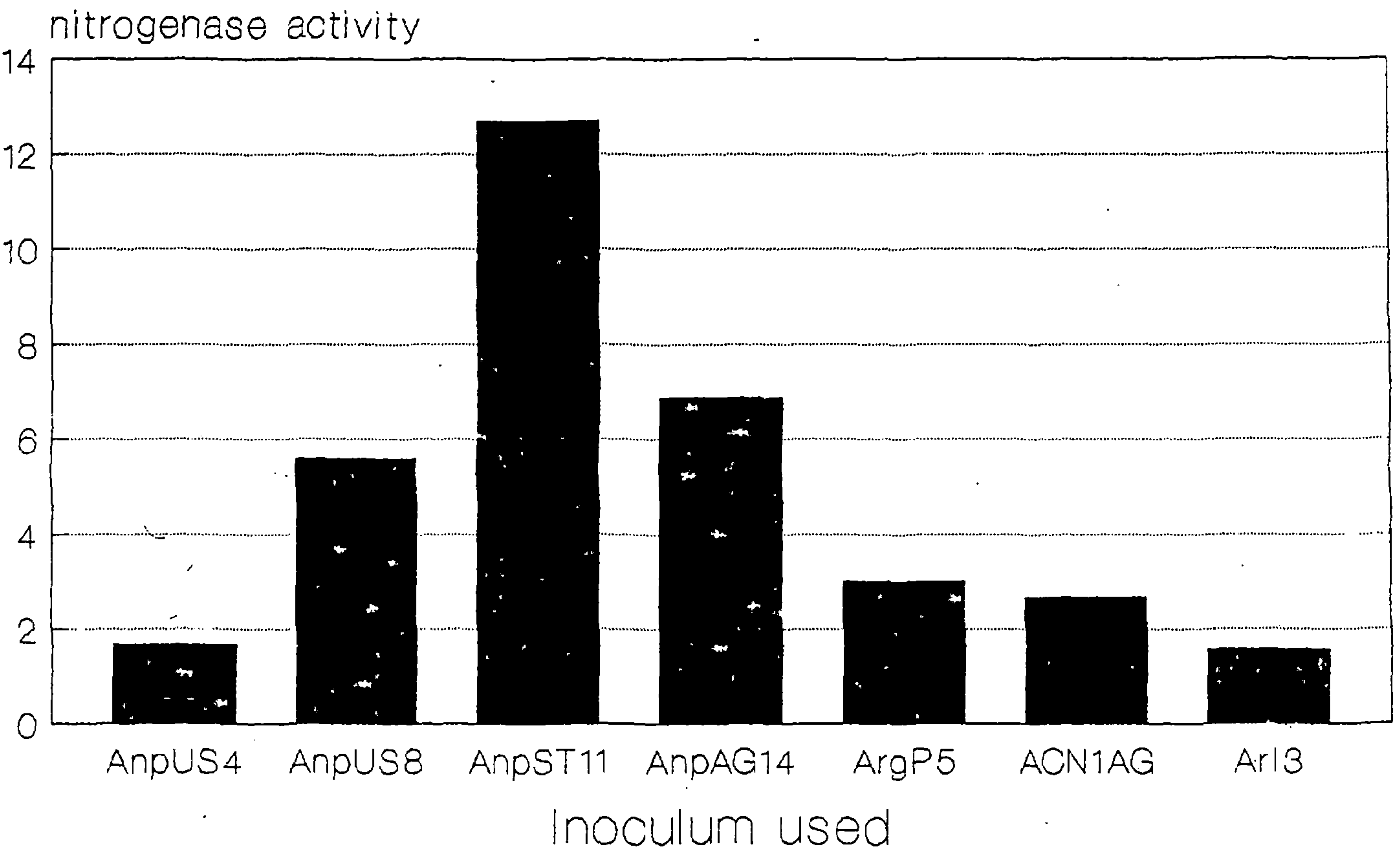
4.2.3.4 Nitrogenase activity of nodulated seedlings

The ARA results of inoculated seeedlings showed them to be effective in fixing atmospheric nitrogen. The details of this work have been presented in Fig 4.8. AnpST11 showed a symbiotic activity better than the other isolates/strains. At this point it should be noted that results of similar nature were observed with field collected nodules from the same site. Remarkably AnpST11 had poor nitrogenase activity in free culture condition while AnpUS4 had the highest activity in culture conditions.

Nitrogenase activity for *Frankia* isolates/strains inoculated in sterile soil indicated the possibility of saprophytic growth/existence in soil (Fig 4.9). AnpUS4 showed efficient performance under this condition. However, this enhanced activity of AnpUS4 might not be true in natural systems due to a variety of interactions. Nevertheless, it does show that AnpUS4 was a poor symbiotic but a better free living nitrogen fixer.

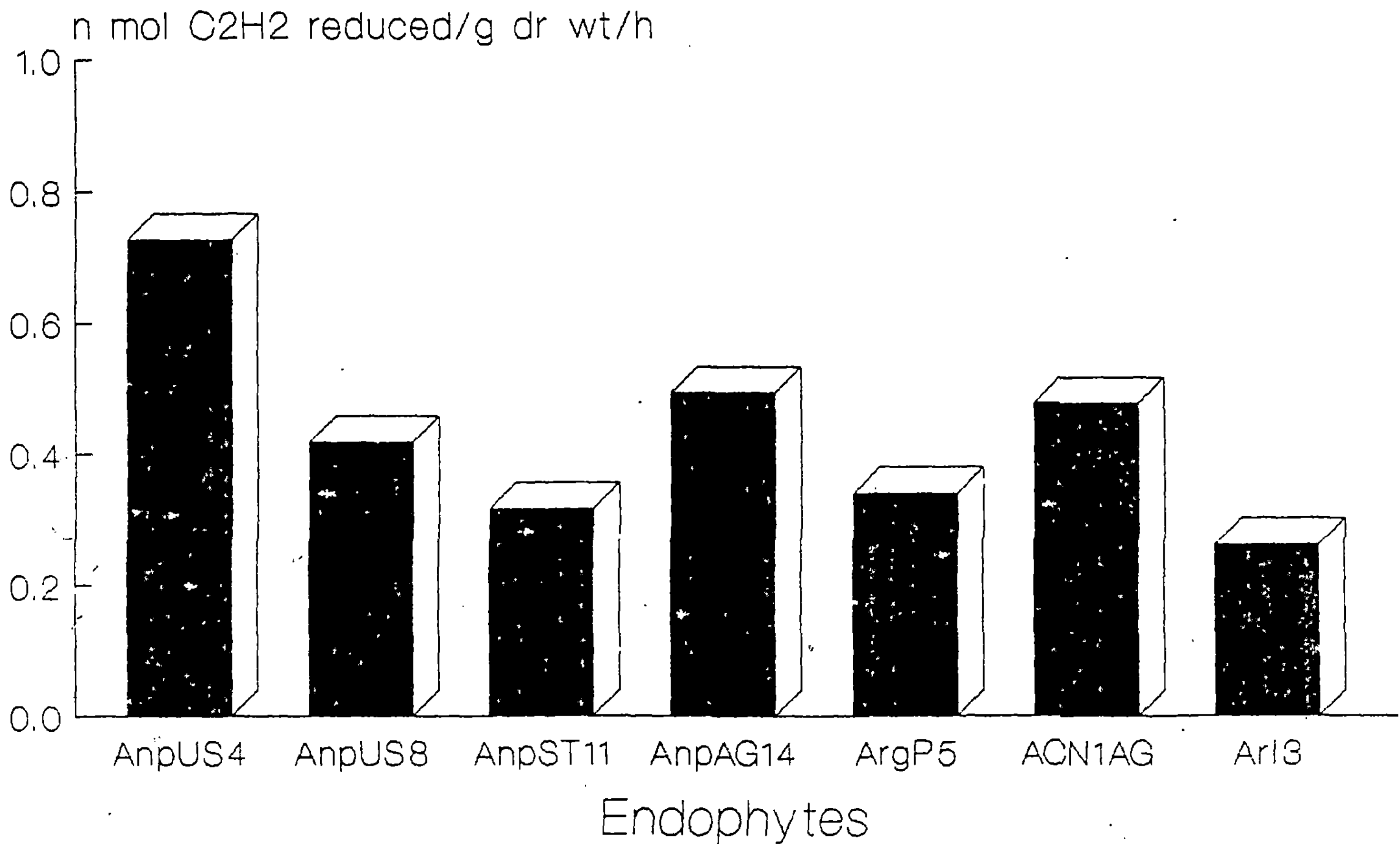
The results of nitrogenase activities demonstrate different performances of an isolate under different conditions. For example, AnpUS4 did well in soil and AnpST11 in symbiotic conditions. Hence for conditions where actinorhizal symbiosis is to be used for enhancing nitrogen status AnpST11 could be an isolate of choice. Whereas, where free living frankiae are to be used for similar purpose AnpUS4 would be a better choice.

Fig 4.8 ARA of nodulated seedlings inoculated with cultures of *Frankia*



ARA: acetylene reduction assay as
nmols C2H2 reduced/seedling/h, 6 wks
after inoculation (SE values not > 0.13)

Fig 4.9 Nitrogenase activity of *Frankia* in sterilized soil



30 d incubation at 28°C
+cont - 0.44; -cont - 0.20
SE values not above 0.3

However, it should be noted that further work is desirable in connection with field performance of AnpST11 after which its competitive efficiency could be established.

4.2.3.5 Addition of nitrogen sources

The experiment was undertaken to assess the uptake of various nitrogen sources by frankiae. Vesicles have been shown to be the sites of nitrogenase activity (Tjepkema *et al.*, 1980). Table 4.9 shows the vesicle counts in DPM and DPM supplemented with nitrogen sources. The fall of vesicle numbers is clearly seen. Although count differences between isolates/strains were observed, the activity of individual vesicles could not be assessed.

It was found that all the nitrogen sources tested suppressed nitrogenase activity drastically in all isolates/strains (Fig 4.10). However, there were no significant differences ($P < 0.05$) between different sources tested. The decline in the activities for all isolates and strains could be due to feedback inhibition. This fall could also be related to membrane potential. A membrane potential of at least 80 mV is required for dinitrogen fixation in whole cells of *Azotobacter* (Gallon and Chaplin, 1987).

4.2.3.6 Addition of metals

Molybdenum stimulated nitrogenase activity of *Frankia* isolates and strains. Naturally nitrogenase synthesized in such conditions contains molybdenum as a constituent of the enzyme.

Table 4.9

Influence of different nitrogen sources on production of vesicles by *Frankia*

frankiae	DPM[a]		Nitrogen source added to DPM					
			Ammonium chloride		Potassium nitrate		Urea	
	V	ARA	V	ARA	V	ARA	V	ARA
AnpUS4	14	+	3	+	4	+	9	+
AnpUS8	12	+	6	+	2	+	7	+
AnpST11	8	+	2	+	8	+	6	+
AnpAG14	14	+	6	+	12	+	11	+
ArgP5	18	+	10	+	7	+	6	+
ACN1[AG]	13	+	2	+	4	+	5	+
ArI3	7	+	2	+	2	+	4	+

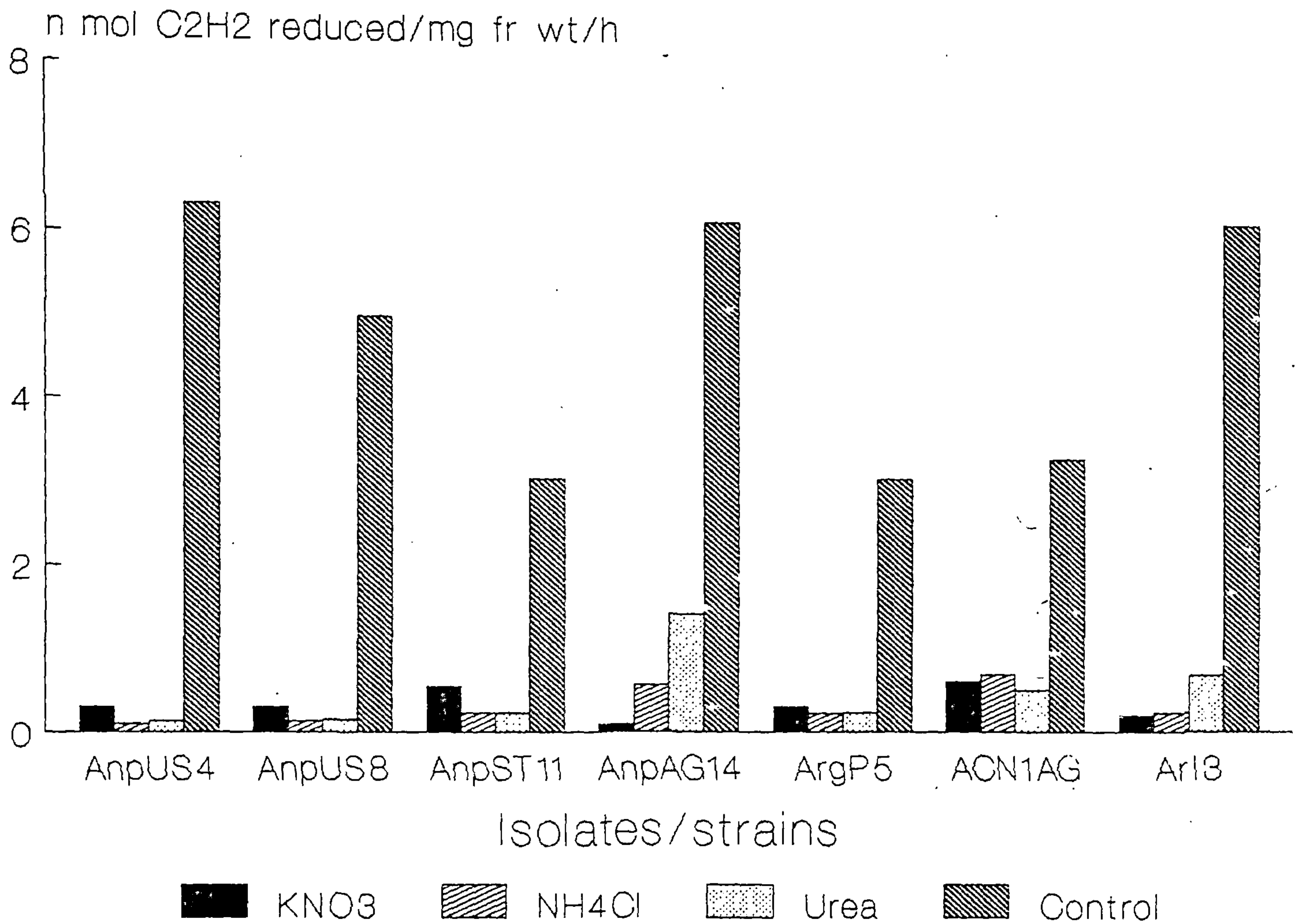
a Baker and O'Keefe, 1984 v vesicle no./colony
+ indicates presence of activity

ARA acetylene reduction assay

Data shown are with 12 d subcultures

Vesicle counts of *Frankia* in presence of N source 30 and 45 d did not exceed the ones for 15 d old subcultures in DPM

Fig 4.10 Effect of different nitrogen sources on the activity of nitrogenase



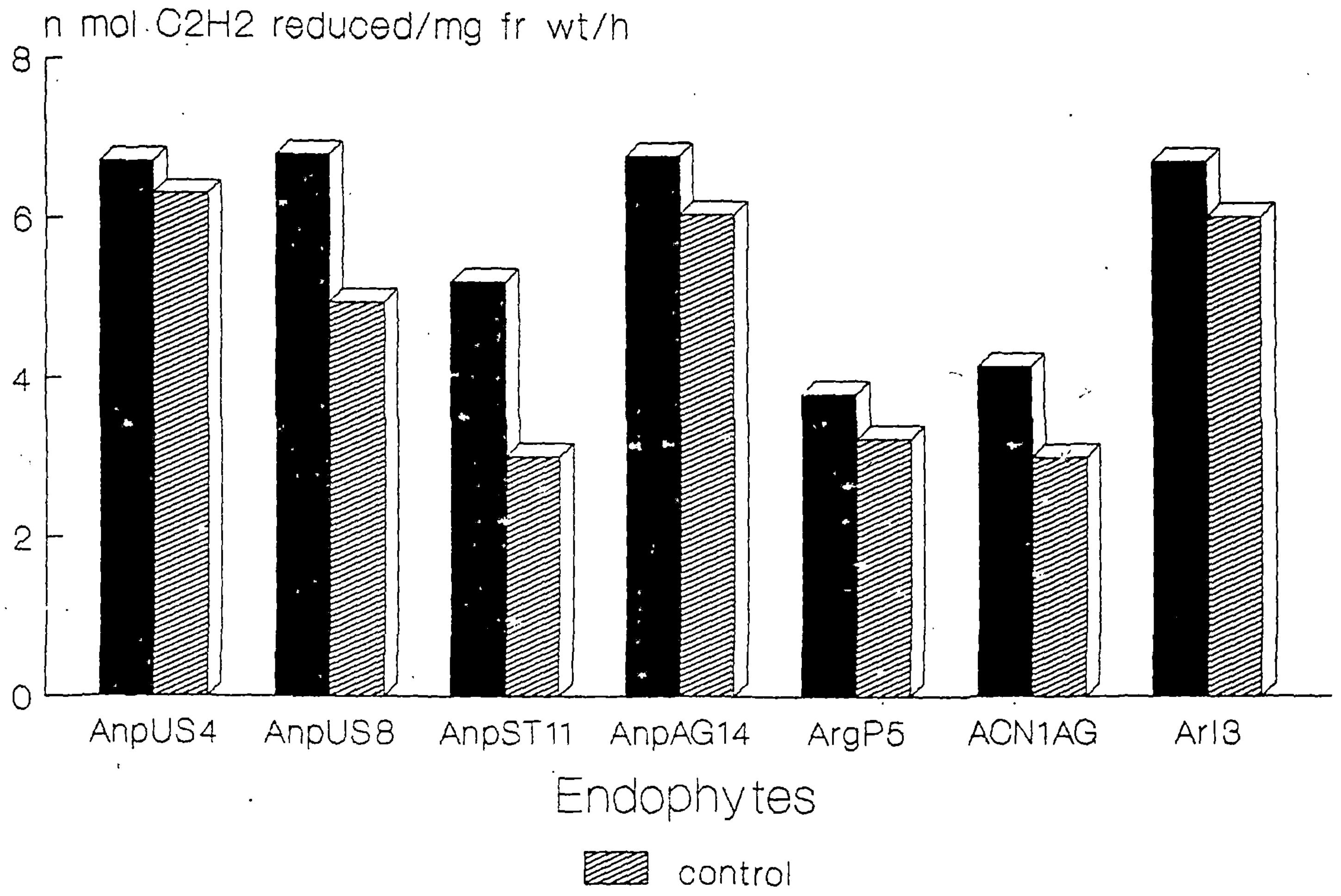
Twelve day old subcultures in DPM
supplemented as shown

Fig 4.11 shows the activity upon addition of molybdenum. The maximum spurt in nitrogenase activity was for AnpST11 followed by AnpUS8. However, all isolates and strains showed an increase of activity after addition of molybdenum. This could also indicate that the concentration of molybdenum in DPM was probably not at the optimum level for the isolates and strains. This is inferred by the stimulation effect and absence of toxic effects.

Supplementing the medium with vanadium too increased reduction of C_2H_2 to C_2H_4 (Fig 4.12). Maximum activity was observed in AnpAG14. A slight increase in activity was observed with AnpUS8. When molybdenum is present in basal dose the effects of vanadium can be attributed to an efficient utilization of molybdenum by the isolates of *Frankia*. It is to be noted that the synthesis of vanadium nitrogenase requires the absence of molybdenum and the presence of vanadium (Bishop *et al*., 1988). However, exclusion of molybdenum produced a sharp decrease in the activity of nitrogenase. Although the cultures were starved the low values ($P < 0.05$) could be due to the recycling of trace elements. The results demonstrated the ability of isolate/strains to synthesize only molybdenum containing nitrogenase under the set of conditions. However, the cells in molybdenum depleted cultures remained viable throughout the experiment.

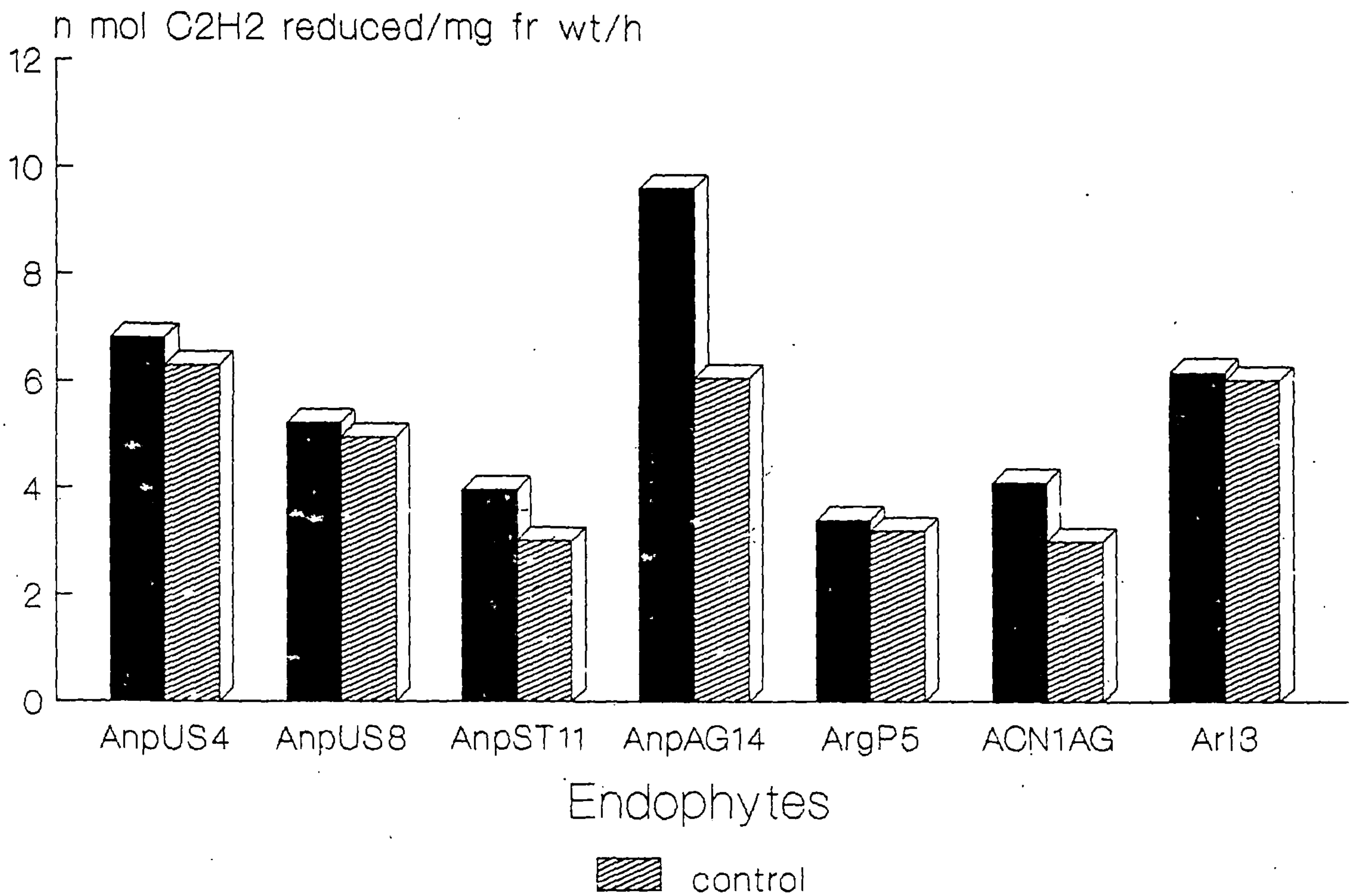
It is an established fact that iron is essential for growth and is needed for various cell reactions. Iron lead to an enhanced activity (Fig 4.13) with AnpAG14 showing the highest

Fig 4.11 Effect of molybdenum on nitrogenase activity



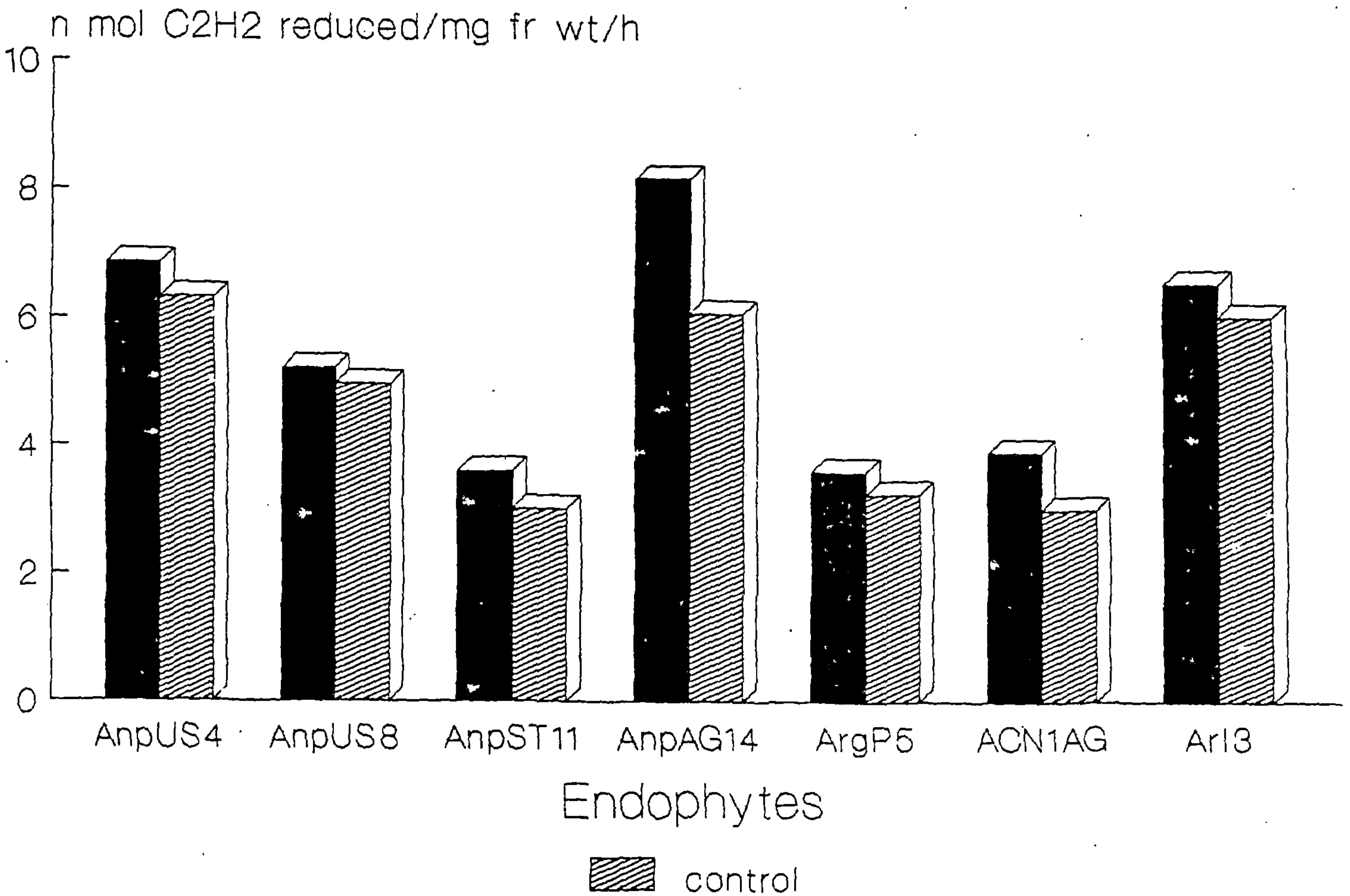
twelve day old subcultures in DPM and
DPM supplemented with molybdenum
SE values not above 0.3

Fig 4.12 Effect of vanadium on nitrogenase activity



twelve day old cultures in DPM and
DPM supplemented with vanadium
SE values not above 0.3

Fig 4.13 Effect of iron on nitrogenase activity



twelve day old cultures in DPM and
DPM supplemented with iron
SE values not above 0.3

activity. It may also enhance the activity by influencing various pathways.

Though the present study points clearly towards stimulation of nitrogenase activity by addition of various metals, presence/absence of the alternate types of nitrogenases could not be inferred. The way out would have been the deletion of structural genes for molybdenum nitrogenase before testing for alternative nitrogenases. But this was not tried since the aim of the present study was to study the diversity among natural populations of *Frankia*.

4.2.4 Detection of Plasmids

Lysis of *Frankia* cells was found to be dependent upon the age of culture. Table 4.10 presents the results of lytic agents used on *Frankia*. An attempt was made to perform the lysis step in the wells in order to obtain higher yields of DNA. However, this method did not give good results.

Fig 4.14 (a) shows AnpUS4 and AnpUS8 possesing plasmids. The molecular weight of this plasmid was about 20 Kb. The band was faint possibly due to the low copy number/cell. Hence various antibiotics were added at various concentrations in order to get enhanced copy numbers and also to establish markers for plasmids. The results of antibiotic resistance have been presented in section 4.2.5.

The two isolates possesing plasmids were from *A. nepalensis*

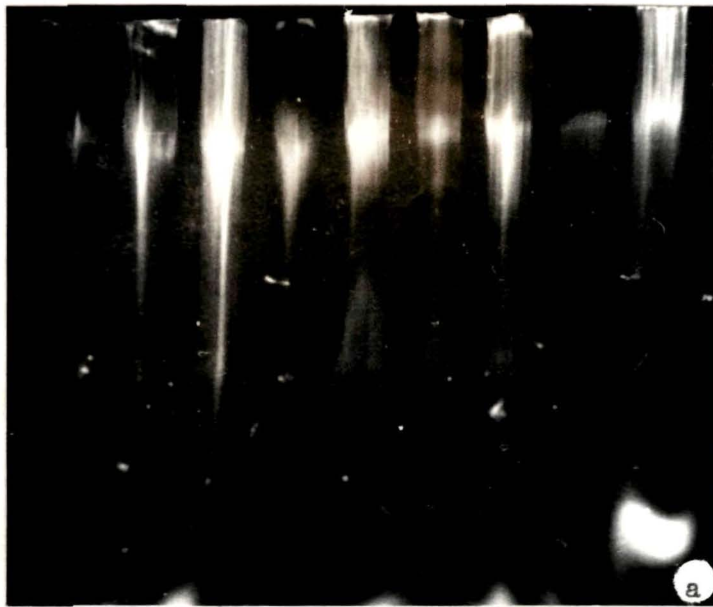
Table 4.10

Standardization of lysis of *Frankia* isolates

Age of sub-culture in DPM	Lytic agent(s)	Lysis
2-12 d	Lysozyme	-
2-12 d	Achromopeptidase	-
2-12 d	Lysozyme and Achromopeptidase	+
2-12 d	Achromopeptidase and RNase	-
2-12 d	Lysozyme and RNase	+
2-12 d	Lysozyme,RNase and Achromopeptidase	+
13-30 d	Lysozyme	-
13-30 d	Achromopeptidase	-
13-30 d	Lysozyme and Achromopeptidase	+
13-30 d	Achromopeptidase and RNase	-
13-30 d	Lysozyme and RNase	-
13-30 d	Lysozyme,RNase and Achromopeptidase	+
31-60 d	Lysozyme	-
31-60 d	Achromopeptidase	-
31-60 d	Lysozyme and Achromopeptidase	-
31-60 d	Achromopeptidase and RNase	-
31-60 d	Lysozyme and RNase	-
31-60 d	Lysozyme,RNase and Achromopeptidase	-

(+) lysis present (-) lysis absent
Incubation temperature 40°C for 1 h
Variations in %ages of lytic agents, temperature and time of incubation too were tried but data not presented here.

Fig 4.14(a) Agarose gel electrophoresis of *Frankia* isolates
Lane(l-r) 1. AnpUS4, 2. AnpUs4, 3.*R. melilote*, 4. AnpUS8, 5. AnpUS8 6. AnpUS4, 7. AnpUS4, 8. *E. coli* and 9. *R. meliloti*


a

growing in Upper Shillong. These plants were separated by not more than two km. Therefore, it is possible that either the two plasmids were same or were unrelated but had approximately similar molecular weight. The later possibility is less likely and there is a need for further investigation to settle this question.

4.2.5 Resistances to antibiotics as markers

Three types of antibiotics were used for the study. Ampicillin is produced by *Penicillium* spp., tetracycline by *Streptomyces* spp. and chloramphenicol by *Streptomyces* spp. However, they vary in their mode of action. Ampicillin which is a derivative of penicillin acts by interfering with a terminal step in bacterial wall synthesis. Tetracycline prevents protein synthesis by binding to the 30S subunit of ribosome. Chloramphenicol also is an inhibitor of protein synthesis (see Maniatis *et al.*, 1982).

Table 4.11-4.12 and Fig 4.15-4.17 show the effect of various concentrations of these antibiotics on *Frankia* isolates and strains. The results showed the frankiae experimented to be sensitive to the antibiotics tested. Till date there exists no report of any antibiotic resistance of *Frankia* nor production of any antibiotics by it.

Majority of the antibiotics are produced by actinomycetes. *Penicillium*, *Streptomyces* and *Frankia* are inhabitors of the

Table 4.11

Light microscopical observation of *Frankia* in medium containing 2 µg/ml Ampicillin

Frankia	Hyphae (15, 30 & 45 d)	Sporangia			Transfer to DPM* after 30 d without Ampicillin	
		15d	30d	45d	growth (30d)	nitrogenase activity (15d)
AnpUS4	+	a(20)	b(25)	d(18)	g	+
AnpUS8	+	a(25)	c(30)	d(12)	g	+
AnpST11	+	b(18)	c(12)	d(17)	g	+
AnpAG14	+	a(22)	b(29)	d(26)	g	+
ArgP5	+	a(16)	b(35)	d(10)	g	+
ACN1AG	+	b(19)	b(18)	d(25)	g	+
ArI3	+	a(12)	b(17)	d(23)	g	+

a 1-10 sporangia
b 11-20 sporangia
c below 50 sporangia
d over 50 sporangia/colony

\+ present
g growth observed

numbers in parentheses: colonies observed

Vesicles seen in few colonies and nitrogenase activity low in ampicillin containing DPM

* Baker and O'Keefe, 1984

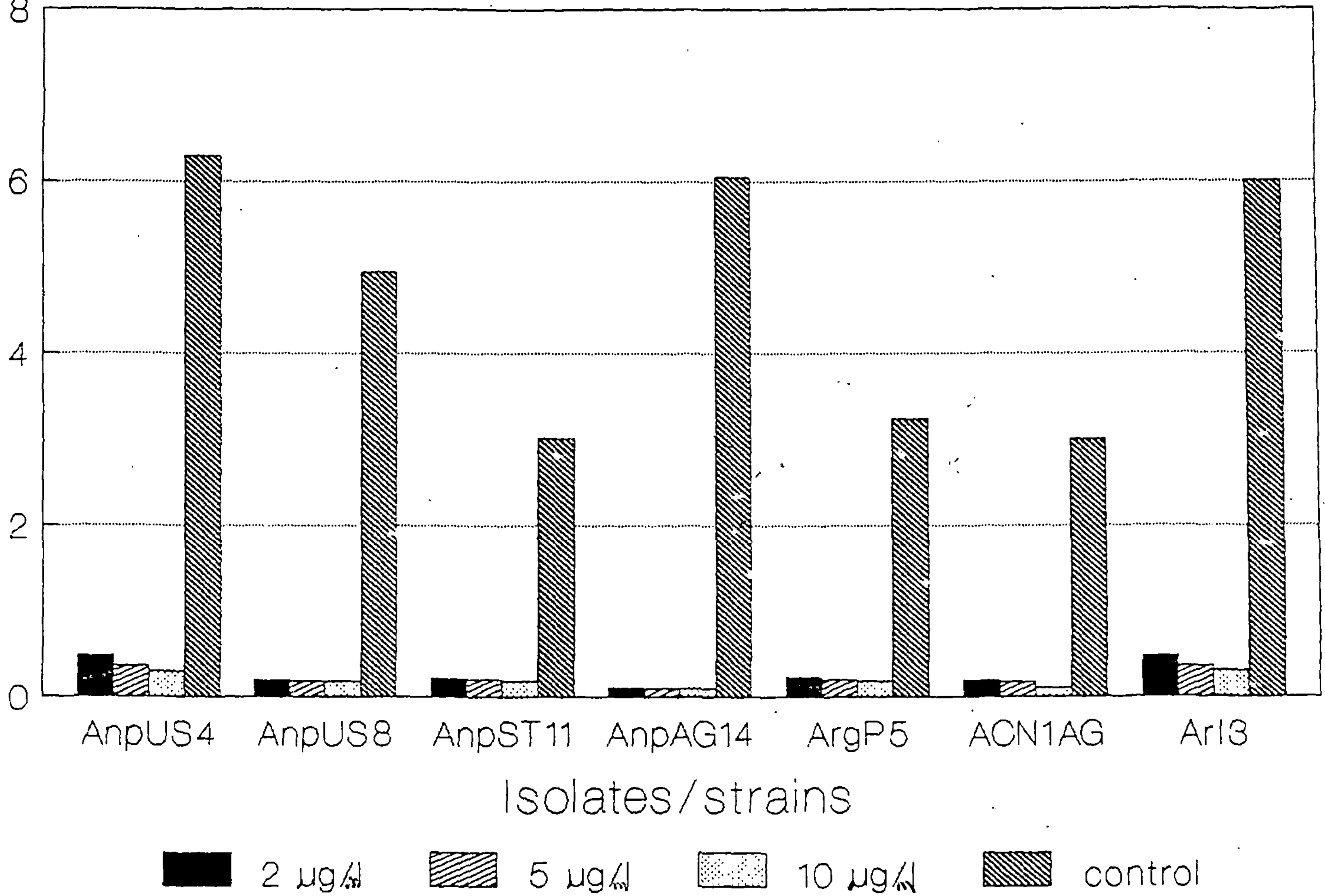
Table **4.12**

Influence of Antibiotics on the growth of frankial isolates and strains

Isolates/strains	Ampicillin	Tetracycline	Chloramphenicol	Control
AnpUS4	0	0	6.6×10^{-3}	0.04
AnpUS8	0	6.6×10^{-3}	0	0.05
AnpST11	0.013	6.06×10^{-3}	0	0.11
AnpAG14	6.6×10^{-3}	6.6×10^{-3}	0	0.09
ArgP5	0	0	6.6×10^{-3}	0.03
ACN1AG	0	0	6.6×10^{-3}	0.06
ArI3	0	0	0	0.03

concentration of antibiotics 2 µg/ml .
increase recorded as gain in weight/unit mass/day
values presented mean of three replicates/organism/antibiotic

Fig 4.15 Effect of ampicillin on nitrogenase activity of *Frankia* cultures



twelve days old cultures in DPM

Fig 4.16 Effect of tetracycline on nitrogenase activity of *Frankia* cultures

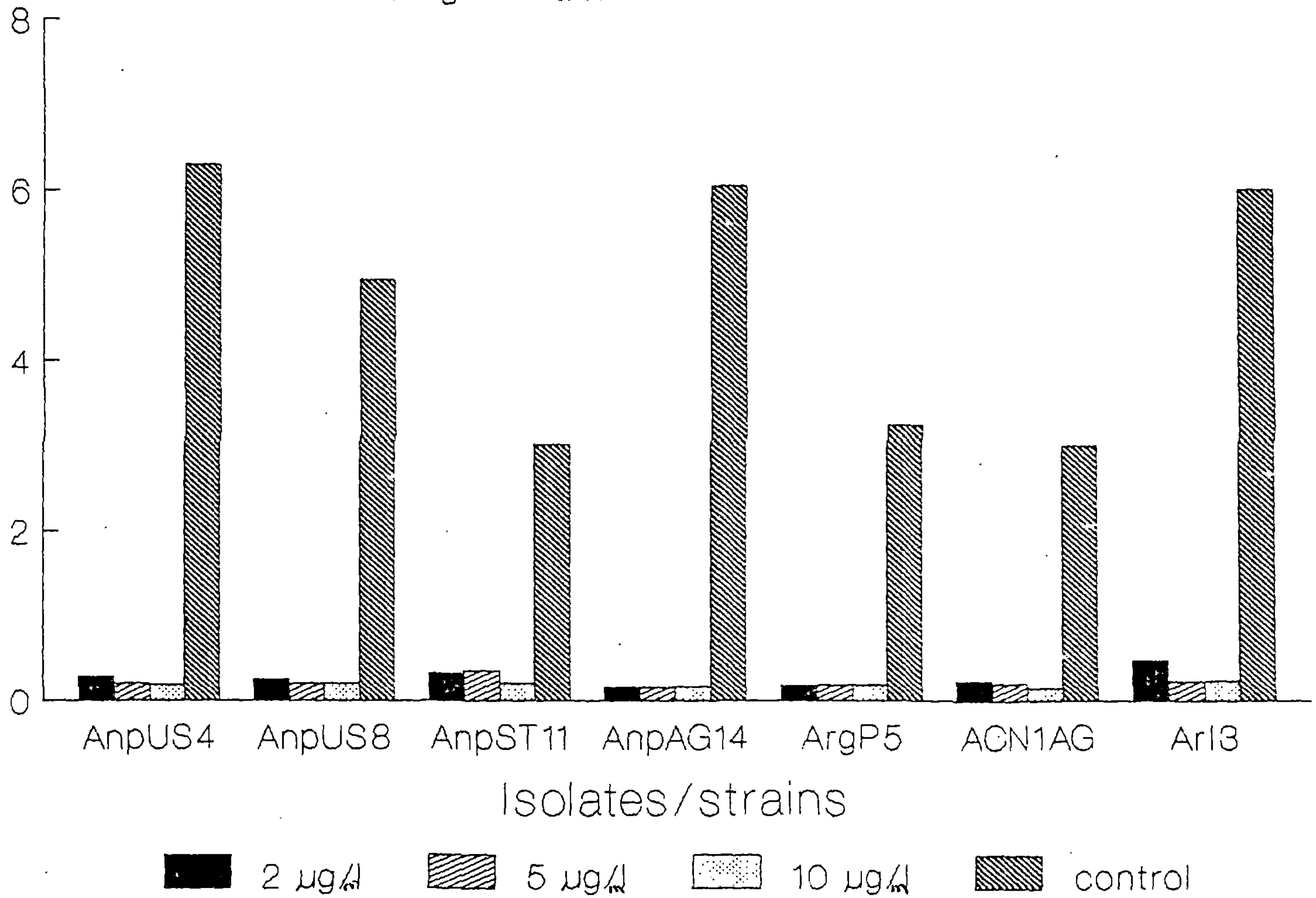
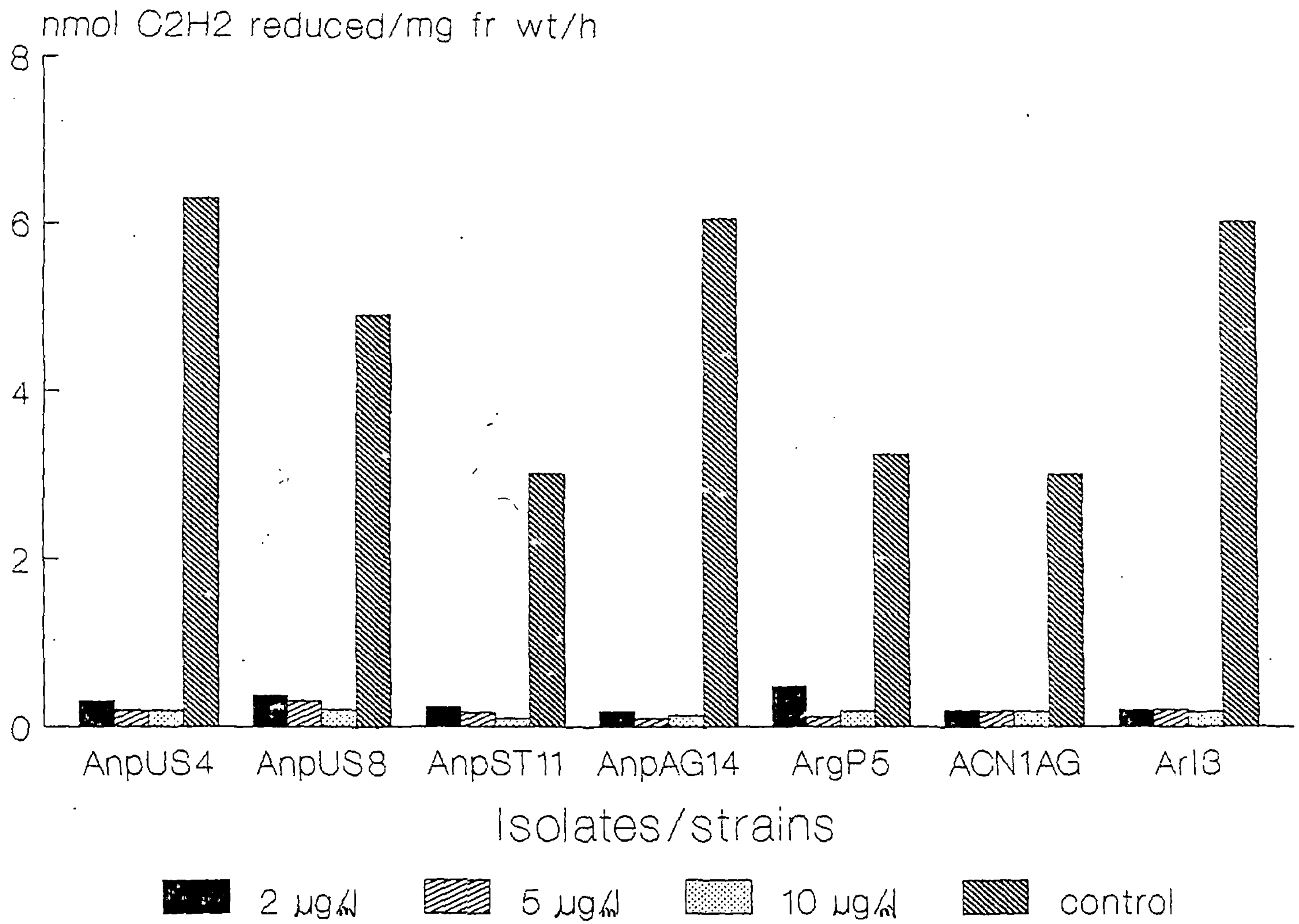

nmol C2H2 reduced/mg fr wt/h
8
6
4
2
0
AnpUS4
AnpUS8
AnpST11
AnpAG14
ArgP5
ACN1AG
ArI3
Isolates/strains
2 µg/l
5 µg/l
10 µg/l
control
twelve days old in DPM

Fig 4.17 Effect of chloramphenicol on nitrogenase activity of *Frankia* cultures


nmol C2H2 reduced/mg fr wt/h
8
6
4
2
0
AnpUS4
AnpUS8
AnpST11
AnpAG14
ArgP5
ACN1AG
Arl3
Isolates/strains
2 µg/l
5 µg/l
10 µg/l
control
twelve days old cultures in DPM

soil. However, the situation in soil is possibly different due to pH, adherence of organisms to soil particles and indirect contact of these antibiotics etc.

In *many* cases the genes for antibiotic resistance have been shown to be located on plasmids (Blenkharn and Huges, 1982; Shannon and Phillips, 1982; Chau *et al.*, 1982). Not only they function as markers but also serve to enhance copy numbers in certain bacteria (see Maniatis *et al.*, 1982). However in the plasmid carrying isolates it was found that antibiotic resistance genes for these antibiotics were not present. The same explanation could be offered for absence of any resistance genes in the chromosomal DNA of all the isolates/strains tested.

Various colonies in these antibiotics too did not produce any variant capable of growing in these antibiotics. However, the viability of these frankiae could not be questioned as they grew again upon transfer to fresh medium and spurt of nitrogenase activity was noticed.

It is concluded that there was variability amongst isolates with respect to presence or absence of plasmids but no diversity was seen with respect to resistance to the three antibiotics tried.

4.2.6 Possible presence of *nif* genes on plasmids

It seems that these plasmids, whether similar or not did not have *nif* genes. This can be stated for two specific reasons -

firstly because these plasmids were just about 20 Kb and only mega plasmids have been found to carry these genes naturally (Simonet *et al.*, 1986) and secondly because the isolates without these plasmids too had *nif* genes as demonstrated by their ability to fix atmospheric nitrogen. These observations made further investigations using DNA-DNA hybridization etc. needless.

The results of the study on genetic diversity demonstrate both similarities and dissimilarities amongst the isolates. They varied in their measurements of hyphal and vesicle diameters, sporangial counts, growth performance in different media, carbon utilization, activity of nitrogenase and the presence of plasmids.

However, all the isolates were similar with respect to sensitivity to the antibiotics tested, in being Sp^- and had similar levels of nitrogenase activity on addition of nitrogen sources.

SUMMARY

The root nodules formed by actinomycete of *Alnus nepalensis* were of '*Alnus* type'. These nodules harboured *Frankia* symbiont which was isolated using various nutrient media. DPM was most suitable medium for the isolation and growth of *Frankia*. Four isolates obtained from *A. nepalensis* growing at various sites were chosen for the study.

The confirmation of these isolates as *Frankia* was carried out using morphological characters, nodulation tests and nitrogenase activity. The isolates showed characteristic

features of *Frankia* by possesing thin hyphae, sporangia and vesicles typical of the symbiont. In addition to exhibiting nitrogenase activity, they nodulated seedlings of *A. nepalensis*. The results obtained therefore demonstrated the isolates to be *Frankia*.

Frankia isolates thus obtained were screened for genetic diversity. Measurements of hyphae and vesicles showed differences with AnpUS4 showing the largest diameters of hyphae and vesicles.

An absence of sporangia were found within the cortical cells of nodules thus classifying them as Sp^{-}.

In all the media used the isolates and strains had loosely arranged hyphae on 15 d and a network of hyphae had formed on 40 d. A difference in sporangial counts was observed with AnpST11 producing more than fifty sporangia per colony in all the media on 40 d. The isolates showed different growth patterns on different media and all grew well in DPM. Utilization of carbon by AnpST11 was exceptionally well in acetate as sole carbon source while other isolates preferred propionate and pyruvate.

Nitrogenase activity varied in both *in vivo* and *in vitro* conditions. AnpUS4 was seen to be an efficient performer in *in vitro* conditions while AnpST11 did well during symbiosis. Addition of metals to the basal medium (DPM) enhanced the activity of nitrogenase to diiferent levels amongst isolates thus indicating the differences in their genetic composition. However,

addition of nitrogen sources to the medium led to a fall in ARA rates, pointing towards the uptake of these sources.

Plasmids were detected in two isolates of *Frankia* from Upper Shillong (AnpUS4 and AnpUS8). Their molecular weights were around 20 Kb. However, it is unlikely that *nif* genes are present on these plasmids - firstly due to their size and secondly due to a similar behaviour by isolates not possessing the plasmids. The use of antibiotics as markers did not yield any significant differences. Neither any difference in the growth and nitrogenase activity of *Frankia* were seen.

Thus it can be concluded from the present study that the frankiae tested were genetically diverse. Variations not only existed amongst isolates but also with reference strains. However, differences narrowed down to nil values with a few parameters. For *in vitro* experiments AnpUS4 seemed to be a good choice and for studies of symbiotic nature AnpST11 was ideal. The utility of AnpST11 would be established after field trials.

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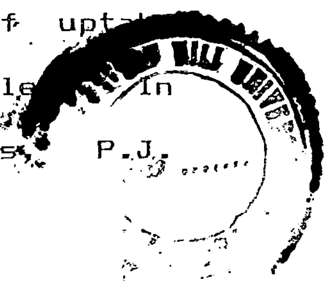

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Appendices

Appendix 3.1

PVP

Polyvinyl pyrrolidone	3.0	g
PBS	100	ml

PBS

NaCl	0.8	g
$Na_2HPO_4 \cdot 7H_2O$	1.16	g
KH_2PO_4	0.2	g
Distilled water	1	l

Appendix 3.2

Qmod

K_2HPO_4	300	mg
NaH_2PO_4	200	mg
$MgSO_4 \cdot 7H_2O$	200	mg
KCl	200	mg
Yeast extract	500	mg
Bacto-Peptone	5	g
Glucose	10	g
Ferric citrate	1	ml
Minor salts	1	ml
$CaCO_3$	100	mg
Lipid supplement	0.5	mg

Distilled water 1 l

(Ferric citrate - Citric acid and Ferric citrate 1% solution
Minor salts(g/l) - H_3BO_3 1.5; $MnSO_4$ 0.8; $ZnSO_4$ 0.6; $CuSO_4$ 0.1; $(NH_4)_6Mo_2O_{24}.\ 4H_2O$ 0.2; $CoSO_4$ 0.01
Lipid supplement - lecithin)

Appendix 3.3

DPM

KH_2PO_4	1.0	g
$MgSO_4$	0.1	g
$CaCl_2$	0.01	g
Sodium propionate	1.2	g
Hoaglands micro elements stock	1	ml
$FeSO_4$ - EDTA stock	1.8	ml
Distilled water	1	l

(Hoaglands micro-elements stock (g/l) - H_3BO_3 0.71; $MnCl_2$ 0.45; $ZnSO_4$ 0.22; $CuSO_4$ 0.02; $Na_2MoO_4.\ 2H_2O$ 0.02)

Appendix 3.4

F

$CaCl_2.\ 2H_2O$	0.1	g
$MgSO_4$	0.2	g
K_2HPO_4	0.5	g
Hydrolysate casein	4.0	g
Glucose	10	g
Yeast extract	0.05	g
Citrate Fe_3	1	ml
Vitamin B (10mg/l)	1	ml
Nicotinic acid (50mg/l)	1	ml
Pyrodoxine HCl (50mg/l)	1	ml
Tween 80	2	ml
Oligo quispel	1	ml

i)	H_3BO_3	1.5	g
ii)	$MnSO_4.\ 7H_2O$	0.8	g
iii)	$ZnSO_4.\ 7H_2O$	0.6	g
iv)	$CuSO_4$	0.1	g

v)	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.2	g
vi)	$CoSO_4$	0.01	g
	Distilled water	100	ml

Distilled water 1 l

(Citrate Fe_3 - Citric acid 1 g; Ferric citrate 1 g in 100ml distilled water)

Appendix 3.5

OS-1

K_2HPO_4	0.15	g
$NaH_2PO_4.2H_2O$	0.13	g
$MgSO_4$	0.05	g
KCl	0.1	g
Yeast extract	0.25	g
Tryptone	2.5	g
Sodium acetate	0.5	g
EDTA	0.01	g
Tween 80	1	ml
Distilled water	1	l

Appendix 3.6

FAA (Formalin - Acetic acid - Alcohol mixtures)

Ethyl alcohol (95%)	50	ml
Glacial acetic acid	5	ml
Formalin	10	ml
Water	35	ml

Appendix 3.7

PBS buffer

Sodium chloride	0.15	M
Sodium phosphate	0.01	M

pH 7.4

Appendix 3.8

Tris-Borate buffer (TBE)

5X (concentated stock solution/lit)

Tris base	54	g
Boric acid	27.5	g
EDTA(0.5M)	20	ml

pH 8.0

Appendix 3.9

Ampicillin - Prepare a 25 mg/ml solution of the sodium salt of ampicillin in water. Sterilize by filtration and store in aliquots at $-20^{o}C$.

Tetracycline - Prepare a 12.5 mg/ml solution of tetracycline hydrochloride in ethanol/water (50% v/v). Sterilize by filtration and store in aliquots at $-20^{o}C$ in containers wrapped in aluminium foil.

Chloramphenicol - Dissolve solid chloramphenicol in 100% ethanol at a concentation of 34 mg/ml. It is stored at $-20^{o}C$.

Appendix 4.0

Maintainence medium* for the symbiont of *Alnus nepalensis*

KH_2PO_4	1	g
$MgSO_4$	0.10	g
$CaCl_2$	0.01	g
Sodium propionate	1.20	g
Yeast extract	0.10	g
Tryptone	0.10	g
Molybdic acid	0.01	g

Hoagland microelements stock solution**	1	ml
$FeSO_4$- EDTA stock**	1	ml
Distilled water	1	l
pH adjusted to 6.9		

* & ** same as in Appendix 3.3