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PCR-RFLP Based Screening of *Frankia* in Alder Nodules Having Different Levels of Nitrogenase Activity

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Abstract

One hundred and thirty nodules from 13 different *Alnus nepalensis* trees from a site in Shillong, India, were screened for nitrogenase activity. Nodules from different trees were found to differ in nitrogenase activity estimates obtained by acetylene reduction assay. In order to analyze whether the differences in nitrogenase activities were due to different *Frankia* strains residing in the nodules, nodule DNA were subjected to a Polymerase Chain Reaction – Restriction Fragment Length Poly-morphism (PCR-RFLP) study. High and low activity nodules from different trees were found to contain *Frankia* belonging to similar PCR-RFLP groups. Therefore, the host plant appears to exert a considerable influence on the nitrogen fixation rates of *Frankia* nodules.

Keywords: *Frankia*, PCR-RFLP, alder, nitrogenase activity

1. Introduction

It was earlier assumed that selecting the best plant clones and *Frankia* strains would suffice for achieving the most efficient symbiotic partnership, but apparently this is not the case. High nitrogen fixing *Frankia* strains frequently

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fail to show high activity after colonizing the best host clone. Previous work on *Frankia* (Reddell and Bowen, 1985; Sougoufara et al., 1992) revealed that the host assumed a greater role than the microsymbiont during the commencement and subsequent development of symbiosis. Past experiments, pertaining to physiological data, point towards a direct involvement of the host, but suffer from lack of molecular evidence.

The aim of the present study was to examine the *Frankia*-actinorhizal host relationship using a molecular approach. Individual nodules from a series of alder trees were screened for nitrogenase activity and PCR-RFLP patterns. Attempts were then made to establish the relationship between the PCR-RFLP patterns and the nitrogenase activities, if any.

2. Materials and Methods

Collection of nodules

A forest stand in Upper Shillong area was selected for this study. It comprised several tree species, including *Myrica nagi*, *Elaeagnus* sp., *Alnus nepalensis* and *Pinus kesiya*. Thirteen trees of *Alnus nepalensis* were randomly selected for the study. Since the trees grew in the same stand, the variability due to soil condition, light availability, moisture and temperature was minimal. Ten young active nodules from each tree were carefully traced to the parent tree and collected during the month of May. They were placed in unused moistened plastic bags and brought to the laboratory on the same day.

Assay for nitrogenase activity

Nitrogenase activity was measured by the Acetylene Reduction Assay (ARA) (Stewart et al., 1968). While this technique may not give very accurate results, it is useful for quick comparative study of the type done by us.

Surface sterilized individual nodules were weighed and placed in sterile glass vials fitted with gas tight rubber stoppers. Ten percent volume of the air in the vial was replaced with pure acetylene. The vial was incubated at 37°C for 3 1/2 hours. One ml of the gas mixture was then injected into a Tracor gas chromatograph fitted with a Porapak T column (6 ft. × 0.125 in., 80–100 mesh). The injection port, column and detector temperatures were 100, 70 and 120°C, respectively. The flow rates of H_2, air and N_2 carrier gases were 20, 120 and 30 ml/min respectively. For each nodule, readings were taken in triplicates. After ARA measurements the nodules were stored in 70% alcohol at –20°C for further use.

The collection of nodules and their ARA were performed on the same day and

the conditions from the time of collection of nodules to their nitrogenase activity assay were constant for all the samples studied, making the comparative inference valid.

Reference strains and Frankia isolates

Reference strains of *Frankia* and laboratory isolates were used in the present study. All were cultured at 28°C in Defined Propionate Medium (DPM, Baker and O'Keefe, 1984). The *Frankia* strains used were $ACN1^{AG}$, $ARgP5^{AG}$ (Lyon, France) and AnpST11 (Shillong, India). *Rhizobium meliloti* strain Rmd1021 was grown in TY with streptomycin (Sambrook et al., 1989) and *E. coli* strain HB101 was grown in LB medium (Sambrook et al., 1989).

DNA isolation, amplification and restriction digestion

The small size of nodules and the presence of plant phenolics makes DNA isolation from alder root nodules difficult. Detailed below is the procedure adopted by us, which yielded good results.

Freshly collected nodules were surface sterilized with 30% H_2O_2 and immediately transferred to 500 µl of cold TEA buffer (Benson et al., 1996; 10 mM Tris, 1 mM EDTA, 20 mM ascorbic acid, pH 7.6). The outer layer was then peeled off. A single nodule was gently crushed once or twice in cold TEA buffer and kept at 4°C for 5 minutes. The buffer was washed off, fresh buffer added, and this step repeated thrice. Finally, 200 µl of ice cold CTAB buffer [100 mM Tris, 20 mM EDTA, 1.4 mM NaCl, pH 8.0, with 2% (wt/vol) polyvinyl-pyrrolidone and 1% (wt/vol) cetyl trimethyl ammonium bromide] were added to the suspension (Rouvier et al., 1996). The mixture was centrifuged, the supernatant discarded, and 200 µl of buffer were added again. The suspension was thoroughly crushed with a hand-held homogenizer. Five µl of 20% sodium dodecyl sulphate (SDS) were added and the solution kept at 4°C for 10 minutes, followed by centrifugation (1200 x g, 5 minutes). The supernatant was extracted twice with equal volumes of chloroform-isoamyl alcohol (24:1). The aqueous phase was transferred to a fresh tube, and the DNA was precipitated with two volumes of ice cold absolute ethanol. The tube was centrifuged (13000 x g, 30 min, 4°C). The pellet was washed twice with 70% ethanol and vacuum-dried. This method yielded a DNA preparation which lacked any visible coloration.

Double-stranded DNA amplifications were performed as previously reported (Simonet et al., 1991). *Frankia*-specific primers in the 16S rRNA and *nif* genes were used in conjunction with universal primers. In the *nif* H-D region, primers FGPH750 [21 mer, 5′ GAAGACGATCCCGACCCCGA 3′ (Simonet et al., 1991)] and FGPD826′ [19 mer, 5′ TTCATCGACCGGTAGCAGTG 3′] were used. In

the 16S rRNA gene primers FGPS989ac [16 mer, 5′ GGGGTCCGTAAGGGTC 3′ (Bosco et al., 1992)], FGPS1490' [22 mer, 5′ AAGGAGGGGATCCAGCCGCA 3′ (Normand et al., 1996)], FGPS485 [15 mer, 5′ CAGCAGCCGCGGTAA 3′ (Normand et al., 1996)], FGPS910' [20 mer, 5′ AGCCTTGCGGCCGTACTCCC 3′ (Normand et al., 1996)] FGPS6 [21 mer, 5′ TGGAAAGCTTGATCCCTGGCT 3′ (Normand et al., 1996)] and FGPS505' [18 mer, 5' GTATTACCGCGGCTGCTG 3' (Normand et al., 1996)] were used. Primer numbers for rRNA genes are as per *E. coli* numbering (Embley et al., 1988) and for *nif* genes as per *K. pneumoniae* numbering (Normand et al., 1988). Positive controls consisted of DNA isolated from ACN1^{AG}, whereas negative controls were ultra pure water. PCR products were purified by the DEAE paper method (Dretzen et al., 1981).

For restriction analysis, 5 µl of the PCR product were digested with 5 U of the respective restriction enzyme in a total volume of 20 µl for 1 hour at the optimal temperature suggested by the manufacturer. The restriction enzymes *Bso*F1, *Bst*U1, *Rsa*1, *Nru*1, (New England Biolabs, USA) *Eco*R1 and *Xho*1 (Bangalore Genei, India) were used for the purpose.

Map locations of restriction sites in the rRNA region

Map locations were inferred from the known sequence of *Casuarina*-nodulating *Frankia* strain ORS020606 (GenBank accession number M 58598). For this sequence and for each enzyme, the IBI MacVector® software (IBI, New Haven, Conn.) gave the locations of the restriction sites and the sizes of the fragments expected. Fragment sizes were calculated by the log molecular weight method using Molecular Analyst® software.

3. Results and Discussion

Assay for nitrogenase activity

Thirteen trees (numbered 1–13) were sampled in the study. Ten nodules were taken from each tree and were numbered 1 to 10. Thus each nodule was assigned an individual number; for example, nodule # 10 from tree # 1 was numbered 1–10 and nodule # 5 from tree # 3 was numbered 3–5.

The nitrogenase activities for the nodules are presented in Fig. 1. Nitrogenase activities for trees 6, 10, 11, 12 and 13 were very low, so they are not shown here.

It was found that significant variability existed in the ARA values among trees. This is seen from ARA values of different trees (Figs. 1 and 3). A "tree effect" was apparent here because each tree had its own range of nitrogen

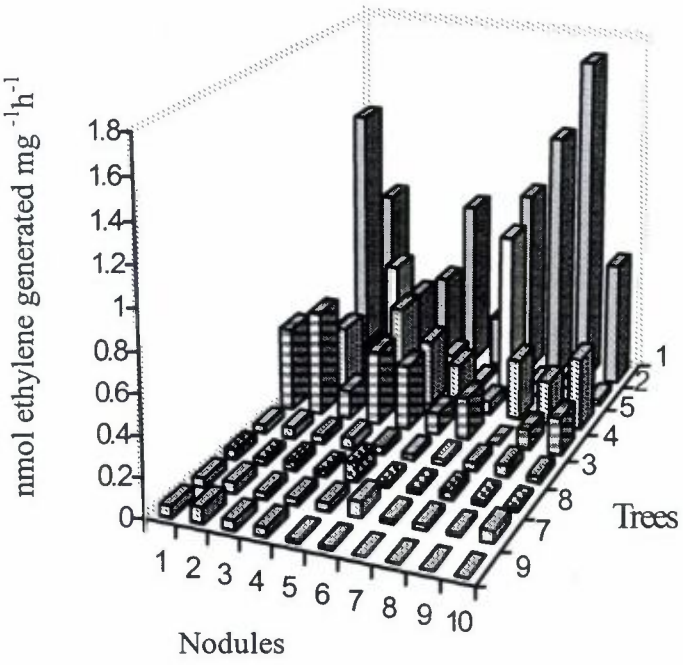

Figure 1. Nitrogenase activities of nodules from different trees. Thirteen trees were sampled from a site in Shillong, India. Ten individual nodules were taken from each tree. Trees with no detectable nitrogenase activity have been omitted.

fixation rates. Natural forest stands of alder would be genotypically heterogeneous since it is an open-pollinated tree.

Variability also existed within single trees. Significant intra-tree variability was found in case of trees 1, and 2 (Fig. 1). This may be due to the presence of different *Frankia* strains as other conditions including the genotype of the host were constant. However, micro climatic differences can not be ruled out.

DNA isolation, amplification and restriction digestion

We used a relatively rapid method for isolating DNA that minimized the amount of actinorhizal plant nodule tissue. Sufficient DNA was obtained from a single nodule to yield a visible 20-kb band on an agarose gel. Combined use of ascorbic acid and polyvinylpyrrolidone ensured near complete removal of plant phenolics, which was crucial because the amount of plant tissue used for DNA

isolation was small, and obtaining a clean preparation was important. Ascorbic acid had been previously used by Benson et al. (1996), who had standardized the protocol for isolation of DNA from a single hypha.

Frankia specific amplification of the distal part of the 16S rRNA gene

The distal segment of the 16S rRNA gene was amplified using primers FGPS989ac and FGPS1490'. Primer FGPS989ac (Bosco et al., 1992) is *Frankia* specific and amplifies DNA from alder and *Casuarina* host specificity groups. In our experiments, the expected 521 bp band was obtained confirming the presence of *Frankia* in the nodules. The amplification product was digested with restriction enzymes *Rsa*I and *Bso*F1, which yielded five distinct restriction patterns (data not shown). Previous reports indicated that the distal region of 16S rRNA in *Frankia* was highly variable. The variability in this region is indicative of differences in genomic groups (Nazaret et al., 1991; Harry et al., 1991; Bosco et al., 1992).

Amplifications of the middle part of the 16S rRNA gene

The middle segment of the 16S rRNA gene was amplified using primers FGPS485 and FGPS910' (Normand et al., 1996). Amplifications yielded the expected 415-bp band. That the band had originated from *Frankia* was tested by searching for *Bst*U1 and *Eco*R1 sites in the amplicons. These sites are absent in plant chloroplast DNA. When the amplified fragments were treated with the two enzymes, digestion was observed (data not shown), though a part remained undigested. It was presumed that the amplification products had originated both from *Frankia* as well as from the plant DNA. Subsequently the rest of the DNA was amplified under more stringent conditions (higher annealing temperature of 65°C, and reduced primer, DNA and *Taq* polymerase concentrations), the aim being to decrease the chances of plant DNA amplification. Single bands were obtained and amplified length polymorphism were seen (Fig. 2A). Two kinds of bands were observed, a 415-bp band and a 330-bp band. However, in some cases there were other minor bands also. Thus the amplicons were purified by eluting the band of interest before restriction analysis. On treatment with the enzyme *Bst*U1, different restriction patterns resulted (Figs. 2B and 2C). The 415 bp band was cleaved to 350, 44 and 16-bp respectively (Fig. 2C). The 330 bp band was cleaved to 190 and 140 bp. In some cases there was no digestion (lane 6, Fig. 2C). Thus RFLP analysis of this region revealed some variability. Although RFLP analysis of the middle segment of 16S rRNA gene is not reported in literature, sequencing results of the total gene have shown that this region is relatively conserved. However,

differences do exist. These differences are relatively rare and may indicate differences in genomic groups. Therefore, it is possible to detect polymorphism by restriction analysis of the middle segment of 16S rRNA gene as well as of other segments of the gene.

Amplification of the proximal region of 16S rRNA gene

The proximal region of the 16S rRNA gene was amplified using primers FGPS6 and FGPS505' (Normand et al., 1996) and the expected 540 bp band was obtained (Fig. 2D). However, a single restriction pattern was observed when different samples were digested with *Alu*1. Two bands having band lengths 280 and 250 bp respectively were seen (lane 4, Fig. 2D). Comparative sequence analysis from different *Frankia* strains revealed that there was no *Rsa*1 site in the proximal region of the 16S rRNA in *Frankia*, whereas the chloroplast 16S rRNA gene is known to have *Rsa*1 site in this region. That the amplicons had originated from *Frankia* DNA was shown when restriction digestion with *Rsa*1 did not result in any digestion (lane 3, Fig. 2D).

nif H-D IGS region

The *nif* H-D IGS was probed with primers FGPH750 (Simonet et al., 1991) and FGPD826' (this study). Instead of the expected single band, a pattern of multiple bands were seen. The pattern differed slightly in different strains and nodules (Fig. 2E). Some bands, like the 400-bp, 250-bp and 89-bp, were present in all the samples tested. By scanning multiple aligned sequences, it was found that *nif*H primer FGPH750 had partial sequence homology with many sites in the *nif*D gene also. Similarly partial match sites for *nif*D primer FGPD826' were present in the *nif*H gene also.

Presence of such repeats in *Frankia* DNA is interesting and is useful for studying DNA polymorphism in closely related strains. Amplification of such regions can generate genomic fingerprints for strains. Sequencing can reveal more variability.

Another way of scanning for differences would be the use of specific restriction enzymes that may differentiate between bands that look similar on the agarose gel. We selected the *nif*H-D region patterns that were identical on the gel and subjected them to digestion with the enzyme *Xho*1. This enzyme has a single site in the distal end of *nif*H and in the proximal part of *nif*D. Therefore, it was expected that *Xho*1 would generate one or more bands. The results indicated that most of the DNAs lacked the restriction sites except some which showed distinct cuts in all bands (lane 4, Fig. 3F). Probably some of the smaller amplicons were contained in the larger fragments.

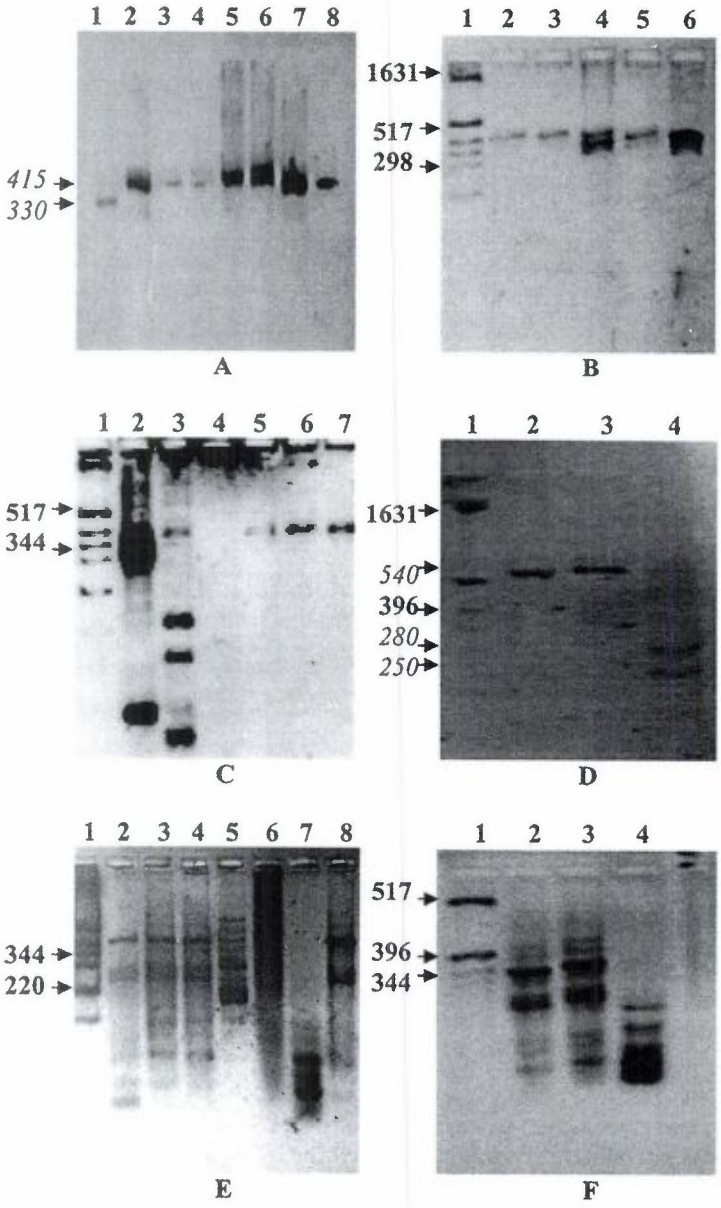

Figure 2. Amplification and restriction analysis of DNA isolated from different nodules of *Alnus nepalensis*. Molecular weights of marker bands are shown in base pairs. Molecular weights of bands referred in the text have been shown in italics. The photographs were generated using BioRad GelDoc1000 and Molecular Analyst software.

In the final analysis, nine PCR-RFLP groups were obtained on the basis of the above results (Table 1).

Table 1. The distinguishing features of the nine groups obtained in the present study

Groups	Restriction patterns				AFLP patterns	
	*Bso*F1	*Rsa*1	*Bst*U1	*Xho*1	in middle 16S	in *nif* region
1	BF1	RI	BU1	X1	AI	NI
2	BF1	RI	BUII	X1	AI	NI
3	BFII	RII	BU1	X1	AI	NI
4	BFII	RII	BU1	XII	AI	NI
5	BFIII	RI	BU1	X1	AI	NI
6	BFIII	RI	BUIII	X1	AII	NI
7	BFIII	RI	BU1	X1	AI	NII
8	BFIV	RI	BU1	X1	AI	NI
9	BFV	RI	BU1	X1	AI	NI

RFLP/AFLP groups revealed by a combination of restriction patterns of *rrn* and *nif* regions are shown. Respective patterns are given numbers I, II, III etc. For instance, restriction pattern I obtained by use of *Bso*F1 is denoted as BF1, pattern II obtained by use of *Rsa*1 is denoted as RII and so on.

Figure 2. Continued.
A: AFLPs obtained during amplification of the 16S rDNA, using primers FGPS 485 and FGPS 910'. Lanes 1 to 8 – amplified DNA from different nodules. B: *Bst*U1 digestion of amplicons obtained as in A). Lane 1 – molecular weight standard, lane 2 – undigested control, lanes 3 to 6 – amplified DNA from different nodules treated with *Bst*U1. The smaller bands in lanes 3 and 5 could not be captured here since the initial quantity of DNA was small. C: *Bst*U1 digestion of amplicons obtained as in A). Lane 1 – molecular weight standard, lanes 2 to 6 – Different amplicons treated with *Bst*U1, lane 7 – undigested control. The DNA in lane 4 may have been under loaded and thus not visible. D: Restriction digestion of DNA obtained by amplification using primers FGPS 6 and FGPS 505'. Lane 1 – molecular weight standard, lane 2 – undigested 540 bp amplicon, lane 3 – amplicon digested with *Rsa* 1, lane 4 – amplicon digested with *Alu* 1. E: Amplification of nodule DNA using *nif* primers FGPH 750 and FGPD 826'. Lane 1 – molecular weight standard, lanes 2 to 8 – amplified DNA from different nodules. F: Restriction digestion of *nif* H-D IGS amplification products. Lane 1 - molecular weight standard, lane 2 – untreated control, lane 3 – amplicon 1 digested with *Xho* 1, lane 4 – amplicon 2 digested with *Xho* 1.

Relation between ARA values and PCR-RFLP groups

The differences in the mean ARA values for different groups were tested statistically using 't' test for each pair of groups. Attempts were then made to establish the relationship, if any, between the observed nitrogenase activities and the PCR-RFLP patterns.

Nitrogenase activities of nodules from individual trees

As far as nodules from a single tree were concerned, significant differences in ARA values were observed in case of nodules originating from Tree 1 and Tree 2. Fig. 3 presents the means of ARA values of nodules from different trees and their PCR-RFLP patterns. In case of Trees 1 and 2, the nodules that showed differences in ARA values did not show any variability in PCR-RFLP patterns. The variation in nitrogenase activities could have been due to one or more of the following reasons:

i. the genetic heterogeneity of the plant material,
ii. the differences in the age of the different nodules,
iii. the differences in effectiveness of the bacterial strains,
iv. the irregular degeneration of the nodules, and
v. the variations in peak enzyme activity/its duration.

Variability between trees with respect to the ARA values and the PCR-RFLP grouping

Fig. 1 shows that different trees possessed nodules that had widely varying nitrogenase activities. Nevertheless each tree exhibited a specific range of ARA values. Apparently this was due to the genotypic differences in the trees. The within tree variations could have arisen because of the differences in the size and age of the nodules and other micro climatic factors.

When each nodule was examined for the *Frankia* strain by PCR-RFLP analysis, it was found that nodules with high as well as low activity fell in the same PCR-RFLP group. As far as nodules from a single tree were concerned, the nodules that showed differences in ARA values did not show any variability in PCR-RFLP patterns, except in two cases. By applying student's 't' test, it was seen that there was no significant difference in the mean ARA values from two different PCR-RFLP groups. At the same time, trees differing in ARA values possibly hosted the same strain of *Frankia*. It appeared that the same strain of *Frankia*, when nodulating different trees, displayed different nitrogenase activities. This suggested that the genotype of the host profoundly influenced the nitrogen fixation rates of nodules.

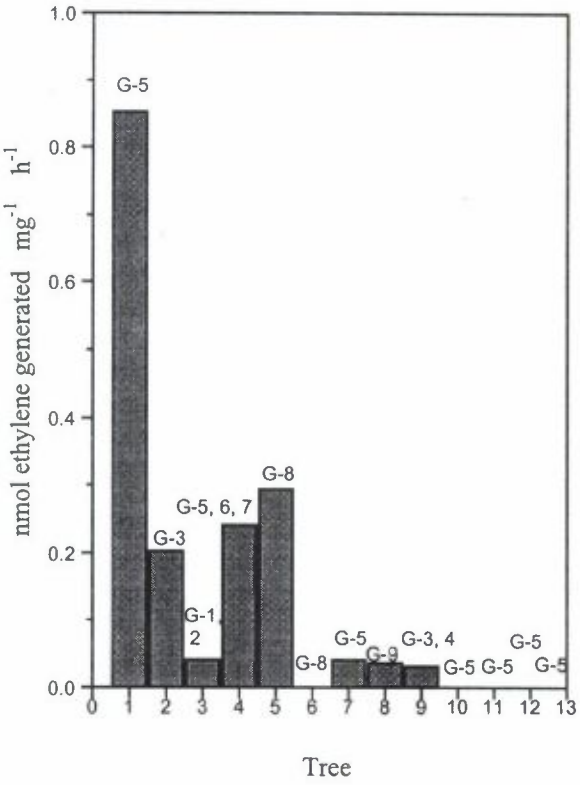

Figure 3. Average ARA values and PCR-RFLP groups obtained for different trees.

It appears that plant-microbe symbiotic interactions are influenced at various levels by many factors. These elements, either in isolation or together, exert their influences on either or both of the symbiotic partners. Nitrogen fixation is overwhelmingly a mutualistic endeavor. The photosynthetic activity of the plants is linked together with the nitrogen fixation activity of bacteria to reduce dinitrogen. There are pressures on both the partners to continue in the symbiotic state though on the surface it may appear that the microbe is more committed towards symbiosis. There is a division of labour between the two organisms, a careful orchestration of which permits optimum benefits to both. Our knowledge of the plant genes involved in symbiosis is fragmentary, as is our information about *Frankia* genes and their expression. We can at best glance at the morphological and physiological features of some of the steps involved and try to extrapolate it to the composite endosymbiotic system.

The symbiotic relationship between *Frankia* and actinorhizal trees is an outcome of mainly three components: the host genome, the *Frankia* genome, and other elements.

The host: The host genotype controls nodule morphology, physiology and nitrogen fixation (Sprent et al., 1987). Kondorosi et al. (1977) showed that *Rhizobium* infection of pea root cells was affected by changes in the cell wall chemistry to the extent that some strains produced effective nodules whereas others produced ineffective nodules on the same host plant. Similar results were obtained in some species of *Alnus* by Dawson and Sun (1981). Ganesh (1993) found a single *Frankia* strain to be highly active in symbiotic condition, as compared to other strains. This strain had low activity in culture but when different host genotypes were nodulated, the nitrogenase activity *in situ* was high. Sougoufara et al. (1992) selected a combination of high and low nitrogen fixing *Frankia* strains and tested them on three *Casuarina* host clones. They discovered that a certain host clone always produced a high level of nitrogen fixing nodules irrespective of the strain used. Similarly, there was a host clone, which always produced the lowest activity nodules.

The microsymbiont: *Frankia* is a direct player in the process of nitrogen reduction. Obviously, the strains that have an efficient nitrogenase system should produce more fixed nitrogen compared to others when all other factors are constant. Ganesh (1993) found that one of his isolates, AnpST11, showed a higher average nitrogenase activity in the symbiotic condition over a random range of host genotypes.

Frankia-host combinations: The microbe and plant may show complete compatibility as far as establishment of infection is concerned, but the resulting association may not provide optimal benefit to either partner. The physiological activity of the two partners must have a say in determining functional compatibility. Both partners can influence the physiology of the other. The host species x *Frankia* strain combination can vary in the degree of effectiveness in nitrogen fixation. Each *Frankia* strain-host combination is thought to be specific. Performance of some host-strain combinations has been evaluated (Monz and Schwintzer 1989; Reddell et al., 1988).

Other elements: The roles of pH of the soil, soluble phosphorus, calcium levels, available nitrogen, conductivity of water, time of collection, climate, light availability, canopy cover, are crucial in determining the outcome of any symbiotic association (Quesada et al., 1997; Han and New, 1998). These factors may influence the host and the microbe individually or may influence the interaction between them. Other factors that may similarly influence the outcome of symbiosis are age of the tree, age of the nodule and presence of other microbial flora in the vicinity of the nodule.

Since we did not find any specific relationship between the strain of

microsymbiont present in the nodule and the nitrogenase activity of the nodule, it would be prudent to conclude that nitrogen fixation in nodules is an outcome of several factors playing a complex role in tandem. A major factor appears to be the host genotype. In support of this, we found that while both Tree 1 and Tree 7 harboured the microsymbiont belonging to the same molecular marker group (G-5), the least activity nodule from Tree 1 had a higher activity compared to the most active nodule of Tree 7. Tree 1 showed a higher average nitrogenase activity compared to others. This could mean that the genotype of Tree 1 supported higher nitrogenase activity in general. Hence our contention that the host genotype is one of the major factors contributing to the level of nitrogenase activity.

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