Electron Microscopy and Molecular Biology of *Frankia*

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(Abstract)

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The present investigations were taken up in the summer of 1992 in order to study some structural and molecular aspects of *Frankia*.

Frankia is a nitrogen fixing , microaerophilic, mesophilic actinomycete which forms symbiotic association with a large number of woody dicotyledonous plants called the actinorhizal plants. These actinorhizal trees play a great role in forest productivity, giving their association with *Frankia* a special significance. Actinorhizal plants include species of *Alnus*, *Casuarina*, *Coriaria*, *Elaeagnus*, *Myrica* etc. From among these, alder (*Alnus glutinosa*) and casuarina (*Casuarina equisetifolia*) were chosen for isolation of *Frankia* in pure culture. Root nodules were collected from casuarina trees growing in Madras, Rameswaram, Thiruchirapally, Thiruvananthaputam etc. while alder nodules were collected from Shillong, Arunachal Pradesh, and Darjeeling. Some of these nodules were used for isolation and rest were stored at -20^{o}C for further studies.

Different media were tried to isolate *Frankia* from root nodules but only Q_{mod} and DPM gave satisfactory results. A novel technique of isolation of *Frankia* directly in the alginate beads was designed and successfully employed in this study. This method is faster and reduces the chances of contamination.

For the confirmation of the identity of the isolated organisms, the isolates were used for nodulation tests. The percentage of nodulation was 55-60%. Plants without young and developing root hairs didn't nodulate. The symptoms of *Frankia* infection were root hair curling, hooking branching etc. Prenodule was seen after 2-3 weeks which developed into brownish nodules (1-1.5 mm in diameter) after 4-5 weeks.

All the isolates showed nitrogenase activity when grown in N_2-free DPM. Among the isolates, CeDI1 showed its superiority in nitrogen fixation as demonstrated by acetylene reduction assay. The value is significant even at $p>0.01$ level.

The isolates of *Frankia* both from alder as well as Casuarina root nodules, when grown under standard culture conditions and studied with SEM and TEM, exhibited cushion like colonies with short wide hyphae.In addition to typical sporangia, intercalary elongated sporangia like structures (SLS), which could be disrupted into spore like units, were also seen. While vesicles were seen *in vitro* for isolates obtained from casuarina root nodules, no vesicles were found inside the nodules. Vesicles were seen *in vivo* in alder nodules.

Hyphal diameter ranged from 0.65 to 0.7µm *in vitro* while it was 0.53 to o.85µm *in vivo*. The mean length of vesicles in different isolates ranged from 2.07 to 2.47µm and diameter ranged from 1.33 to 1.47µm. The sporangia length and diameter ranged from 9.1 to 10.3µm and 3.66 to 4.28µm respectively.

Ultra sections of chemically fixed cells of *Frankia* showed the presence of 'void area' which possibly represented the lipid lamellae. These may act as nitrogenase enzyme protection mechanism.

Presence of sporangia *in vivo* is a rare event. Nodules collected from different parts of India were screened for sporangial occurrence but *in vivo* sporangia were found only in the nodules collected from Hyderabad.

Frankia is known to contain high G+C percentage in its genomic DNA. Nucleoside analysis on PepRPC column through FPLC of four endophyte isolates gave G+C values of around 70 mol%. Isolates under study were indistinguishable by their G+C contents. However, published reports have indicated a general range of G+C mol% for *Frankia* to be in the same range. This high G+C mol% may provide *Frankia* better radio protection.

For total DNA isolation of *Frankia* isolates, enzyme induced techniques for lysis were tried. It was found that 10mg/mL of lysozyme supplied with a pinch of achromopeptidase gave best results. A successful lysis followed by standard phenol, chloroform extraction and ethanol or iso-propyl alcohol precipitation gave a good yield.

For a comparative study, DNA from field collected nodules too was isolated. Use of lysozyme and achromopeptidase followed by lauryl sarcosine gave satisfactory results. But for DNA amplification, an extraction buffer supplemented with CTAB was found to be suitable, though the amount of DNA yield was less.

PCR amplification of a part of 16S rRNA gene with a *Casuarina* and *Alnus* compatible *Frankia* specific probe further proved the identity of the isolates, while amplification of *nif*D and *nif*K primers confirm the presence of *nif*D and *nif*K genes in the genome of the isolates.

To compare the isolates at the molecular level, restriction digestion with *Nci*1 and comparison of RFLP pattern of amplified *rrn* regions was done. The isolates and nodules of Arunachal Pradesh yielded similar pattern.

In concluding, the following points are highlighted:

1. *Frankia* isolates from nodules of casuarina and alder were obtained.

2. A rapid technique for isolation through Ca-alginate beads was developed.

3. Nodulation tests, morphological and anatomical studies under SEM and TEM, G+C mol% content and DNA amplification using *Frankia* genus specific probes for 16S rRNA gene and *nif* genes confirmed the isolates to be *Frankia*.

4. One of the isolates had statistically significant higher nitrogenase activity compared to the reference strains.

5. Marginal morphological variability was seen amongst the isolates.

6. No variability at the molecular level could be detected using restriction enzyme *Nci*1 for distal region of 16S rRNA gene.