ISOLATION AND CHARACTERIZATION OF FRANKIA FROM ROOT NODULES OF *CORIARIA NEPALENSIS*,A NON-LEGUME IN THE MURREE FORESTS

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ABSTRACT

Frankia CN5 isolated from *coriaria nepalensis* Wall. was found comparable to some of its previously reported strains in morphological and physiological characteristics. The isolate produced reddish brown to whitish brown pigmentation on solid medium. Though Qmod medium proved to be the best for isolation but it showed maximum growth on P+N and Tween-80 media, in 28 days. In the absence of combined nitrogen, the strain CN5

fixed nitrogen showing acetylene reduction assay (ARA) and was found highly sensitive to higher concentrations of NaCl (500mM in yeast extract-dextrose medium). This strain gave the infective and effective results for nodulation on the root system of *C. nepalensis*. The light microscopy revealed the occurrence of septate and branched hyphae bearing sporangia and vesicles.

INTRODUCTION

Biological nitrogen fixation (BNF) is restricted to the procaryotic microorganisms such as certain cyanobacteria, eubacteria and actinomycetes. *Frankia* spp., members of actinomycetes, form nitrogen fixing nodules on the roots of non-legumes including mostly perennial shrubs and trees. Nodulation plays an important role by which major portion of nitrogen fixed is transferred to the host plants.

The identification of genus *Frankia* as a member of actinomycetes was initially based on ultra-structural studies of the endophytic material (backing 1970). Later on, the classification was confirmed by the description of *Frankia* pure cultures (Callaham et al. 1978; Quispel and Tak 1978). *Frankia* generally grows in the form of branched septate hyphae with terminal and/or intercalary sporangia and vesicles. The first description of pure *Frankia* culture was from Pommer (1959) but the successful isolation of the endophyte was achieved from *Comptonia peregrina* by Callaham et al. 1978. *Frankia* is considered an actinomycete if 1) it is able to induce an effective symbiosis with its host plant, 2) it is capable of vesicle and/or sporangium formation and 3) it can fix nitrogen (Baker et al. 1980 and Hahna et al. 1988). The shape of vesicles and the presence of spores can often be influenced by the host plants and existence of overlapping cross inoculation groups as well as non-infective *Frankia* strains making host specificity as inappropriate criterion of classification (Lichevalier 1984). At present, the possibilities are investigated of using DNA homologies (Normand and lalonde 1986 and Bloom et al. 1989); nif gene restriction patterns (Benson and Hahna 1983) and isozymes (Gardes et al. 1987); in combination with infectivity on different plants for the taxonomy of *Frankia* spp. A large number of isolates have been obtained from the actinorrhizae of *Alnus* (Baker et al. 1979 and Hafeez et al. 1984); *Casuarina* (Iqbal et al. 1986); *Eleagnus* (Baker and O'Keefe 1984) and *Hippophae* (Burgers et al. 1984). Shipton and Burrgraaf (1983) reported that the growth of *Frankia* isolates ceased between-18 to-27 bars. The isolates of *Frankia* from *Casuarina* and *Allocasuarina* and that from *Purshia trident* are more tolerent to NaCl than isolates from species not normally growing under sodic conditions. This provides optims that these strains could establish in saline soils if introduced with any species of host plants that could tolerate these soils (Dawson and Gibson 1987). An ultimate criterion of authenticity for *Frankia* is its ability to form effective root nodules on respective host plant. Some non-infective strains like CN-4 (Chaudhry and Mirza 1987) were isolated from *C. nepalensis* but untill now no isolation of effective and infective strain has been reported from this host. The present study was, therefore, aimed at isolation and characterization of *Frankia* strain from root nodules of *C. nepalensis* growing in the Murree forest areas.

MATERIALS AND METHODS

A survey was carried out to find out the extent of nodulation on the root system of *C. nepalensis* growing in different forest areas of Murree. The individual plants were dug out carefully with the help of spade and hoe and the whole root system was washed thoroughly to remove the soil particles for counting the

number of nodules per plant.

ISOLATION

Actinorrhizal nodules of *C. nepalensis* collected from Chitta More and Jhika Gali were used for isolation of endophyte. These were surface sterilized with 0.1% HgCl2 for 2-3 minutes and then washed (2-3 times) with sterile distilled water. The surface sterilized nodules were cut into small pieces by sterile surgical blade and incubated indiviudually in the tubes contianing 7-10 ml of Qmod or P+N media at 28° ± 1C. The outgrowths attached to the cut exposed surfaces of the nodule pieces were visible within 1-2 weeks. The outgrowths were tested for their actinomycetous nature (*Frankia*) and purity by observing under the light microscope after dilution and subculturing (Lalonde and Calvert 1979; Hafeez et al. 1984).

A preliminary assessment of the isolate (flocky, sticky, settled at the base or adhering to the sides of the tubes) was made visually. Pigmentation was also observed. The diameter and shape of colonies were measured by culturing the isoalte on Qmod and P+N solid media. Necessary Photographs were taken to compare with other strians like CAQPI,CAQP2, Anl and An2. To study the three morphological forms i.e. septate hyphae, vesicles and sporangia, light microscope fitted with camera (Nikon, Japan) and differential phase contrast photo-microscope was used.

NITROGENASE ACTIVITY

Nitrogenase activity of pure strain (CN5) was measured by ARA reduction assay (Hardy et al. 1968). The colonies were taken in air tight tubes from which 10% air was replaced with acetylene and then incubated at 37°C for one hour. Gas samples were taken from these tubes and analysed on FID system of gas chromatograph (Hitachi, model-163) for the production of ethylene. The carrier gas (N2) flow rate was precisely maintained at 40 ml/min. at column,temperature of 50 ± 0. 2°C. Pora pack R,pakcked loop steel column, 1.5m long and 3mm in ID were used for the separation.

GROWTH EXPERIMENTS

The isolate was cultured on different media (Tween-80, yeast extract-dextrose, P+N, Qmod and P-N) by inoculating two months old culture. All the inoculated tubes containing 10ml/tube of the respective media (20 tubes for each medium) were kept at 28°C. After 21-and 28-days, the colonies were harvested by centrifugation at 2100g for 3-5 minutes and washed 3 times with 50mM phosphate buffer (pH7.0). The contents of base soluble proteins were determined according to Moss and Bond (1957).

SENSITIVITY TO DIFFERENT SALT (NaCl) CONCENTRATIONS

Isolate was cultured on yeast extract-dextrose medium modified to contain different salt concentrations (0,50,100,150,200 and 500mM). Tubes containing 10ml medium were inoculated with 0.2 ml of one month old culture and incubated at 28°C in the dark. 21-days and 35-days old cultures were harvested by centrifugation at 2100g for 3-5 minutes and washed 3 times with phosphate buffer (50mM,pH7.0). The protein profile was determined according to Jefferey and Gibson (1987).

INFECTIVITY TRIALS

Pre-soaked seeds (24 hours) of *C. nepalensis* were surface sterilized (0.2% HgCl2 for five minutes) and rinsed 2-3 times with autocalved distilled water. Seeds were then

transferred to petridishes and allowed to germinate in the incubator. Hoagland solution of 1/4th strenght was used to moisturize the whatman filter papers.

After a week's germination the petridishes were separated in two groups A and B.The inoculum (prepared according to Burgraaf and Shipton 1981) was added to the seedlings in the petridishes in group A while group B served as control The seedlings were allowed to grow in dark for one more week before transplanting into sterile sand and gravel cultures. Sand cultured seedlings were placed under two conditions (a) Sunshine (b) growth room (light intensity 7000 lux, temperature 25 ± 1^{0}C.) In each assembly 3 weeks old seedlings (45 from group A and 15 from B control) were transplanted into pots. In all the 3 systems nitrogen free Hoagland (1/4th strenght) solution was replaced with fresh one after every week. Pre-inoculated (Group A) seedlings were again inoculated with four weeks old isolate (CN5). A preliminary assessment of nodulation and harvesting was made 8 weeks and 13-weeks respectively after transplanting.

RESULTS AND DISCUSSION

The suvery of the various forest areas of Murree revealed that the maximum number (91.4%) of nodulation was found on plants growing at Chitta More and minimum (40.0%) at Lower Topa (table 1).

Nodulation in *Coriaria nepalensis* growing in various areas of Murree hills forest.

Site	Plants		Nodulation (%)
	Examined	Nodulated	
	(No.)	(No.)	
Murree-Road Kohalla	15	10	66.7
Chitta More	35	32	91.4
Jhika Gali	25	18	72.0
Lawrence College Road	9	5	55.6
Lower Topa	5	2	40.0

The variation in nodulation may be due to the distribution of different infective *Frankia* strains and soil composition.

The compact flocky outgrowth was cultured and recultured for purifying the strain (CN5). The colonies turned light brown after 3-4 weeks in the liquid Qmod while on the solid medium of P+N these were reddish brown; whereas on Qmod Agar the colonies were brown at the periphery with whitish brown centre, On the solid medium the colonies ranged in diameter from 2-8mm within one month. The isolate was found to contain all the presumptive *Frankia* features such as branched, septate hyphae, vesicles and sporangia, by light and phase-contrast microscopy. The sporangia were both terminal and intercalary. These were irregular in shape on old cultures and a large number of bursted sporangia with non-motile spores were also observed (table-2).

The isolate obtained on liquid medium (Qmod) after 2 weeks of inoculation produced compact flocks adhered to the nodule pieces settled at bottom of the tubes. However, it produced reddish brown to whitish brown pigmentation on the solid medium. The colonies were transparent at the periphery and dense at the centre which may be due to the presence of a large number of vesicles, sporangia and spores. These findings are in close agreement with Baker et al. 1879; Hafeez et al. 1984 and Chaudhry and Mirza 1987. CN5 showed all the characteristic features of *Frankia* isolates like branched, septate hyphae, irregular sporangia and spherical vesicles as previously reported by many workers (Chaudhry and Mirza 1987; Iqbal et al. 1986 and Hafeez et al. 1984).

Table - 2 *Frankia* Strain CN5 from *Coriaria nepalensis* showing host source, cultural characterisitics and maintenance medium.

Characteristics	Explanation
Host Plant	*Coriaria nepalensis* Wall.
Sporangia	Irregular,rounded,pear shaped or club shaped
Vesicles	Showing spherical vesicles
Pigmentation	Reddish brown or whitish brown
Isolation medium	Qmod liquid
Isolation time	1-2 weeks
Activity	Acetylene reduction
Nodulation	Nodulate the *C.nepalensis* Wall.
Colony diameter	2-8mm on solid medium within two months
Hyphae	Branched and septate

When the isolate was grown on nitrogen free medium (P-N) having sodium propionate as the C source it showed ARA at the rate of 89.59nmoles C_2H_4 proteins mg.-1hr-1 clearly establishing the nitrogen fixing activity and potential of the strain.

The isolate showed moderate acetylene reduction rate thereby indicating the presence of moderate number of vesicles. This view supports the hypothesis that vesicles are the site of nitrogen fixation like heterocysts in Cyanobacteria (Tisa and Ensing 1987). The ARA of CN5 confirmed that it is a nitrogen fixer (Diem et al. 1982; Akkremans et al. 1984 Zhang et al. 1984).

The growth yield of the isolate measured after 21- and 28- days of inoculation, based upon the protein contents of the colonies harvested by centrifugation at 2100g for 3-5 minutes, is indicated in table -3.

Table - 3. Growth yield of *Frankia* on different media after 21 and 28 days

Media	Growth yield in mg proteins/L after	
	21 days	28 days
Qmod	11	38.00
Tween-80	19	45.00
P+N	8	38.00
P-N	7	23.00

Value are the mean of five determinations

The above table clearly shows that the maximum growth (19.00 mg/l) is the Tween-80 and the minimum in P-N media after 21 days. However, the increase in growth rate after 28 days was two and a half times higher than that after 21 days. This may be due to the fact that the surfactants like Tween-80 and triton-100 act as moderate membrane solublizers and affect the functioning of protein annulus lipids in the cell membrane (Helerius and Simons 1975). The lower concentrations of these compounds might act in enhancing the uptake of nutrients by changing the lipid fluidity of the plasma membrane (Burgers 1984).

The growth of CN5 was found to increase (6.00 mg proteins/L) by 50mM NaCl as compared to control; whereas the higher concentrations i.e. 100, 150 and 200mM decreased it progressively. The highest concentration of 500mM NaCl in hibited considerably the growth of *Frankia* (table 4).

Table 4. Growth yield of *Frankia* on nitrogen supplimented medium after 21 and 35 days at 29°C at 0, 50, 100, 150, 200 and 500 mM NaCl

Concentration Grade	Growth yield in mg Proteins/L after	
	21 days	35 days
OmM	26.00	29.00
50mM	32.00	35.00
100mM	24.00	27.00
150mM	22.00	20.00
200mM	12.50	8.00
500mM	0.35	0.35

Values are the average of five determinations

This pattern of growth yield represents the extent of sensitivity of NaCl. The *C. nepalensis* isolate grew well as or almost with 100mM of NaCl as it did at OmM, while the higher concentration i.e. 150mM retarted the growth. The highest concentration (500mM) completely hindered it as reported by Jeffery and Gibson 1987.

After 13-weeks of inoculation, the nodules were visible on the root system of *C. nepalensis*. The nodules on the plants grown in gravel culture and sun shine sand culture were more prominent and bigger in size as compared to those of growth room sand culture. At the harvest, none of control plant showed nodulation. The nodulation percentage among different cultures varied. It was 24% in growth room sand 60% gravel and 64% in sun shine sand cultures. This strain could be considered as true endophyte after satisfying Koch's postulates (Koch 1982). The plants were dark green in sun shine sand culture as compared with other two cultures indicating some sort of correllation between photosynthesis and nodulation activities in the plants.

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