

## ISOLATION OF FIVE *FRANKIA* STRAINS FROM ACTINORHIZAL NODULES OF *CASUARINA GLAUCA*

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### Abstract

Five strains of *Frankia* isolated from root nodules of *Casuarina glauca* collected from different localities of Islamabad were characterized by the occurrence of branched and septate hyphae bearing sporangia and vesicles typical of *Frankia*. The strains showed marked differences in their growth requirements, growth rate, nitrogen fixing ability, production of pigments and infectivity of host seedlings. CAQP1 and CAQP2 strains infected *C. glauca* seedlings while CAQP3, CAQP4 and CAQP5 failed to induce nodules on the host.

### Introduction

Microsymbionts that are responsible for the formation of actinorrhizae have been classified in the genus *Frankia* of actinomycetes. The first success in isolation of *Frankia* and its cultivation *in vitro* was achieved in *Comptonia peregrina* by Callaham, *et al.*, (1978). Since then a number of *Frankia* strains have been isolated from the actinorrhizae of *Alnus* (Baker, *et al.*, 1979. Hafeez *et al.*, 1984), *Casuarina* (Diem *et al.*, 1982), *Colletia* (Akkermans *et al.*, 1984), *Hippophae* (Burggraaf, *et al.*, 1981) and *Myrica* (Baker, *et al.*, 1981). Diem, *et al.*, (1982) have isolated 5 *Frankia* strains from root nodules of *Casuarina equisetifolia* by serial dilution technique. Gauthier, *et al.*, (1981) reported the isolation of 2 actinomycete strains from *C. equisetifolia* which reduced  $C_2H_4$  *in vitro* but were unable to nodulate the host seedlings. Later Diem, *et al.*, (1983) reported successful isolation of an effective nodulating strain of *Frankia* from *Casuarina* spp. In Pakistan *Casuarina glauca* and *C. equisetifolia* are common species that have been introduced of which *C. glauca* is quite common in Islamabad. The present studies report the isolation and characterization of 5 *Frankia* strains from *C. glauca*.

### Materials and Methods

*C. glauca* root nodules collected from different localities of Islamabad were used. Nodules surface sterilized in 0.1%  $HgCl_2$  were crushed into suspension, 1 g nodule/10 ml sterile distilled water from which serial dilution of  $10^{-2}$  –  $10^{-4}$  were spread over QMOD solid medium, 0.2 ml/Petri plate. The dishes were incubated at 28-30°C. The colonies of *Frankia* were visible in 3 weeks. The isolates were purified by repeated subculturing from isolated colonies by dilution and plating. Five pure strains viz., CAQP<sub>1</sub>, CAQP<sub>2</sub>, CAQP<sub>3</sub>, CAQP<sub>4</sub> and CAQP<sub>5</sub> were separated and propagated in liquid and solid media.

Surface sterilized nodules were cut into small pieces and inoculated on QMOD agar slant. After 2 weeks of incubation the apparently axenic nodule parts were transferred into tubes containing 10 ml of liquid QMOD medium and incubated at 29°C. *Frankia* colonies attached with the cut exposed surfaces of the nodule pieces were visible within 3 weeks. Flocks of *Frankia* were disintegrated by passing through a syringe needle (26G x 11mm) and purified by subculturing onto QMOD and NH<sub>4</sub>-propionate medium (Lalonde & Calvert, 1979).

Isolates cultured on QMOD, P + N (Propionate + NH<sub>4</sub>Cl) and P – N (Propionate -NH<sub>4</sub>Cl) medium (Hafeez, *et al.*, 1984) were harvested by centrifugation and washed once with phosphate buffer (50 mM, pH 7.0). One month old cultures of the isolates were inoculated with 0.1 ml (base soluble protein) of washed cells in Erlenmeyer flasks, containing 50 ml of medium. The content of base soluble protein was determined according to Moss & Bond (1957).

One month old culture of each strain was washed twice by centrifugation at 2700 rpm for 5 min with 50 mM phosphate buffer, pH 7.0 and homogenized by forcing through a syringe needle, 26G x 11 mm. Eight week old seedlings of *C. glauca* were inoculated by immersing the roots in nitrogen free solution containing *Frankia* isolates and grown in test tubes (Gibson, 1963) filled with ½ strength nitrogen free Hoagland solution (Hoagland & Arnon, 1938) and modified Leonard's bottle jar assemblies (Vincent, 1970). In sand culture experiment the seedlings were inoculated with suspension of washed, homogenized 1 month old culture by adding it in sand near the roots of the seedlings. Nodulation was recorded 3 months after inoculation.

Nitrogenase activity of pure cultures of *Frankia* and of the nodulated plants was determined by acetylene reduction assay. Cells of *Frankia* isolates grown in stationary Erlenmeyer flasks with P – N medium were harvested and 5 ml of concentrated cells taken in 16.6 ml air tight tubes with rubber stopper and incubated with 10% acetylene at 29°C. After 1 h 0.1 ml of gas samples were taken from each tube and analyzed chromatographically on Hitachi, 163 gas chromatograph (Hardy, *et al.*, 1968).

## Results

**Morphology:** Strains CAQP<sub>1</sub>, CAQP<sub>2</sub> and CAQP<sub>4</sub> were pigmented with distinctly pinkish red orange, red and pink colonies respectively, while strains CAQP<sub>3</sub> and CAQP<sub>5</sub> were non-pigmented. Growth of the colonies on solid media was slower than in liquid media. Colonies of *Frankia* did not cause turbidity in the liquid medium and remained undisturbed even when shaken. The strains grew and remained at the bottom of the test tube in QMOD medium, while in NH<sub>4</sub>-Propionate and Tween-80 medium the colonies were sticky and adhered to the walls of the test tube. The hyphae were septate and branched. The vesicles were spherical, formed terminally on short parental hyphae. The sporangia of different sizes with irregular shape were always formed terminally.

Table 1. Yield\* and nitrogenase activity of *Frankia* isolates.

<i>Frankia</i> isolates	Yield			Nitrogenase activity**
	P - N	(mg protein l <sup>-1</sup> )* P + N	QMOD	(nmoles C <sub>2</sub> H <sub>4</sub> mg <sup>-1</sup> protein hr <sup>-1</sup> ) P - N
CAQP <sub>1</sub>	4.19 ± 0.52	8.95 ± 0.86	16.84 ± 1.54	323 (278-380)
CAQP <sub>2</sub>	3.72 ± 0.31	6.59 ± 0.55	14.70 ± 1.33	166 (150-179)
CAQP <sub>3</sub>	6.00 ± 0.70	9.16 ± 1.05	22.50 ± 1.81	60 ( 42- 90)
CAQP <sub>4</sub>	5.40 ± 1.10	7.44 ± 0.81	17.70 ± 1.21	35 ( 29- 45)
CAQP <sub>5</sub>	3.20 ± 0.58	1.90 ± 0.48	12.88 ± 1.29	200 (180-230)

\*After 21 days of growth. Values are mean of 5 determinations.

\*\*Values are mean of 5 determinations. Maximum and minimum values are given in parentheses.

*Growth yield on various media:* All the 5 isolates showed growth on Propionate medium that lacked combined nitrogen (Table 1). Maximum growth of P - N medium was observed in strain CAQP<sub>3</sub> whereas CAQP<sub>5</sub> showed minimum growth. Growth rate of all the 5 isolates was higher in QMOD medium than on P - N and P + N media.

*Nitrogenase activity:* All the 5 strains grew on nitrogen free media with propionate as carbon source and were able to fix nitrogen *in vitro* (Table 1). Strain CAQP<sub>1</sub> exhibited high acetylene reduction rate of 323 n mole C<sub>2</sub>H<sub>4</sub> mg protein<sup>-1</sup> h<sup>-1</sup> while strain CAQP<sub>4</sub> showed the lowest rate (Table 1).

*Infectivity tests:* Infectivity of *Frankia* strains tested on host seedlings showed nodule formation after 40 days of inoculation. Strains CAQP<sub>1</sub> and CAQP<sub>2</sub> were infective (Table 2) in water as well as in sand culture experiments while strains CAQP<sub>3</sub>, CAQP<sub>4</sub> and CAQP<sub>5</sub> failed to nodulate the host seedlings.

## Discussion

The morphological characteristics of all the 5 strains isolated from *Casuarina glauca* root nodules are similar to the *Frankia* strains isolated from other types of actinorhizae in having prokaryotic septate and branched hypahae, irregular sporangia and spherical vesicles. Strains isolated from other actinorhizal plants (Callaham *et al.*, 1978; Diem *et al.*, 1982) always exhibited hyphae and sporangia but vesicles appeared only on QMOD and N free medium.

Table 2. Nodulation of *Casuarina glauca* by *Frankia* isolates.

<i>Frankia</i> isolates	Water culture			Sand culture		
	No. of plants Inoculated/ Nodulated	Nodulation %	Effectivity*	No. of plants Inoculated/ Nodulated	Nodulation %	Effectivity*
CAQP <sub>1</sub>	20 (11)	55	+	20 (17)	85	+
CAQP <sub>2</sub>	20 ( 5)	25	+	20 (16)	80	+
CAQP <sub>3</sub>	20 ( 0)	—	—	20 ( 0)	—	—
CAQP <sub>4</sub>	20 ( 0)	—	—	20 ( 0)	—	—
CAQP <sub>5</sub>	20 ( 0)	—	—	20 ( 0)	—	—
Control	20 ( 0)	—	—	20 ( 0)	—	—

\*As determined by acetylene reduction assay.

Production of insoluble pigments have been reported for the isolates from *Elaeagnus* (Baker *et al.*, 1979), *Casuarina* (Gauthier *et al.*, 1981), *Alnus* (Hafeez *et al.*, 1984). The strains from *C. equisetifolia* isolated by Diem *et al.*, (1983) were non-pigmented. During the present investigation isolates CAQP<sub>1</sub>, CAQP<sub>2</sub> and CAQP<sub>4</sub> produced pigments while CAQP<sub>3</sub> and CAQP<sub>5</sub> were non-pigmented. Pigmented strains did not cause turbidity in the medium indicating the insoluble nature of the pigments.

All the 5 strains formed spherical vesicles on various media. They were able to grow in nitrogen free medium, though at different rates. The measurement of their nitrogenase activity (Table 1) confirmed that these strains were free living N<sub>2</sub>-fixers like various other *Frankia* strains (Gauthier *et al.*, 1981; Diem *et al.*, 1982). Non formation of vesicles in the medium with combined nitrogen supports the view that vesicles are involved in nitrogen fixation like heterocysts in cyanobacteria (Tjepkema *et al.*, 1980).

Theoretically the 3 strains unable to nodulate the host seedlings could not be considered as endophytes. Isolates of *Frankia* from *C. equisetifolia* did not nodulate their own host (Diem *et al.*, 1982). Lechevalier & Lechevalier (1983) considered free-living actinomycetes which produce vesicles and sporangia as members of *Frankia* even if they had no known nodule forming capacity. Failure of *Frankia* to nodulate host plant may be linked to the use of inappropriate isolation procedures (Diem *et al.*, 1983). The inability of CAQP<sub>3</sub>, CAQP<sub>4</sub> and CAQP<sub>5</sub> strains to infect host plant may not necessarily be related to the isolation techniques since similar techniques were used as has been successfully used by Lalonde *et al.*, (1981). Out of the 5 isolates strain CAQP<sub>4</sub> is pigmented and non-infective. It falls under the category "A" according to the classification proposed by Lechevalier (1984) which is based upon morphology, physiology, cell chemistry and infectivity of the host plant. However, the morphological characteristics and infectivity

of other strains are not in conformity with the categorization of Lechevalier (1984). There is therefore a need for more critical investigations for the categorization of *Frankia* isolates.

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