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FRANKIA AND *RHIZOBIUM* STRAINS AS INOCULUM FOR FAST GROWING TREES IN SALINE ENVIRONMENT

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Abstract

Frankia strains isolated from actinorhizae of 7 *Casuarina/Allocasuarina* species were screened for nodulation and nitrogen fixing ability on *Casuarina glauca* and *C. obesa* plants under controlled environmental conditions. Five out of 13 strains were found to induce root nodules on *C. glauca* with none on *C. obesa*. Similarly various rhizobial strains were screened for nodulation and nitrogen fixation on 4 *Acacia* species. *Frankia* strains CcOl and CcI3 and *Rhizobium* strains Abal, Ar2-1 and PMA63/1 were checked for sodium chloride tolerance *in vitro*. *Frankia* strain CcO1 showed tolerance for upto 500 mM NaCl concentration (Ec_e 47 dSm^{-1} approx.), whereas strain Cc13 was sensitive to even 100 mM NaCl concentration (Ec_e 9 dSm^{-1} approx.). The 3 rhizobial strains showed tolerance upto 300 mM NaCl concentration (Ec_e 28 dSm^{-1} approx.). A morphological and dry weight analysis for the initiation and development of sporangia in *Frankia* strains CcO1 and Cc13 grown on modified basal propionate medium showed increase in dry weight and number of sporangia formation.

Indigenous population of rhizobial cells was found to be very low, 2 cells g^{-1} of saline soil with an Ec_e level of 5-7 dSm^{-1}. The *Frankia* population was completely absent in the saline soil, indicating the requirement for inoculation of the host plants by their respective microorganisms.

Introduction

Salinity and sodicity of soils is a serious problem of agricuture and environment. An extensive area of arable land is excluded from cultivation due to salinity and this has greatly affected productivity. Of the 13 million hectares of salt-affected land in Pakistan, the saline soils have a high concentration of sodium chloride, high pH, low content of available nitrogen and phosphorus and very low in organic matter. Various approaches are being followed to ameliorate and economically utilize these soils. Nitrogen can be added either as fertilizer or in the form of biologically fixed nitrogen. The most effective methodology is to initiate plant succession on such soils. The use of fast growing nitrogen fixing trees (NFTs) in agroforestry can make a major contribution to sustainable agricuture by restoring and maintaining soil fertility, combating erosion and providing fuel wood (Budowski & Russo, 1997; Franco & Faria, 1997). Many *Casuarina* and *Acacia* species are reported to be tolerant to high soil temperatures and salt cocentrations (Dommergues, 1997; Doran & Hall, 1982; Laurd & El-Lakany, 1984). Some *Casuarina* and *Acacia* species have been introduced in Pakistan, but early field observations have shown that many of the trees are not nodulated in the field (Chaudhry *et al.*, 1981; Masutha *et al.*, 1997). This may be due to the absence or non-compatibility of specific strains of *Frankia* and *Bradyrhizobium* endemic in local soils.

Experiments were therefore carried out to find the most compatible strains of *Frankia* and *Bradyrhizobium* for their specific hosts for improved nitrogen fixing ability, to study their survival in the local soils under stress conditions of high salinity and to introduce the symbionts in the local soils by large scale inoculum production.

Materials and Methods

Actinorhizal and legume nodule collection: Root nodules of *Casuarina* species were collected from 3 different sites in Pakistan viz., Punjab Forest Research Institute (PFRI), Faisalabad; Bio-Saline Research Station (BSRS), Lahore; and Quaid-i-Azam University (QAU), Islamabad. Root nodules of *Acacia* species were obtained from highly saline area of BSRS, Lahore.

Isolation of the endophyte: Isolation of *Frankia* strains from the root nodules of *Casuarina glauca* was carried out by two different methods: (1) Nodules were surface sterilized with 3% osmium tetroxide (OsO_4), washed thoroughly with sterile distilled water, crushed in a drop of 0.1 M Phosphate buffer under aseptic conditions and used to inoculate Qmod medium (Lalonde *et al.*, 1981). The inoculated medium was incubated at 28-30°C and growth of actinomycete was monitored. (2) Isolation of the endophyte was also carried out by modified sucrose gradient method (Baker & O'Keefe, 1984). It involved the use of sucrose gradient of 1.0, 1.6 and 2.5 M in a Teflon centrifuge tube. The surface sterilized nodule suspension made in 0.1 M phosphate buffer was poured on top of the gradient. The gradient was run at a speed of 5,000 x g in a swinging rotor at room temperature for 20 min. Fraction at the interface between 1.6 and 2.5 M was collected and mixed in basal propionate (BAP) agar media (Meesters *et al.*, 1985), while it was still warm (35-40°C). The plates were incubated at 28-30°C, for 25-30 days and typical star shaped hyphal colonies of *Frankia* were aseptically picked singly and inoculated in BAP broth. All the strains were sub-cultured on BAP broth supplemented with sodium and potassium phosphate, Fe-EDTA and trace elements and incubated at 28°C. The strains were sub-cultured every two months. The mature and fully developed *Frankia* colonies were repeatedly passed through an 18 gauge syringe needle to separate and homogenize them before being used for sub-culturing and inoculation.

Isolation of rhizobial strains from root nodules of *A. ampliceps, A. aneura* and *A. senegal* was carried out. The nodules after surface sterilization with 0.2% $HgCl_2$ and washed thoroughly with sterile distilled water were crushed in a drop of 0.1 M Phosphate buffer aseptically and inoculated on yeast extract mannitol (YEM) agar plates (Vincent, 1970) which were then incubated at 28-30°C till the colonies appeared on the plates. For rhizobial characterization, the strains were sub-cultured on YEM media supplemented with congo red or bromo thymol blue (BTB) to monitor growth rate and acid production. The purified cultures were authenticated by their infectivity on their host plants grown in growth pouches containing N-free nutrient solution.

Nodulation studies: Seeds of *C. glauca* and *C. obesa* obtained from Australian Tree Seed Center, Canberra, Australia were surface sterilized with 0.2% $HgCl_2$, washed thoroughly with sterile distilled water and sown in sterile sand. The seedlings were irrigated with nitrogen-free nutrient solution. Pure *Frankia* strains isolated from 7 different

Casuarina spp., used in the study were obtained from the culture collection of different laboratories around the world.

The roots of one month old *C. glauca* and *C. obesa* seedlings were dipped in a suspension of *Frankia* strains for 18-24 hours and two inoculated seedlings were sown in each leornard jar assembly containing sterile sand. There were 3 replicates for each treatment. Controls without inoculation were also kept. The assemblies were watered with nitrogen-free nutrient solution and the plants were grown under controlled environmental conditions, with a 14 h photoperiod of 4,750 LUX, day and night temperature of $28 \pm 2^{\circ}C$ and humidities of 70% and 50%, respectively. Plants were harvested after 2 months of inoculation to be screened for nodulation.

Cross inoculation ability of locally isolated and exotic strains of *Bradyrhizobium* was checked on *A. ampliceps, A. nilotica, A. aneura, A. saligna* and *A. sterophylla*, in leonard jars and growth pouches. Seeds of *Acacia* were surface scarified with concentrated H_2SO_4 for 10 minutes followed by repeated rinses in sterilized water. The seeds were allowed to imbibe in the last rinse for 2 hours. Four seeds were planted and thinned to one after establishment. There were four replicates per strain. One ml broth inoculum (10^9 cell) of each strain was applied per plant. The experiment was conducted under controlled environmental conditions with a photoperiod of 4,750 LUX under 16 hours light and 8 hours dark with 28°C day temperature and 22°C night temperature and day and night humidities of 70% and 50%, respectively. Plants were harvested 6 weeks after applying inoculum and presence or absence of nodules was monitored. Data on number of nodules and their fresh weight was also collected.

Nitogenase activity: Acetylene reduction assay (ARA) of fresh root nodules obtained from *C. glauca* plants was done according to the method of Hardy *et al.*, (1968). The excised nodules were enclosed in 13 ml vacutainer tubes fitted with air tight suba seals. Using a pressure lock gas syringe (Precision Sampling Corporation), 0.1 atm of air was replaced by 0.1 atm of acetylene. Nodules were incubated for one hour at room temperature. Control tubes were kept (a) without nodules in acetylene and (b) with nodules and no acetylene. The gas samples (100 μl) were analyzed on a gas chromatograph (Carlo-Erba Model 180) fitted with a 1 mx2 mm steel column filled with Porapak R (80-100 mesh) and a H_2 flame ionization detector (FID). Nitrogen was used as a carrier gas at a flow rate of 30 ml min^{-1}.

Sodium chloride tolerance of Frankia and Rhizobium strains: The two most efficient nitrogen-fixing *Frankia* strains CcO1 and Cc13 were selected for *in vitro* NaCl tolerance studies. These strains were previously isolated from *C. cunninghamiana*. A cell packed volume of 0.5 ml of each strain was inoculated in 10 ml of BAP broth in screw capped tubes containing NaCl at a concentration of 0,100,300,500,700 and 900 mM (approx. Ec_e 0,9,28,47,66,84 dSm^{-1}, respectively). The amount of soluble proteins present at zero time i.e., at the time of inoculation was estimated by Lowry's method (Lowry *et al.*, 1951). The cultures were incubated without shaking at 28°C for 45 days and data on protein content was recorded (Table 3). Similarly, the selected efficient rhizobial strains i.e., Abal, Ar2-1 and PMA63/1 were also checked for their *in vitro* NaCl tolerance. The rhizobial cells (10^6 cells ml^{-1}) were inoculated in triplicate in 100 ml YEM broth supplemented with NaCl concentrations ranging from 0-500 mM. The

cultures were grown at 28°C with continuous shaking for 3-5 days and then the viable cell counts were made.

Effect of salinity on Frankia and Rhizobial population: Two soils at different salinity levels (Ece, 5-7 and 15 dSm^{-1}) were analyzed for indigenous populations of rhizobia nodulating *Acacia ampliceps*, as well as that of *Frankia* strains nodulating *C. glauca*, by plant infectivity test and by using most probable number (MPN) methods (Asad *et al.*, 1991).

Moreover, effect of different salinity levels, ranging from Ec_e 0.8 to 35 dSm^{-1}, on nodulation, dry weight, nitrogenase activity and rhizosphere population of locally isolated rhizobial strains Abal, Ar2-1 and exotic rhizobial strain PMA 63/1 with an initial population of $2.6x10^6$ cells ml^{-1}, was checked on *Acacia ampliceps* under controlled environmental conditions in leonard jar assemblies. Four replicates were kept along with negative controls and the observations were recorded 3 times after every 15 days.

Sporulation studies: *Frankia* strains CcO1 and CcI3 were used to study enhanced sporulation using modified BAP medium (Burleigh & Dawson, 1991a). The experiment was carried out in 25 ml screw capped glass tubes as well as in 100 ml conical flasks containing 10 ml and 50 ml of the medium, respectively. The tubes were incubated without shaking at 28°C, whereas the flasks were shaken at 100 rpm. A set of test tube samples were oven dried at 65-70°C, at 8 days interval upto 32 days. Three replicates were kept for each reading. Strains grown in the flasks were used for morphological studies as well as to compare the rate of increase in growth between shaken and unshaken cultures.

Results and Discussion

Frankia-Actinorhizal Symbiosis: Hypertrophies were observed in few plants of *C. glauca* and *C. obesa* growing at Pakistan Forest Research Institute. Only a few *C. glauca* plants growing in saline soil of BSRS had root nodules that were hard and woody. On the other hand *C. glauca* plants at QAU had enormous number of root nodules. *Frankia* strain CGQU was isolated from root nodules collected from Quaid-i-Azam University area.

The *Frankia* isolates identified and characterised on the basis of their growth on specific Qmod media showed typical star shaped colonies with characteristic thin septate hyphae, numerous microscopic rounded vesicles and a number of pear shaped sporangia (Hafeez *et al.*, 1984; Hameed *et al.*, 1994; Torry & Callaham, 1982; Zhang *et al.*, 1984). The locally isolated strain CGQU formed effective nitrogen fixing root nodules upon inoculation of *C. glauca* seedlings. Four out of 11 exotic *Frankia* strains also formed nodules on *C. glauca* plants upon inoculation, showing cross infectivity, as most of these strains had previously been isolated and characterised from different *Casuarina* species. None of the *Frankia* strains formed nodules on *C. obesa*. The local isolate CGQU formed highest number of nodules but its nitrogenase activity was very low as compared with the nodules induced by exotic *Frankia* strains CcO1 and CcI3 showing higher nitrogenase activities (Table 1).

Table 1. Screening of *Frankia* strains for effective nodulation on *Casuarina glauca*.

Frankia Strains	Nodulation	Nodule No. Plant^{-1}	Nodule fresh wt.(mg Plant^{-1})	C_2H_2 reduction activity (μmol.g^{-1} fresh nodule wt.h^{-1})
LOCAL				
CGQAU	+++	13	140.0	1.7
CGBSRS	-	-	-	-
EXOTIC				
CM21	-	-	-	-
CM22	-	-	-	-
CcL-17	-	-	-	-
Alll-1	-	-	-	-
CcO1	++	9	106.0	36.0
Cc13	+++	11	110.0	4.1
Ce24	-	-	-	-
53024	-	-	-	-
Ino-3593	-	-	-	-
JCT-287	+	4	30.0	- ND -
F-49	+	6	37.0	- ND -

- = Absence of nodules, + = 5 to 10 nodules, ++ = 10 to 20 nodules, +++ = > 20 nodules, ND = not determined.

The exotic strain CcO1, showed high salt tolerance *in vitro* (Table 3), of upto 500 mM NaCl concentration (Ec_e 47 dSm^{-1} approx.) whereas strain Cc13 was sensitive to even 100 mM NaCl concentration (Ec_e 9 dSm^{-1} approx.). The locally isolated strain CGQU could not survive even under very low NaCl concentrations. Miettinen (1993) have also reported that *Frankia* strain isolated from *C. equisetifolia* was tolerant to salt level of only 16 dSm^{-1} under free living condition. *Frankia* strain CcO1 grown on modified basal propionate medium, in an effort to enhance sporulation (Fig.1a,b), resulted in an increased total biomass within a given period of time. Enhanced *Frankia* growth of nearly 13% in terms of increase in dry weight was observed. Enhanced *in vitro* sporulation in *Frankia* strain Cc13 was also reported by Burleigh & Dawson (1991b). High nitrogenase activity and better hyphal biomass *in vitro* at high salt concentrations, suggests *Frankia* strain CcO1 to be a better endophyte for *C. glauca* plants.

Rhizobium-Legume Symbiosis: Isolations of the rhizobial endophyte made from the root nodules of *Acacia ampliceps, A. senegal* and *A. aneura* were characterised on the basis of growth rate as well as acid or alkali production in the medium. The strains isolated from *A. ampliceps* Abal, Aba2 and Ar2-1 showed maximum growth within 72 hours of incubation producing large amounts of exopolysaccharides in the medium and turned the BTB medium colour yellow due to their acid producing characteristic. These strains were therefore, grouped as fast growing *Rhizobium* strains. Milnitsky *et al.*, (1997) and Zou *et al.*, (1995) have also isolated fast growing *Rhizobium* strains from *A. ampliceps*.

Table 2. Screening of locally isolated and exotic (*Brady)rhizobium* strains for nodulation in different *Acacia* species.

Strains	*A. ampliceps*		*A. aneura*		*A. nilotica*		*A. saligna*		*A. sterophylla*	
	Nod.	Wt.	Nod.	Wt.	Nod.	Wt.	Nod.	Wt.	Nod.	Wt.
Exotic										
14631.1	8	20	0	0	5	12	0	0	14	20
19631	15	30	0	0	0	0	13	10	9	10
NA2538	0	0	0	0	7	10	24	40	20	130
Amm1	9	16	0	0	7	8	0	0	0	0
TAL1436	8	18	ND	ND	3	4	ND	ND	ND	ND
TAL1446	7	13	ND	ND	9	15	ND	ND	ND	ND
TAL1521	8	15	ND	ND	8	10	ND	ND	ND	ND
PMA63/1	18	32	ND	ND	5	12	ND	ND	ND	ND
PMA311/1		9	9	ND	ND	0	0	ND	ND	ND
Local										
Aa2	0	0	10	20	0	0	0	0	20	20
Abal	14	30	0	0	0	0	0	0	32	30
Aba2	9	21	ND	ND	0	0	ND	ND	ND	ND
AS1	10	30	0	0	6	8	0	0	2	10
AS2	3	1	00	0	8	14	0	0	10	50
AS3	0	0	0	0	8	10	6	2	0	0
Ar2-1	20	36	ND	ND	5	6	ND	ND	ND	ND

Nod. = Number of nodules plant^{-1}

Wt. = Fresh weight of nodules (mg plant^{-1})

ND = Not determined

Strains isolated from *A. senegal*, AS1, AS2 and AS3, as well as isolated from *A. aneura* Aa2 reached at maximum growth after 120 hours, produced lesser amounts of exopolysaccharides and turned the medium blue, indicating them as slow growing *Bradyrhizobium* strains. All the exotic strains listed in Table 2 were characteristically slow growing *Bradyrhizobium* strains, except strain PMA 63/1.

Infectivity tests on 5 *Acacia* spp., by locally isolated and exotic Bradyrhizobial strains showed nodule induction in *A. ampliceps* and *A. nilotica* by most of the strains tested. No nodulation was observed on *A. aneura* except by a local isolate Aa2. Two exotic and one local isolate formed nodules on *A. saligna* (Table 2). Nodule induction and cross inoculation among wide range of rhizobial strains suggest that the microorganisms can be effectively utilized as inoculum for a range of *Acacia* species in the field to increase soil fertility.

The three rhizobial strains Abal, Ar2-1 and PMA63/1 showed tolerance of upto 300 mM NaCl concentration with maximum increase in viable cell count of approximately $3x10^9$ cells ml^{-1} for each strain (Table 4). The indigenous population of rhizobi-

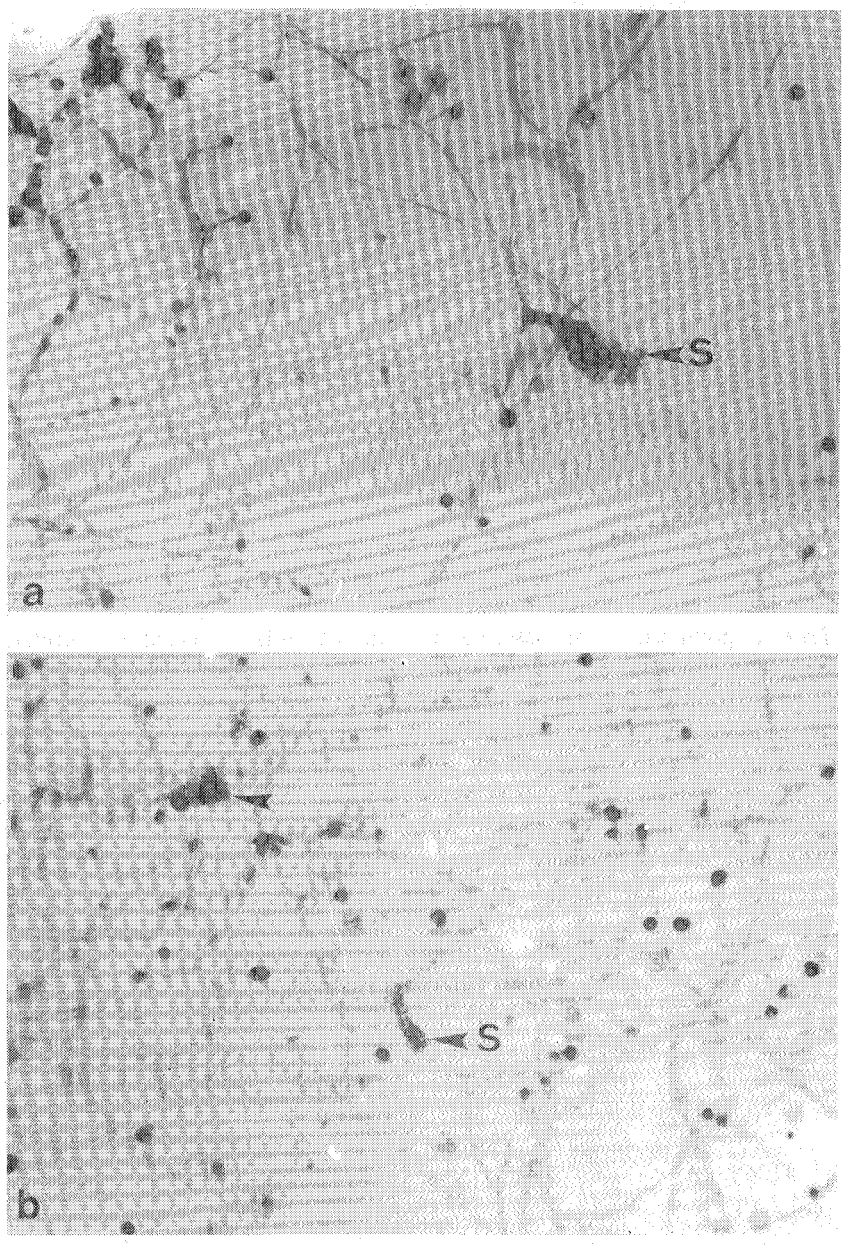

Fig.1. Photomicrograph showing colonies of *Frankia* strain CcO1, (a) typical mature sporangium (S) and (b) the presence of many young sporangia induced by modified medium (Burleigh & Dawson, 1991a). X 1800.

Table 3. Sodium chloride tolerance of *In vitro* grown *Frankia* strains.

NaCl conc. (mM)	Ec_e Level (dSm^{-1})	Total Protein increase ($\mu g\ ml^{-1}$)* CcO1	Cc13
0	0.8	7.4±0.5	2.3±0.5
100	9	7.5±0.2	0.0
300	28	8.8±0.2	0.0
500	47	9.8±0.6	0.0
700	66	0.0	0.0
900	84	0.0	0.0

*Increase in total protein content over the initial inoculation.
Mean values with standard deviation are given (N=4).

al cells estimated in 2 soils at different salinity levels was found to be only 2 cells g^{-1} for *A. ampliceps* in soil 1 with an Ec_e levle of 5-7 dSm^{-1}, whereas soil 2 with an Ece level of 15 dSm^{-1} showed complete absence of any rhizobial population. Moreover, no native *Frankia* population was detected in both the soils. A negative correlation is shown by native rhizobial population towards increasing salinity levels, indicating the need for inoculation of the legume plants in saline soils. In another experiment, the initial observations after 15 days showed higher cell counts of all the strains at salinity levels of 25 and 30 dSm^{-1}, respectively, whereas, the number decreased after 45 days for same salinity levels in sand. Local rhizobial strain Abal nodulated the host plant at lower salinity level of upto 10 dSm^{-1}, while local rhizobial strain Ar2-1 and exotic strain PMA 63/1 formed effective nodules upto 15 dSm^{-1}. Zou *et al.*, (1995) had also

Table 4. Sodium chloride tolerance of *in vitro* grown rhizobial strains.

NaCl conc. (mM)	Ec_e level (dSm^{-1})	Rhizobial strains Abal	Ar2-1	PMA63/1
0	0.8	1.7	1.06	1.4
100	9	2.0	2.1	2.1
200	18	2.1	2.4	2.3
300	28	3.3	3.6	3.5
400	38	0.8	1.5	1.1
500	47	0.7	0.4	0.5

Initial viable cell count at the time of inoculation for each strain was kept at x 10^6 cells ml^{-1}. Mean values with standard deviation are given (N=3).

reported that rhizobial strains are more tolerant to salinity than bradyrhizobia. The nodule number decreased towards 25 dSm^{-1} and no nodulation was observed at 30-35 dSm^{-1}.

The infectivity tests on *Acacia* and *Casuarina* species by their respective microorganisms indicate the presence of cross infection among various strains of Brady) rhizobium and *Frankia* on their hosts. The most compatible *Rhizobium* and *Frankia* strains for efficient nitrogen fixation, evaluated through these studies, have been used for large scale inoculum production and tested in the field on their respective host plants.

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