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Isolation, Characterization and Selection of Salt Tolerant Rhizobia Nodulating *Acacia catechu* and *A. nilotica*

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Abstract

Bacterial strains were isolated from root nodules of two tree legumes, *Acacia catechu* and *A. nilotica*, growing in barren soils in the sub-Himalayan tract. Their morphology, physiology and biochemistry was studied according to the description given for the family Rhizobiaceae in Bergey's Manual of Systematic Bacteriology (Jordan, 1984). Morphology, biochemistry and physiology of the strains was similar and indicated that they belong to the species *Rhizobium loti*, which has now been assigned to *Mesorhizobium loti* (Jarvis et. al., 1997). Strains MTCC 2379 (isolated from *A. catechu*) and MTCC 2381 (isolated from *A. nilotica*) were highly salt-tolerant. There was marked difference in nodule number, root length, shoot length, average nodule weight, plant dry weight and nitrogenase activity between salt-tolerant and salt-sensitive strains. In saline conditions salt-tolerant strains were much more effective.

Keywords: *Rhizobium*, salt-tolerance, *Acacia catechu*, *Acacia nilotica*, saline soil, nodulation, nitrogenase

1. Introduction

It has been estimated that the earth's forest and world lands annually account from 28% of the total estimated nitrogen fixation in terrestrial eco-

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systems (Burns and Hardy, 1975). A large fraction of these lands occur in arid and semiarid regions affected by saline soil. In India about 7.5 million hectares of land are saline or alkaline. These soils usually are barren and devoid of vegetation. There is an urgent need to reclaim these soils. One of the processes is by planting salt-tolerant fast growing tree legumes (Felker and Bandurski, 1981). Leguminous trees, including species of *Acacia, Albizzia, Prosopsis, Pithecollobium, Butea* and *Dalbergia,* are known to occur naturally in saline environments (Yadav and Singh, 1970; Felker and Bandurski, 1981) and are recognised to increase the fertility of soil.

There are marked differences between strains of rhizobia in adaptations to saline conditions (Yadav and Vyas, 1971; Sprent, 1984). However, in the legume-*Rhizobium* symbiosis, the microsymbiont is much more resistant to salinity than the host plant (Bhardwaj, 1975; Singleton et al., 1982; Abd Alla, 1992). Some strains of rhizobia, especially those isolated from arid lands can actually grow in solutions with salinity as high as the salinity of sea water (Singleton et al., 1982; Abd Alla and Abdel Wahab, 1995). The ability of salt-tolerant rhizobial strains to induce better nodulation under stress conditions must be explored. The present study has been aimed to investigate bacteria associated with two tree legumes namely *Acacia catechu* and *A. nilotica* growing under naturally saline conditions, particularly as they relate to salt-tolerance.

2. Materials and Methods

Bacterial strains were isolated from root nodules of *Acacia catechu* and *A. nilotica* growing under naturally barren conditions at the banks of river Ganga in the sub-Himalayan tract, in the Hardwar district, 200 km northeast of New Delhi, in the state of Uttar Pradesh (U.P.). The soil is alkaline with a pH of 7.8±0.2. The area comes under deciduous rain forests but due to high evaporation rate the soil is saline.

Bacteria were isolated from nodules according to Vincent (1970). The cultures were incubated on yeast extract-mannitol (YEM) agar at 28°C and stored on slants of this medium at 4°C. Only strains with characteristics of Rhizobiaceae (Jordan, 1984) were retained. Ten strains were isolated from root nodules of *Acacia catechu* and 15 strains from *A. nilotica.* On the basis of salt-tolerance one strain from each plant species was selected for further study. Both the selected strains were deposited in Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh (India) under the accession numbers MTCC 2379 (isolate from *A. catechu*) and MTCC 2381 (isolate from *A. nilotica*).

Isolates were tested for nodulation on their original hosts. Seedlings were grown in earthen pots (22×15 cm) containing sterilized garden soil. Pots were kept in a seed germinator at 32°C during day and at 27°C during night, with illumination of 14 hd^{-1} and watered with sterile water as per requirement. Nodulation was checked after 60 days.

Average doubling (generation) time were estimated from absorbance at 420 nm, recorded every 2 h in YEM broth at 28°C. Growth on Hofer's alkaline medium (YEM agar with pH adjusted to 11 and 1 ml of 1.6% bromothymol blue solution) was checked after inoculation with bacteria from a log phase culture in YEM. After incubation for 48 h, growth and colour change were observed. Strains were tested for their ability to grow on glucose peptone agar after a 48 h incubation period at 28°C. YEM agar plates containing sodium citrate in place of mannitol supplemented with 1 ml of 1% bromothymol blue solution were incubated for 48 h at 28°C. Ability to produce 3-ketolactose and hydrogen sulfide was determined according to the method of Gaur et al. (1973) and Zobell and Feltham (1934), respectively. Catalase and oxidase activity was determined by the methods of Graham and Parker (1964) and Kovaks (1956), respectively. All the tests were done in triplicate.

The suitability of culture media for large scale production of *Rhizobium* depends upon the efficiency of utilization of carbon source. This was tested in YEM broth in which the yeast extract was reduced to 50 mg l^{-1}. Filter-sterilized solutions of carbohydrates (80 µl of 10 % w/v solution) were added to 5 ml of medium. The medium was then inoculated by addition of 80 µl of actively growing bacterial suspension containing approximately 10^8 cells ml^{-1}. Growth was observed after incubation for 7 d at 28°C.

DNA base composition was calculated by the thermal denaturation temperature determined with a ECIL digital spectrophotometer model GS5701, as given by Marmur and Doty (1962). Extraction and analysis of cellular fatty acids was determined according to the method of Nishimura et al., 1994. Whole cells were collected by centrifugation in the late log phase (18 h), washed with distilled water and freeze dried. Cellular fatty acids were extracted from alkaline saponified materials, esterified with diazomethane and analysed by gas-liquid chromatography.

Antibiotic resistance was detected by using antibiotic discs (HiMedia, Bombay) on YEM agar plates, each inoculated with 1 ml of exponentially growing cultures in YEM broth. The resistance to an antibiotic was detected by an inhibition zone around the disc. Antibiotics used were: erythromycin, 15 µg; streptomycin, 10 µg; bacitracin, 10 µg; carbenicillin, 100 µg; neomycin 30 µg; chloramphenicol, 30 µg; ampicillin, 10 µg; doxycycline, 30 µg; penicillin G, 10 units; kanamycin, 30 µg; methicilin, 5 µg and cephalothin, 30 µg.

Study on salt tolerance

Seeds of salt-tolerant genotypes of *Acacia catechu* and *A. nilotica* were collected from a Govt. Forest Department nursery, Bahadrabad, Hardwar (U.P.), India. The seeds were sterilized by immersing in H_2SO_4 for a period of 10 min and 1 h for *A. catechu* and *A. nilotica* respectively. Seeds then were washed 10 times with sterilized distilled water and sown in 22×15 cm earthen pots, containing autoclaved garden soil. Each seedling was inoculated with one ml of log phase culture isolates (10^8 cells/ml). The soil, in these pots, was saturated by watering with saline water (500 ml of water), containing 0 to 200 mM NaCl per pot. Uninoculated plants were also maintained on water with varying NaCl concentration as control. Plants were grown at 32°C in day and 27°C during night with 14 h day light. The plants were irrigated with 100 ml sterilized water per pot each day.

Strains from *A. catechu* and *A. nilotica* were tested in pure cultures for their tolerance to different salt concentrations. Growth of selected isolates was studied in YEM broth containing 0 to 800 mM NaCl, in triplicate, in a shaking incubator at 200 rev/min and 28°C. Cells grown in YEM broth for 18 h (log phase) were used as inoculum (2% v/v, 10^7 c.f.u./ml). After 60 days the plants were harvested to analyse shoot length, root length, nodule number, average nodule weight and plant dry weight. The nodular nitrogenase activity was determined by the method of Hardy et al. (1968).

3. Results

Both of the isolates were motile, gram-negative rods of 0.5–0.9×1.2–3.0 μm, and they were commonly pleomorphic under adverse growth conditions (Sadowsky et al., 1983). After 3–5 days colonies formed on YEM agar plates were circular, convex, white coloured with low convex elevation, smooth surfaces, entire margins, mucoid and 2–4 mm in diameter. Bacterial isolates failed to grow on Hofer's alkaline medium and GPA. The strains were unable to utilize citrate. The generation time for strain isolated from *A. catechu* (MTCC 2379) was 5.8 h and for *A. nilotica* isolate (MTCC 2381) it was 6.0 h, indicating that both the strains are fast growers (Jordan, 1984). Both the isolates produced nodules with their original host. In majority of cases inoculated plants showed increase in dry weight over their uninoculated controls.

Both the strains were not able to produce 3-ketolactose, as there was no yellow ring formation, and also there was no hydrogen sulfide production, due to absence of black stain on lead acetate paper. However, both of the strains were catalase- and oxidase- positive (Table 1). Both of the strains were able to utilize D-arabinose, D-glucose, D-xylose and maltose but were unable to utilize

Table 1. Characteristics of the strains isolated from *Acacia catechu* (MTCC 2379) and *Acacia nilotica* (MTCC 2381).

Characteristic	MTCC 2379	MTCC 2381
Gram reaction	–	–
Generation time (h)	5.8	6.0
Growth on HAB	–	–
Growth on GPA	–	–
Utilization of citrate	–	–
Production of 3-ketalactose	–	–
Production of H_2S	–	–
Catalase	+	+
Oxidase	+	+
DNA base composition (mol %)	60.0	63.3

+ = positive reaction; – = negative reaction.

Table 2. Utilization of carbon sources by strains MTCC 2379 and MTCC 2381.

Carbohydrate	MTCC 2379	MTCC 2381
D-arabinose	+	+
D-glucose	+	+
Inositol	+	+
Lactose	–	+
Maltose	+	+
Mannitol	+	+
D-xylose	+	+
L-xylose	–	–
Starch	–	–
Glycerol	+	+

+ = positive reaction; – = negative reaction.

L-xylose, starch and glycerol as carbon source (Table 2). The G+C contents of DNA was 60.0 and 63.3 mol % for strain MTCC 2379 and MTCC 2381, respectively. The major fatty acids in strains MTCC 2379 and MTCC 2381, were straight chain unsaturated fatty acids of C18:1 type, 82.6% and 91.8% of the total respectively. Other fatty acids were 13:O ISO 3OH (0.188 and 0.3%),

16:O (5.7 and 3.84%), 17:O ISO (3.16 and 1.96%), 18:O (0.132 and 0.081%), 19:O 10 Methyl (0.476 and 0.0513%) and 19:O cyclo (8.12 and 0.972%) in the strains MTCC 2379 and MTCC 2381, respectively. Strains were resistant to penicillin-G, ampicillin, erythromycin, bacitracin, carbencillin, cephalothin, kanamycin, methicillin and sensitive to neomycin, streptomycin, and doxycycline. On the other hand, strain 2379 was resistant to 2381 was sensitive to chloramphenicol.

Studies on salt tolerance

Two isolates, MTCC 2379 and MTCC 2381 were highly salt-tolerant. Two other strains ACH-7 (from *A. catechu*) and ANH-6 (from *A. nilotica*) were also salt-tolerant to 200mM NaCl, but could not illicit nodule formation on respective hosts at this concentration. MTCC 2379 and MTCC 2381 were salt-tolerant in explanta to 700 mM NaCl.

Salinity reduced the total nodule number per plant, and there were significant differences between salt-tolerant and the salt-sensitive strains. Tolerant strains showed better survival, nodulation and nitrogen fixing ability than sensitive strains under saline conditions. *A. catechu* and *A. nilotica* failed to nodulate at high salinity (50 mM NaCl) when inoculated with salt-sensitive strains. However, the two hosts nodulated at this salt concentration when inoculated with MTCC 2379 and MTCC 2381, respectively. *A. catechu* showed an 87% and *A. nilotica* a 26% reduction in the number of nodules as compared to controls at 50 mM NaCl.

The relative salt induced reduction in growth of plants inoculated with salt-tolerant strains was much less in comparison to that of plants inoculated with salt-sensitive strains. However, increasing salt concentrations reduced shoot length, root length, fresh weight, dry weight (Fig. 1) and nodule number per plant.

In the nodules of *A. catechu* there was no significant reduction in nitrogenase activity with increase in salinity from 0 to 50 mM NaCl, whereas in *A. nilotica* there was a 21% reduction under the same conditions with salt-tolerant, MTCC 2379 and MTCC 2381 strains, respectively. Salt-sensitive strains showed complete loss of nitrogenase activity at 50 mM NaCl (Fig. 2).

4. Discussion

Fast growing *Acacia* species are being used in land reclamation because of their productivity, salt-tolerance, multiple uses and capacity to form nitrogen-fixing symbiosis with *Rhizobium* or *Bradyrhizobium* (Zou et al., 1995). There appear to be three distinct groups of *Acacia* species, based on their symbiosis

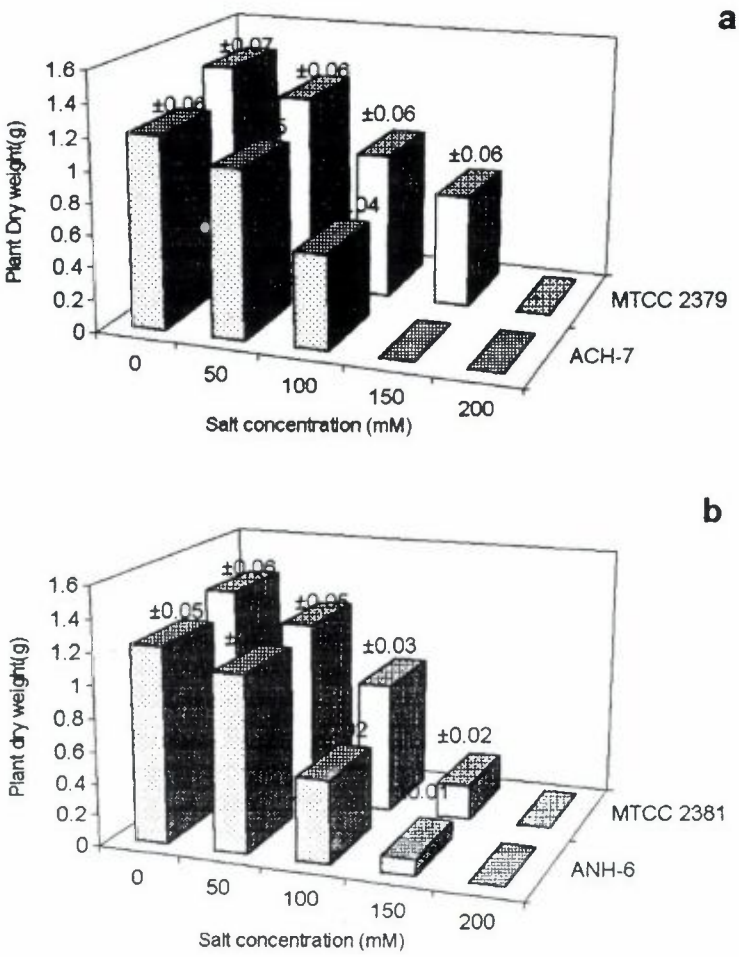

Figure 1. Plant dry weight at different salt concentrations of soil after 60 days. (a) *A. catechu* inoculated with strains ACH-7 and MTCC 2379, (b) *A.. nilotica* inoculated with strains ANH-6 and MTCC 2381.

with rhizobia: one group nodulated by *R. loti*, one group nodulated by *Bradyrhizobium* sp. (Acacia) and one group nodulated by both of these species (Dreyfus and Dommergue, 1981). *A. catechu* and *A. nilotica* being the common Acacias found in this region, root nodulating bacterial strains were isolated from the nodules of these tree legumes and characterised. Two strains were selected for detailed study on the basis of salt-tolerance. The cellular fatty acid profile of both the strains, MTCC 2379 and MTCC 2381, followed the similar pattern as described by Jarvis and Tighe (1994) for *M. loti*. The

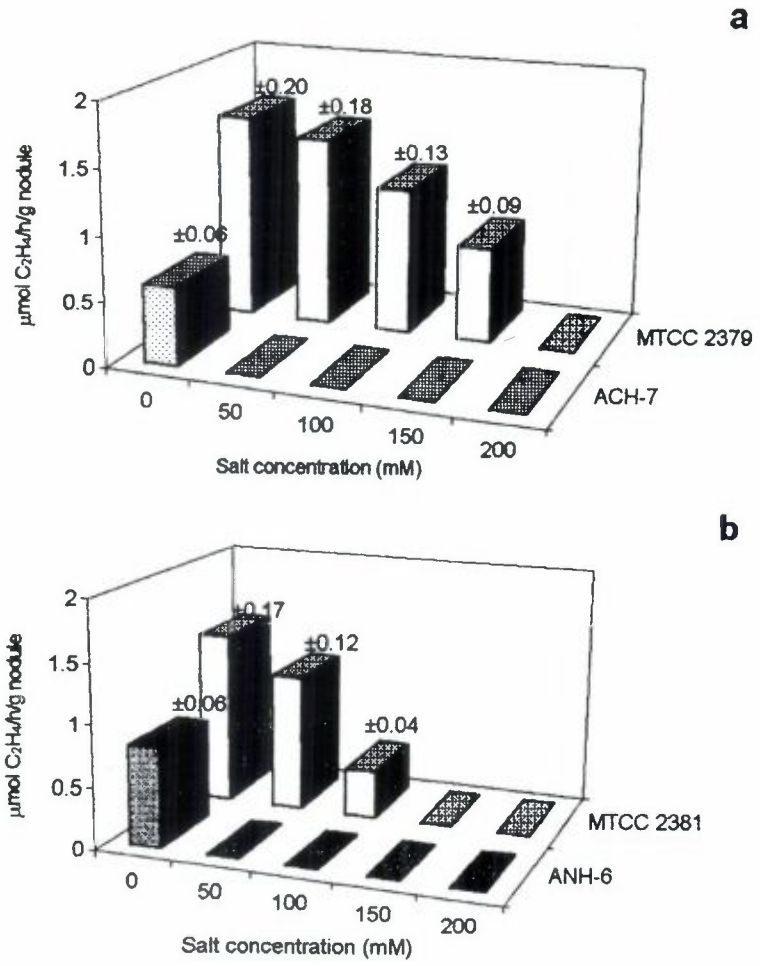

Figure 2. Nitrogenase activity at different salt concentrations of soil after 60 days. (a) *A. catechu* inoculated with strains ACH-7 and MTCC 2379, (b) *A. nilotica* inoculated with strains ANH-6 and MTCC 2381.

morphological, physiological and biochemical characteristics, of both the strains showed that they belong to the species *Mesorhizobium loti.*

Biological nitrogen fixation and fertility of saline soil can be increased by planting salt-tolerant tree legumes along with inoculation with salt-tolerant rhizobia. The adverse effects of salt on nodulation, plant growth and nitrogen fixation, have been reported for *Acacia* (Craig et al., 1991; Hafeez et al., 1988). Inoculation of tree legumes with appropriate *Rhizobium* species increases the plant dry weight (Bala et al., 1990) was proved correct by this study as the

salt-tolerant strains were much more effective in saline conditions. Salt-tolerant strains, MTCC 2379 and MTCC 2381 were tolerant to 700 mM NaCl in explanta but could not elicit nodule formation at this concentration. Recently Lal and Khanna (1995) reported that some rhizobia can tolerate salt concentration under explanta conditions upto 850 mM but could not establish symbiosis at this concentration. The results confirm the study of Singleton and Bohlool (1984) that nodule formation is a very sensitive process in effective *Rhizobium* symbiosis under saline conditions and is also affected by saline conditions to some extent.

Salt-tolerant *Rhizobium* alongwith respective tree legumes can thus be used to obtain maximum benefits of biological nitrogen fixation in reclamation of saline soils.

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