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Morphological and biochemical characterization of *Rhizobium* isolates obtained from fenugreek (*Trigonella foenum*)

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ABSTRACT Fenugreek (*Trigonella foenum graecum* L.), a seed spice, is known for its dietary protein source, medicinal properties and symbiotic nitrogen fixation by *Rhizobium* bacteria present in its root nodules. The study was carried out to characterize *Rhizobium* strains isolated from root nodules of fenugreek. The isolates were identified and characterized on the basis of colony morphology and biochemical traits *viz.* gram staining, catalase, and oxidase tests, and utilization pattern such as citrate utilization activity, nitrate reduction and methyl red test. The *Rhizobium* isolates were rod-shaped, gram negative, acid and mucous producing. They were found to be sensitive to temperature and pH, at 27°±1°C and 6.8±0.2, respectively. It utilizes starch as sole carbon source.

Key words: Fenugreek, *Rhizobium* isolates, morphological and biochemical analysis

Fenugreek is an important legume crop for consumers as a popular spice in Indian cuisine and has long been used in both Ayurvedic and Chinese traditional medicine to induce labour and laction. It aids in digestion and as a general health and wellness tonic [1]. Its crop contributes an important nutrient N for the soil. Fenugreek is also a good soil renovator and widely used as a green manure. Fenugreek was reported to fix 48% of its total N_2 during the growing season. It is also a good source of atmospheric nitrogen fixation by *Rhizobium* present in its root nodules [2]. In the present study, we have isolated isolates of *Rhizobium* from root nodules of fenugreek and studied for their characterization by morphological and various biochemical tests.

MATERIALS AND METHODS

The fresh and healthy pink root nodules of fenugreek were collected from the plants grown in field. The collected nodules were washed in running water to remove adhering soil particles. The nodules were immersed in 0.1% mercuric chloride for 5 minutes for surface-sterilization and repeatedly washed in sterile water 3-4 times. The nodules were placed in 75% ethanol for three minutes and again washed in sterile water. They were crushed in 1 ml sterile distilled water with the help of flat sterile glass rod and made the uniform suspension. Nodule extract was serially diluted by adding one ml of extract in 9 ml of sterile distilled water (10^{-1}-10^{-5}). From each dilution, 0.1 ml suspension was spreaded using sterilized glass spreader on to each plates (Triplicates) on (Yeast Extract Mannitol Agar CRYEMA with congo red dye 0.025 µg/ml), under aseptic conditions. Plates were then incubated in an inverted position at 27°±1°C in incubator and observed daily [3]. After incubation the bacterial colonies were picked in sterile distilled water and the culture was purified by taking single colony through subsequent streaking.

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Colony morphology was evaluated by streaking a loop of the initial inoculum on CRYEM agar media and incubated at 27°±1°C for 3-4 days [4]. Rhizobium colonies obtained were white, circular, translucent and glistening.

This test was performed to determine the capability of microorganism to utilize glucose as the sole carbon source for its growth. The pure bacterial culture was streaked on Glucose Pepetone Agar (GPA) medium containing (Bromo Cresol Purple (BCP) dye and incubated at 27°±1°C for 48 hr to grow. Presence was observed according to Vincent [4].

Rhizobial isolates were cultured on Yeast Mannitol Agar (YEMA) media containing Bromothymol Blue (BTB) as the acid/base indicator and incubated at 27°±1°C for 3-4 days. For an acid-producing isolates, the medium colour was changed from green to yellow, whereas with alkaline production changed from green to blue.

This test was performed to determine capability of microorganism to use starch as carbon source [5]. Starch agar media were inoculated with isolated *Rhizobium* culture and incubated at 27°±1°C for 24 hr. After 24 hr drops of iodine solution were spread on 24 hr old cultures grown on Petri- plates. Formation of blue colour indicated non-utilization of starch and *vice versa*. Iodine test was used to determine capability of microorganisms to use starch.

This test was performed to determine the presence of oxidase enzyme in bacterial isolates. Kovac's reagent (1% N, N, N^1.N^1-tetramethyle-p-phenylene diamine) was dissolved in warm water and stored in dark bottle. A strip of filter paper was dipped in this reagent and air-dried. With the help of sterile wire loop, one-day-old rhizobial colonies from agar plates were transferred on this filter paper strip and observed for production of colour according to Kovaks [6]. The oxidase positive colonies turned lavender coloured which became dark purple to black in colour within 5 min.

This test was performed to determine the presence of catalase enzyme in bacterial colonies. Forty-eight hour old *Rhizobial* colonies were taken on glass slides and flooded one drop of hydrogen peroxide (3%) and observed for liberation of effervescence of oxygen around the bacterial colonies.

Loopful culture of rhizobia were aseptically streaked on Simmons Citrate Agar slants and incubated at 27°±1°C. Appearance of blue colour on the slant as the growth of rhizobia gives positive test for citrate utilization, whereas negative means no growth of microoraganism on the slant and medium remains green.

Nutrient broth with 1% potassium nitrate in the test tubes was inoculated with the bacterial isolates and incubates at 27°±1°C for 3 days. Mixture 0.5 ml each of solution sulphuric acid and alpha naphthylamine was added to the culture broth. Development of red colour indicates the presence of nitrite in the medium. Nutrient broth without culture serves as the control.

When the pure rhizobial cultures were grown in nutrient broth for 3 days at 27°±1°C and a few drops of methyl red solution were added to the culture medium, red colour developed as the positive test and broth remain unchanged, indicating negative test.

The ability of *Rhizobium* isolates to grow on YEM broth having different concentrations of NaCl (0.1, 0.5, 1.5, 2.5, 3.5, and 4.5, and 5.5% (w/v), YEM broth was used as the rol. To determine the effect of pH on growth of *Rhizobia*, YEM broth was prepared and pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. All the broth tubes were incubated at 27°C for 48 hr and YEM broth were used as the control [7]. After inoculation on CRYEMA media, the plates were kept at 10°, 15°, 20°, 25°, 30°, 35° and 40°C to analyze the effect of temperature. The control plates were incubated at 27°C. The isolates were considered salt tolerant, resistant to acidity and temperature when growth was similar to that of the control.

RESULTS AND DISCUSSION

A total of 18 bacteria were isolated from root nodules of fenugreek, collected from semi-arid and arid regions of Rajasthan. All isolates were gram negative and rods-shaped [8]. The pathogenecity by authentication test confirmed that 15 isolates

Table 1. **Biochemical activities of *Trigonella foenum graecum* rhizobial isolates of rhizobia**

	ATFG-1	ATFG-2	BTFG-3	ATFG-4	ATFG-5	ATFG-6	ATFG-8	ATFG-9	ATFG-12	BTFG-13	BTFG-14	BTFG-15	BTFG-16	BTFG-17	BTFG-18
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization test	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+
Nitrate reduction test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl red test	-	-	-	-	+	-	+	+	+	+	+	-	+	-	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GPA with BCP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
YEMA with BTB dye	+Y	+Y	+Y	+Y	+Y	+Y	+Y	+Y	+Y	+Y	+Y	+Y	+Y	+Y	+Y

Note: +ve, Positive test; -ve, negative test; +y, colour of media changed from green to yellow (Y)

were able to nodulate the host plant. Uninoculated plants were used as the control and rest of 3 isolates did not show nodule formation. After authentication test, 15 isolates that nodulated on host plant were used for further morphological and biochemical characteristics.

All isolates showed the same colony characteristics, after 48 hr of incubation. The colonies were white, translucent, circular in shape, producing mucou, raised and 3-4 mm in diameter. Similar result was reported by Vincent [4] and Holt *et al.* [9]

All isolates showed growth in 3-4 days and turned the yeast mannitol agar media containing bromothymol blue to yellow, showing that they were acid producers [10]. All the isolates were catalase and oxidase positive as confirmed by liberation of effervescence of oxygen around the bacterial colonies and change in colour of oxidase strip, respectively. All isolates were able to utilize citrate except three isolates namely, ATFG-4, ATFG-6 and ATFG-12. All isolates gave positive nitrate reduction test. Of all isolates, except seven gave positive result of methyl red test. None of the isolates showed growth on glucose peptone agar medium containing bromocresol purple dye. Starch hydrolysis activity gave positive results for all isolates and a clear zones around the colonies were seen and colonies were with yellow colouration, whereas blue colour appeared on no growth areas. This indicates that the isolates have the potential to hydrolyze starch present in medium.

De Oliveira *et al.* [5] also observed that *Rhizobium* strains can utilize starch obtained from different sources. Biotic and abiotic stress change to soil *i.e.* soil temperature, physical and chemical composition and moisture content in soil which vary within small areas and these variations affect the populations of soil inhabitants. Therefore, differences in response towards salinity, pH and temperature are expected. It is known that salt stress significantly reduced nitrogen fixation and nodulation in legumes. Hashem *et al.* [11] proposed that salt stress may decrease the efficiency of *Rhizobium* legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand by decreasing survival and proliferation

of Rhizobia in soil and Rhizosphere or by inhibiting very early symbiotic events, such as colonization, thus directly interfering with root nodule formation. In present study, all isolates were able to grow in salt concentrations (0.1%) of NaCl (Fig. 1). However at higher concentrations, tolerant isolates decreased rapidly and only four isolates showed moderate growth at 5.5% NaCl. Similar results were reported by Abdelmoumen and El Idrissi [12], Ali *et al.* [13] and Nagales *et al.* [14]. Thrall *et al.* [15] reported that increasing salt concentrations may have a detrimental effect on rhizobial populations as a result of direct toxicity as well as through osmotic stress. The pH is an important parameter for growth of organisms. Slight variations in pH of medium might have enormous effects on growth of organism.

Our results indicated that Rhizobium bacterial cells were able to grow only at pH 6.8±0.2 and kept at 27°±1°C. Only four isolate were grown at pH 5.0 and no growth was observed in medium with pH 4.0, 9.0 and 10.0 (Fig. 2). Some Rhizobial isolates can be shown more sensitive to low pH than their host and this affects the establishment of symbiosis, limiting survival and persistence of rhizobia. The effect of temperature is varying with host species, with cultivars within species, as well as among *Rhizobium* strains [16] Optimum temperature for survival of rhizobia was 27°±1°C which serves as the control. Three isolates

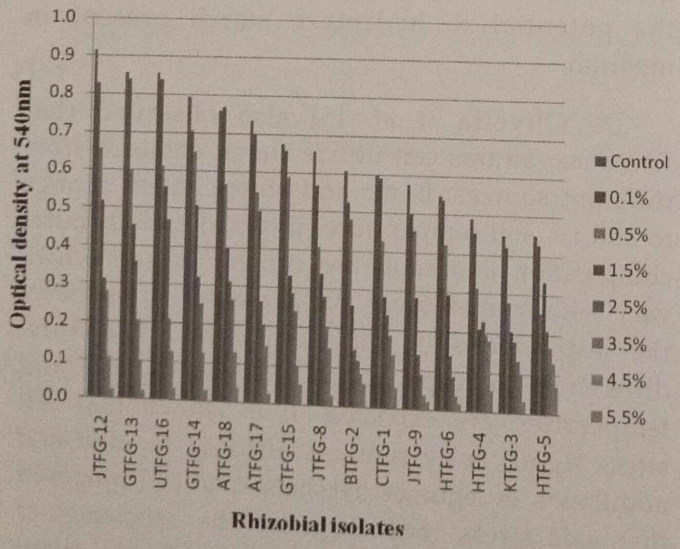

Fig. 1. Variation in growth of *Rhizobial* isolates at different NaCl concentration

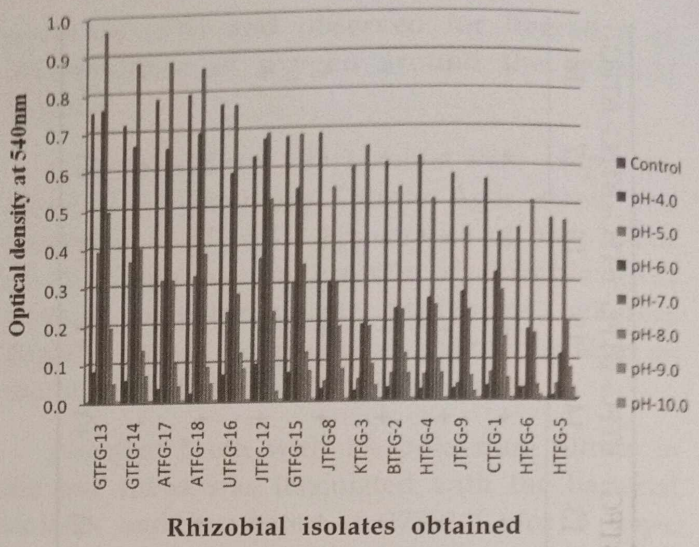

Fig. 2. Growth of *Rhizobium* isolates in different pH level

showed medium growth at 10°C, whereas remaining showed no growth. At 15°C four rhizobial isolates showed medium growth, whereas remaining ones showed no growth. At 20°-30°C maximum isolates showed growth, whereas at 35°-40°C minimum isolates showed growth.

Nevertheless, survival under higher temperatures does not mean efficiency in nitrogen fixation. Rhizobial strains obtained from hot and dry environments that grew up to 45°C lost their ineffectiveness. Screening of *R. leguminosarum* bv *phaseoli* showed that some strains were able to nodulated *Phaseolus vulgaris* at high temperature (35°C and 38°C) but nodules formed at higher temperatures were ineffective and plants did not accumulate nitrogen in shoots [17]. Thus, medium with pH 6.8±0.2 and temperature 27°±1°C are optimum parameters for growth of organisms. Similar result was also observed by Gauri *et al.* [18], Baljinder Singh *et al.* [8]; Kucuk *et al.* [19]; Baoling *et al.* [20].

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