

## ISOLATION AND CHARACTERIZATION OF RHIZOBIAL STRAINS FROM ALFALFA (*MEDICAGO* SP.)

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This study was conducted to isolate and characterize the Rhizobial strains from alfalfa (*Medicago* sp.). Of six strains isolated from alfalfa plants, only four were found capable of nodulation. They were confirmed to be *Rhizobium meliloti* by examining their growth rate, morphological characteristics, and carbon source utilization potential. A comparison among the local strains and exotic strain TAL-1373 was also made. Some of the local strains proved equally efficient as the exotic strain, TAL-1373, for their capability of nodulation, nitrogen fixation and carbon source utilization.

Key words: alfalfa, Rhizobial strains

### INTRODUCTION

Nitrogen being an essential component of proteins and nucleic acids plays an important role in improving the crop production. It constitutes 78% of the atmosphere. Only a few plants can utilize this free atmospheric nitrogen. Many higher plants can utilize N when it is available in combined form with other gases like hydrogen and oxygen, resulting from a process known as nitrogen fixation. A small amount of nitrogen is fixed naturally in the atmosphere through thunderstorm. A significant amount of nitrogen is contributed to the biosphere by industrial nitrogen fixation which of course is very expensive source because of high energy costs. Chemical fertilizers produced at the expense of natural environment require the use of fossil fuels like oil, gas etc. The depletion of these non-renewable fossil fuels is supposed to make the extensive use of nitrogen fertilizers economically not feasible and environmentally unsafe in the near future. To reduce energy costs, and for sustainable agricultural production more emphasis is being given on biological nitrogen fixation (Hardarson, 1993). Among the nitrogen fixing systems, *Rhizobium-legume* symbiosis has been recognized the most effective system. It has the potential to fix up to 300 kg nitrogen per hectare (Peoples et al., 1995) and improve the crop production limited by soil nitrogen deficiency.

*Rhizobium* inhabiting the root nodules of a legume crop is said to have a mutualistic relationship with it. If legume provides some nutrients to the *Rhizobium*, it also rewards the legume with ammonia converted from N<sub>2</sub> through an enzyme nitrogenase (Beringer et al., 1979). Rhizobia inhabiting the soil initially invaginate a root hair. Through an infection thread they migrate to the cortex and cause further nodulation, a characteristic specific to legumes. Rhizobial strains show great variation in their inoculation potential, a pre-requisite to effective nodulation. Hence, the selection and isolation of superior *Rhizobium* strains showing high nodulation under different agro-environmental conditions is considered necessary.

*Medicago sativa* (alfalfa) is the most important winter forage crop in Pakistan. Being a leguminous crop, it is cultivated in

rotation with wheat crop to improve the soil fertility through nitrogen fixation and to procure high quality forage for livestock. Different alfalfa cultivars greatly vary in their response to compete Rhizobial strains in terms of their nodulation and nitrogen fixing efficiency (Bromfield 1984; Materon, 1991). Therefore, the isolation of Rhizobial strains from *Medicago* sp. and their characterization was the main objective of this study.

### MATERIALS AND METHODS

This study was conducted at the Biofertilizer Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, for isolation and characterization of Rhizobium strains from alfalfa.

i) Isolation of Rhizobia from Alfalfa Plants: Specimens of alfalfa plants were obtained from the fields adjacent to NIBGE. Nodules were carefully detached from well washed alfalfa roots, surface sterilized by dipping in 0.1 % HgCl<sub>2</sub> for 2-3 minutes and rinsed with distilled water for 2-3 minutes. They were crushed with the help of a forceps in a drop of distilled water and the suspensions were streaked in yeast extract manitol (YEM) agar plates containing congo red (Vincent, 1970). The plates were incubated at 28±12°C for three days. Afterwards the cultures were purified by sub-culturing them on separate plates. The pure cultures were confirmed based on morphological parameters of different colonies and by gram staining technique (Rao, 1999).

ii) Re-inoculation Test for Confirmation of Isolates: A confirmatory test was performed to examine the re-inoculation capability of isolates. This experiment comprised eight treatments with three replicates and was laid out in a completely randomized design with factorial arrangement. Alfalfa seeds obtained from the Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan, were initially grown in agar media in test tubes. After germination, the plants were transferred to glass tubes having vermiculite media provided with Hoagland nutrient solution. Each treatment involved a separate rhizobial strain.

Hi) Carbon Source Utilization: Nine carbon sources viz. arabinose, galactose, glucose, raffinose, sucrose, maltose, mannitol and xylose were used as alternate carbon sources for yeast extract mannitol media (Vincent, 1970). The

concentration of a carbon source was 1% (w/v). The isolates were streaked on plates in triplicate and the presence or absence of their growth was observed after 3-5 days.

## RESULTS AND DISCUSSION

The average nodule number formed by alfalfa plants in different treatments inoculated with Rhizobial strains indicated significant differences among themselves (Table 1). The maximum number of nodules per plant was recorded for alfalfa plants inoculated with MS-I. It was significantly higher than with all other treatments inoculated with local Rhizobial strains but at par with those receiving TAL-1373 (exotic strain) inoculation. In alfalfa plants receiving MS-2,

inoculated plants, fresh nodule weight per plant was significantly less than that of TAL-1373 (exotic strain).

Nodule dry weight per plant ranged from 1.17 mg (MS-2) to 3.53 mg (MS-I). However, the differences between the nodule dry weights of MS-2 and MS-3 were non-significant. Similarly, nodule dry weight in MS-3 and MS-4 inoculated alfalfa plants also differed non-significantly.

Nitrogenase activity of alfalfa nodules ranged from 1.69 nmole C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> (MS-3) to 2.83 nmole C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> per plant. The maximum nitrogenase activity in MS-I inoculated alfalfa strains was, however, at par with those inoculated with MS-4 and TAL-1373. The intermediate position for nitrogenase activity, significantly higher than that of MS-3

Table 1. Effect of Rhizobial inoculation on growth, nodulation and N<sup>2</sup> fixation in alfalfa plants

Strains	No. of nodules/ plant	Fresh weight of nodules/ plant (mg)	Dry weight of nodules/ plant (mg)	ARA nmole C <sub>2</sub> H <sub>4</sub> h <sup>-1</sup>	ARA nmole C <sub>2</sub> H <sub>4</sub> h <sup>-1</sup> dry weight of nodules
MS-I	8 a*	5.60 b	3.53 a	2.83 a	266 e
MS-2	4 be	2.63 c	1.17 d	2.26 b	646 b
MS-3	5 b	2.83 c	1.33 cd	1.69 c	424 c
MS-4	3 c	2.67 c	1.40 c	2.83 a	673 a
TAL-1373	8 a	6.00 a	3.13 b	2.83 a	300 d
Control (un-inoculated)	0	0	0	0	0

\*: Treatment means sharing the same letters differ non-significantly.

MS-3, and MS-4 inoculation, average number of nodules per plant was significantly less than that of TAL-1373 and was recorded as 4, 5, and 3 respectively. Nevertheless, alfalfa

(minimum value), was recorded for MS-2 inoculated alfalfa plants. Similar results have been reported by Nelson (1987). Specific nitrogenase activity of alfalfa plants varied from

Table 2. Utilization of various carbon sources by different Rhizobial strains isolated from alfalfa plants

Strains	A	Gal	Glu	Mal	Man	R	S	X	Mol
MS-I	+++	+++	+++	+++	+++	+++	+++	+++	+++
MS-2	+	+	+	+++	+++	+++	+	+	+++
MS-3	+++	+++	+++	+++	+++	+++	+	+++	+++
MS-4	+	+	++	+++	+++	+++	+	+	+++
TAL-1373	+++	+++	+++	+++	+++	+++	+++	+++	+++

A: arabinose; Gal: galactose; Glu: glucose; Mal: maltose; Man: mannitol;

R: raffinose; S: sucrose; X: xylose; Mol: molasses; +: poor growth;

++: moderate growth; +++: excellent growth.

plants in the control (uninoculated) treatment did not cause nodulation. Nelson (1987) inoculated pea plants with isolates of high and low effectiveness which resulted in significantly different number of nodules per plant.

Among the treatments involving inoculation with local strains, the maximum fresh weight (5.6 mg) of nodules per plant, significantly higher than those of all other treatments was recorded for MS-I. Fresh weight of nodules per plant in MS-2, MS-3 and MS-4 inoculated plants differing non-significantly from each other was recorded as 2.63, 2.83 and 2.67 mg respectively. However, in all local Rhizobial strain

266 nmole C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> g<sup>-1</sup> (MS-I) to 673 nmole C<sub>2</sub>H<sub>4</sub> h<sup>-1</sup> g<sup>-1</sup> dry weight (MS-4) of nodules. In MS-2, MS-3 and MS-4 inoculated alfalfa plant nodules, specific nitrogenase activity was significantly higher than that observed for TAL-1373 and the differences among themselves were also significant. Nonetheless, in MS-I treated plants specific nitrogenase activity was significantly less than that of TAL-1373. These findings have been supported by Ahmadi and Yazdi (1994) and Roomi et al. (1994).

Isolates varied greatly in the utilization of different carbon sources (Table 2). The indigenous Rhizobial strains MS-I and the exotic strain TAL-1373 showed full growth in

response to all carbon sources tested in this study. MS-3 also showed excellent growth for all carbon sources except sucrose. However, MS-2 and MS-4 behaved alike and showed excellent growth in maltose, mannitol, raffinose and molasses. In glucose, MS-4 showed moderate growth while for carbon sources i.e. arabinose, galactose, sucrose and xylose both MS-2 as well as MS-4 exhibited poor growth. During a similar study, Sadowsky et al. (1983) and Anand and Dogra (1991) observed that fast growing Rhizobial strains utilized a wider variety of carbohydrates than the slowly growing strains. Hafeez et al. (1993) and Moawad and Bahlool (1993) also utilized different carbon resources as a helpful tool to characterize the isolates.

Although the Rhizobial strains isolated from alfalfa plants varied greatly in their nodulation, nitrogenase activity and utilization of different carbon sources, yet some of them were found almost efficient in terms of these characteristics as the exotic strain TAL-1373. Further exploration and characterization of efficient Rhizobial strains for practical use and introduction in different agro-ecosystems is still desired.

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