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ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF *RHIZOBIUM MELILOTI* FROM ROOT NODULES OF ALFALFA (*MEDICO SATIVA).*

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ABSTRACT

The Rhizobium a nitrogen fixing bacteria is the essential feature of leguminous plants. Increased cultivation of legumes is essential for the regeneration of nutrient-deficient soils and providing needed nutrients to humans and animals. The present study was aimed to isolate the beneficial nitrogen fixing rhizobium from root nodules of Alfalfa (*Medico sativa*) plant. Total of Fifty (50) nodules samples were collected equally from five different localities of District Quetta Balochistan, Pakistan and were subjected to culture on differential media Bromothymol Blue (BTB) added Yeast Extract Mannitol (YEM). After series of biochemical and Sugar Fermentation tests twenty Five (25) samples were identified as Sinorhizobium meliloti. The organism was present in all areas. This study confirms the presence of Rhizobia in leguminous fodder in the area.

Key words: Root nodules, alfalfa, Rhizobium meliloti, bromothymol blue.

INTRODUCTION

Soil contains many types of microorganisms such as bacteria, actinomycetes, fungi, and algae, which are important because they affect the physical, chemical, and biological properties of soil. Amongst the soil bacteria a unique group called Rhizobia has a beneficial effect on the growth of plants. It can live either in the soil or within the root nodules of host legumes. The bacteria colonize within root nodules, where it converts atmospheric nitrogen to ammonia and provides organic nitrogenous compounds to the plants. In legumes and few other plants, the bacteria live in small outgrowths on the roots called nodules. Within these nodules, the bacteria do nitrogen fixation, and the plant absorbs the ammonia (Oblisami, 2005). The soluble form of nitrite and nitrate can be assimilated by plant roots and utilized in synthesizing proteins and nucleic acids. This form of nitrogen can be converted to ammonia by plants, animals and microorganisms. Animals return nitrogenous wastes to the environment as uric acids (Atlas, 1998). Low soil pH does not allow the rhizobial cells to survive in adequate numbers in free living state. Consequently it becomes inevitable to inoculate the crop in adequate rhizobium (Deka and Azad, 2006).

Legumes are herbaceous woody plants that produce seeds in pods; examples of legumes include peas, beans, alfalfa, vetches and clovers. Alfalfa is a cool season perennial legume living from three to twelve years, depending on variety and climate of the target area. The plant has a deep root system making it very resilient, especially in drought conditions. It has been proven that plant productivity increases when the *Rhizobia* are present in rhizosphere. It provides the major biological source of fixed nitrogen in agricultural soils (http://filebox.vt.edu).

A well established practice for maintaining soil fertility has been the cultivation of leguminous plants which replenish atmospheric nitrogen through symbiosis with rhizobia in rotation with non leguminous plants. This study was aimed to isolate and identify sinorhizobium species from root nodules of the target area and to create awareness among farmers to cultivate leguminous plants for better agriculture growth.

MATERIALS AND METHODS

Study Area: This study was conducted in the district Quetta Balochistan, Pakistan located at 30^0 12' 38'' N 67^0 1'8" E and comprises approximately 2653 Km^2. The city is situated at an average elevation of 1654 meters above sea level making it Pakistan's only high-altitude major city. It is the capital of the province inhabiting more than 2.5 million populations.

Procedure: Total of Fifty 50 fifty samples (Ten from each location) of root nodules from alfalfa (*medico sativa*) were collected randomly from five different localities of Quetta viz, Killi Sheikhan, Sabzal Road, Brewery Road, Sariab Road and Spini Road Quetta (Table-1). Healthy root nodules were washed with tape water thrice before streaking on agar plate as described by (Ben-Gweirif *et al.,* 2005). The nodules were sterilized externally using 95 % alcohol for 1-4 minute, followed by washing with calcium hypochlorite solution (10g/150ml distilled water) and crushing in a drop of

sterile water. A loopful ground material was transferred to 5 ml of sterile water, of which 0.1 ml sample was spread onto the surface of Yeast Extract Mannitol Agar (YEMA). Plates were then incubated at 28^0C for 48 hours. Well isolated typical single colonies were restreaked on freshly prepared YEMA plates in order to obtain pure cultures.

Morphological Characteristics: Circular, Raised with smooth edges and musky odor of the colony were observed under low power microscope. Similarly using Gram staining technique as described by (Arora, 2003) pink colored Gram negative rods were observed.

Biochemical Tests: All the collected samples were processed through different biochemical tests viz, Catalase Test, Indole Production Test Methyl Red Test, Vogas Proskauer Test, Citrate Utilization Test as described by (Lowe,1962) and Starch hydrolysis Test, Gelatin liquefaction Test and Motility Test as mentioned by (Arora, 2003) and ONPG Test (O-Nitrophenyl-D-Galacto-Pyranoside) as described by (Cappuccino, 2007).

Sugar Fermentation Tests: The isolates were also examined for fermentation of the various sugars including Glucose, Mannitol, Galactose, Raffinose, Trehalose, Mannose, Xylose, Cellebiose. One percent 01% aqueous stock solution of the test sugars was prepared in small tubes while for sialicin 4% sugar solution was prepared and sterilized as mentioned by (Hugh, 1953).

Growth on Bromothymol Blue (BTB) added Yeast Extract Mannitol (YEM) Medium: The YEM medium was enriched with BTB @ (25 µg /ml) to selectively identify rhizobium meliloti as quoted by (Vincent, 1970). All the samples were subjected to grow on BTB added medium. The positive samples showed moist and gummy colonies after incubation for 48 hrs at 28°C and surrounding medium plate were yellow due to acid production by the Sinorhizobium meliloti.

Statistical analysis revealed that there is non significant association ($p > 0.05$) among different locations of Quetta for the occurrence of rhizobium spp in the root nodules of alfalfa.

RESULT AND DISCUSSION

Out of fifty (50) samples of root nodules from alfalfa (*medico sativa*), twenty five 25 were found positive for the presence of Sinorhizobium meliloti, after screening through a series of various biochemical and Sugar fermentative tests.

Twenty five (25) samples were characterized biochemically as Sinorhizobium meliloti. Gram Negative rods with circular, Raised and smooth edges colony with musky odor were observed. These findings are in line with Hussain *et al., (*2002); Oblisami (2005) who also isolated the Sinorhizobium meliloti from alfalfa with same Characteristics.

All the positive samples were also streaked on Bromothymol blue (BTB) added Yeast Extract Mannitol (YEM) selective media for further confirmation. Similarly the positive samples from all target areas showed hazy appearance in the motility media and also were positive for Catalase, motility and O-nitro phenyl-β-D-Galacto-pyranoside (ONPG) Tests. The samples were found negative for Methyl Red (MR), Voges-Proskauer (VP), Indole, Citrate utilization test, Hydrogen Sulphide production, Urea hydrolysis tests and Gel liquefaction tests (Table-2). Our these findings are in close agreement with Elsheikh and wood (1989); Javed and Asghari (2008) who also characterized the rhizobium from soil and sunflower root nodules with the same positive biochemical tests. Similarly Oblisami (2005) also studied the nodulation pattern in forage legume bacteria by screening through the same tests results and Singh *et al., (*2008) characterized rhizobium strain from the roots of *Trigonella foenumgraecum*.

Table-1- Isolation of rhizobium meliloti from root nodules of Alfalfa from different locations of Quetta.

Sr No	Area	No of Samples	Positive	Negative
1	killi Sheikhan	10	6	4
2	Sabzal Road	10	5	5
3	Sariab Road	10	4	6
4	Brewery Road	10	5	5
5	Spiny Road	10	5	5
	Total	**50**	**25**	**25**

Table-2- Biochemical characteristics of Rhizobial isolates from alfalfa in Quetta, Pakistan.

Sr. No	Tests Performed	Results
1	Catalase Test	Positive
2	Motility Test	Positive
3	ONPG (O-nitro phenyl-β-D-Galacto-pyranoside)	Positive
4	Methyl red Test	Negative
5	Voges-Proskauer (VP)	Negative
6	Indole Test	Negative
7	Citrate utilization Test	Negative
8	Hydrogen sulphide Production	Negative
9	Urea hydrolysis Test	Negative
10	Gel liquification Test	Negative

The positive samples were also subjected to various Sugar fermentation tests and were found positive to Glucose, Galactose, Mannose, Trehalose, Xylose,

Raffinose, Cellibiose and Mannitol confirming the bacterial specie (Table-3). These findings corroborate with the results of Oblisami (2005); Michael (2006); Singh (2008) and Erum (2008) who also reported these sugar tests positive during isolation and characterization of rhizobium meliloti.

It can easily be concluded from this study that soil of different location of Quetta City is equally suitable for the rotational cultivation of alfalfa and other leguminous fodder. There is dire need to create awareness among agriculture farmers to cultivate certain leguminous fodders in the area that will not only improve their socio-economical status but will also be helpful to put on pedestal the national economy.

Table-3-Sugar Fermentation tests for identification of Sinorhizobium meliloti from the root nodules of alfalfa.

1	Glucose	Positive
2	Galactose	Positive
3	Mannose	Positive
4	Trehalose	Positive
5	Xylose	Positive
6	Raffinose	Positive
7	Cellibiose	positive
8	Mannitol	Positive

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