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ISOLATION, SCREENING OF SYMBIOTIC NITROGEN FIXING RHIZOBIUM BACTERIA FROM DIFFERENT RHIZOSPHERE SOILS OF LEGUMES

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ABSTRACT

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. Totally i isolated 30 strains of Rhizosphere soil microorganisms and further characterized by using different methods to screen to the Biological nitrogen fixing microbes and using different legume plants soils. Main aim of this study is to use as biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or to soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants.

KEYWORDS: Rhizobium bacteria, Symbiotic Nitrogen fixation, Rhizosphere, legume plants, etc.

INTRODUCTION

Soil is a very good Nutrient media for the growth of different variety of microorganisms. 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.[3] 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.[1] 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.[2] 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.

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.

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or to soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants. Very often microorganisms are not as efficient in natural surroundings as one would expect them to be and therefore artificially multiplied cultures of efficient selected microorganisms play a vital role in accelerating the microbial processes in soil. Use of biofertilizers is one of the important component of integrated nutrient management, as they are cost effective and renewable source of plant Nutrients to supplement the chemical fertilizers for sustainable agriculture.

MATERIALS AND METHODS

1. Collection of soil Sample

Collect the different rhizospheres soil sample and different plants root nodules of legume plants from the different area for the isolation Nitrogen fixing bacteria rhizobium. Different soils like it varies depending on the colour and area some black, red and sandy soils. The different root nodules of the legume plant like chick pea, ground nut, methi, soya etc for the isolation Nitrogen fixing bacteria i,e Rhizobacteria.

2. Isolation and screening of rhizobium bacteria by using different Methods

a) Isolation of Nitrogen fixing Rhizobium Bacteria

Collected rhizoshere soil and sample and different root nodules for the isolation of rhizobium bacteria. These samples are serially diluted and further inoculated on to the different cultural media for the growth of rhizobium bacteria. Collected rhizospere soil and healthy pink coloured root nodules from the legume plants are washed with tape water thrice before streaking on agar plates. The nodules were strelized externally using 95% alcohol for 1-4min followed by washing with calcium hypochlorite solution (10g/150ml distilled water) and crushing in a drop of sterile water. A loop full ground material was transferred to 5ml of sterile water of which0.1 ml of sample was spread on to the surface of YEMA(Yeast Extract Mannitol Agar media) agar plates. Plates were then incubated at 28oC for 48 hours. Well single colony was selected and restreaked on YEMA agar slants. These isolates were used to further study the morphological, cultural and Biochemical characteristics.

b) Screening of rhizobium bacteria by using morphological and cultural

Isolated colonies on YEMA media are further morphological characterized. YEMA media is suitable for the growth and identification of rhizobium. 2.5 ml of Congo red dye is mixed with litre of YEMA medium to prepare Congo red Yeast extract mannitol agar medium (CRYEMA). Bacterial colonies on the YEMA media streaked on the CRYEMA medium and pertiplates are incubated at 280C for 5-7 days. Rhizobial cells uptake congored very weakly so they form white, circular, entire, raised, convex colonies.

c) Growth on Bromothymol Blue(BTB)

The YEMA medium was enriched with BTB@(25µg/ml) to selectively identify *rhizobium meliloti* as quoted by.[9] All the samples were subjected to grow on BTB added medium. The positive sample show moist and gummy colonies after incubation for 48hours at 28°C and surrounding medium plates were yellow due to acid production by the sinorhizobium meliloti.

3. CHARACTERIZATION OF NITROGEN FIXING BACTERIA *RHIZOBIUM*

i) Morphological and staining characters of Rhizobium

The rhizobium bacteria are gram negative, aerobic, non spore forming and motile rods. In general the colonies were circular, convex, whitish pink and glistering with entire margin.

S.No	CHARACTERS	RESULTS
1.	Shape	Circular
2.	Size of clony	3.1mm
3.	Colour/Pigmentation	Whitish pink and glistering
4.	Elevation	Convex
5.	Margin	Regular/entire
6.	Opacity	Opaque
7.	Motility	Motile
8.	Bacterium shape	Rod shape
9.	Oxygen demand	Aerobic
10.	Spore formation	Non spore forming.
11.	Gram's nature	Gram negative.

ii) Cultural characteristics of Rhizobium bacteria

a) YEMA media with Congo red

All isolates of rhizobium bacteria show growth in 2 days and turn the YEMA media containing congo red rhizobium forms white, translucent, glistening, elevated and comparatively small colonies.

b) YEMA media with BTB

All isolates showed growth in 2 days and turned the Yeast Extract Mannitol Agar media containing BTB to yellow colour showing that they were fast growers and acid producers and they are moist and gummy colonies.

4. Biochemical characteristics of Rhizobium Bacteria

Further characterization of rhizobium bacteria is by different biochemical tests were carried out in growth medium at 28ºC for 48hours incubation at pH 6.8-7.0.

i) Acid from Glucose

Mannitol in the YEM agar replaced by equal amount of glucose and bromothymol blue (25mg/ml) was added to it, this modified media was inoculated with the strains and incubated. Change in colour around was observed(shown in Table-2)

ii) Catalase Activity
Isolated strains were 48 hrs old cultures and add one drop of hydrogen peroxide and observe for liberation of effervescence of oxygen around the bacterial colonies according to[7] (Shown in Table-2)

iii) Citrate Utilisation test
Citrate utilization test is used to citrate utilization ability was determined by replacing mannitol from YEM agar with equal amount of sodium citrate and bromo thymol blue (25mg/ml). The petriplates with modified media were inoculated and then incubated for 48hrs[7] Table-2.

iv) Gelatin hydrolysis
Log phase cultures from YEM broth were swabbed on the surface of YEM agar plates containing 0.4%(w/v) gelatin to examine gelatinase activity. The plates were incubated at 28^0C for 7 days[8] (Table-2).

v) Starch hydrolysis
Starch hydrolysis test was performed to determine the ability of microorganisms to use starch as a carbon source[4] this medium was inoculated with rhizobium and analysed for starch utilization. Iodine test is used to determine the capability of microbes to use starch .Few drops of iodine solution (0.1%) were spread on 24 hrs old cultures grown in petriplates. formation of blue colour indicated non-utilization of starch and vice versa (Table-2).

vi) Methylene blue test
Methylene blue dye was added to the growth medium at a concentration of 0.1% then inoculated with rhizobium and incubated at 28 ^{0}C for 2-7 days.

Chemical composition of Yeast extract Mannitol (YEM) agar media: Table-1

S.no	Components	Quantity(g/L)
1.	Mannitol	10.0
2.	K_2HPO_4	0.5
3.	$MgSO_4.7H_2O$	0.2
4.	NaCl	0.1
5.	Yeast extract	0.5
6.	Agar	20.0
7.	Distilled water	1L

Preparation of indicator

a. Congo red
Add 10ml of stock solution (dissolve 250mg of Congo red in 100ml Water) to 1 Litre.

b. Bromothymol blue (BTB)
Weigh 25 mg of BTB and dissolve in 1ml of water and filter and use.

Table-2: Different Biochemical characteristics of all the isolates.

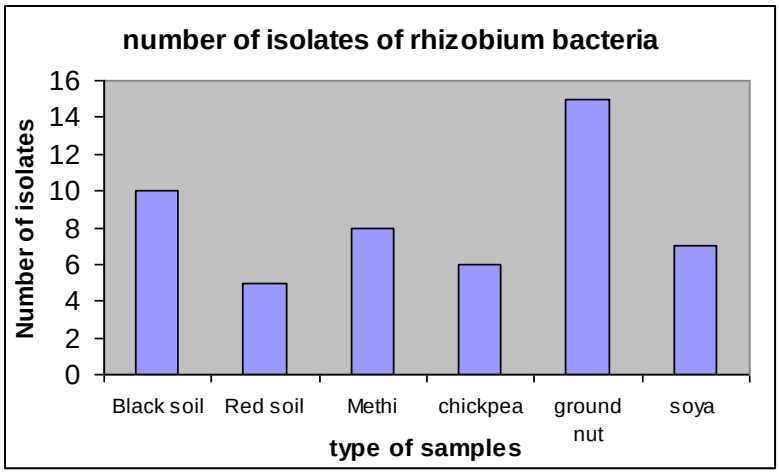
Isolate No	Methylene Blue	Acid from Glucose	Catalase activity	Citrate Utilization	Gelatin hydrolysis	Starch hydrolysis
1	-	+	+	-	-	+
2	-	+	+	-	-	+
3	-	+	+	-	-	+
4	-	+	+	-	-	+
5	-	+	+	-	-	+
6	-	+	+	-	-	+
7	-	+	+	-	-	+
8	-	+	+	-	-	+
9	-	+	+	-	-	+
10	-	+	+	-	-	+
11	-	+	+	-	-	+
12	-	+	+	-	-	+
13	-	+	+	-	-	+
14	-	+	+		-	+
15		+	+	-		+
16	-	+	+	-	-	+
17	-	+	+	-	-	+
18	-	+	+	-	-	+
19	-	+	+	-	-	+
20	-	+	+	-	-	+
21	-	+	+	-	-	+
22	-	+	+	-	-	+
23	-	+	+	-	-	+
24	-	+	+	-	-	+
25	-	+	+	-	-	+
26	-	+	+	-	-	+
27	-	+	+	-	-	+

28	-	+	+	-	-	+
29	-	+	+	-	-	+
30	-	+	+	-	-	+

RESULTS AND DISCUSSIONS

Totally we isolated 30 strains of Nitrogen fixing rhizobium bacteria from different rhizosphere soils and root nodules of different legume plants of methi, chickpea, groundnut plant and soya. All the isolates are symbiotic nitrogen fixing bacteria and some bacteria they are rhizosphere microorganisms they present around the roots of the plants.

Nitrogen fixing bacteria from different samples



All the legume plant are used for the sample collection and screening of Rhizobacteria. Different soils and legume plant containing microbes as shown in graph total number of isolates and corresponding to the type of sample used. Total i isolated nearly 50 isolates and further screened and finally 30 isolates are used further identification studies.

As per chart maximum numbers of isolates are isolated from groundnut plants and these plants form root nodules, compare to the other legume plants and soils. root nodule formation is due to the symbiotic nitrogen fixation takes place because the the Soil & atmospheric nitrogen abosrbes and convert into ammonia and suppy to the roots of the legume plants & take the nutrients from the plants this association is Symbiotic nitrogen fixation and Rhizobacteria.

Figure 1: YEMA media with Bromom thymol blue(Dye)

YEMA media with Bromothymol Blue is the selective media for isolation and screening of Rhizobacteria example is Rhizobioum and symbiotic Nitrogen fixing bacteria and we isolated these strains from different soils of legumes plants and Rhizosphere soils.in this media rhizobacteria only grows and colour of the media turns yellow indicates the Rhizobacteria grows and turns to yellow.

SUMMARY AND CONCLUSION

Although Rhizobium is the most researched and well known among these, there are a number of microbial inoculants with possible practical application in upland crops where they can serve as useful components of integrated plant nutrient supply systems. Such inoculants may help in increasing crop productivity by way of increased biological nitrogen fixation (BNF), increased availability or uptake of nutrients through solubilization or increased absorption, stimulation of plant growth

through hormonal action or antibiosis, or by decomposition of organic residues.

This is the one of the important topic for the isolates of these rhizobacteria are used for further as a important in the agriculture as a Biofertilizers have an important role to play in improving the nutrient supplies and their crop-availability in upland crop production.

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