research article | International Journal of Plant Sciences, (July to December, 2009) Vol. 4 Issue 2 : 521-523

Study of native bioinoculants from the mung bean of district Sagar (M.P.) India

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Accepted : April, 2009

SUMMARY

Exploration and isolation of efficient and multifunctional native strains from legumes can yield a roubst bioinoculants. In order to obtain such strains isolation of *Rhizobium* from the root nodules of mung bean (*Vigna radiata*) collected in 2007 from different fields of district Sagar (M.P.) was carried out. The isolation was done by serial dilution of nodule content on yeast extract mannitol agar (YEMA). A total of 50 isolates were isolated. Primary identification of isolates was done on the basis of gram staining and the presence of poly-â-Hydroxy butyrate granules. Among these, a total of 23 isolates were identified as *Rhizobium*. These were further characterized by different biochemical test *i.e.* oxidative fermentation of lactose, growth on nitrogen free medium, phosphate solubilization test, motility, nitrate reduction test, utilization of different sugars and their ability to grow at pH- 4.0 - pH- 10.0 etc. All the isolates grew well on medium containing 0.2% to 4.5% NaCl and pH 4.0-10.0 pH; they were also able to grow at different temperatures thus showed salt, pH and temperature tolerance. While only 13 isolates showed phosphate solubilization activity. Maximum phosphatase activity (phosphate solubilization) was noted in Two *Rhizobium* isolates of mung bean (*i.e.*, M21 and M34) found with good activity of phosphatase, using Pikovskaya's medium. Significance of the test *Rhizobium* strains will be discussed in the present communication.

Key words : *Rhizobium*, Mung bean, Isolates, Legumes, Phosphatase

Mung beans originate from India and India remains a leading producer of this legume. Most mung beans are olive green in colour but they can also be yellow, brown, or mottled black. They are an excellent source of folic acid and a good source of magnesium, phosphorus and thiamin. Mung beans are an important food in rural areas of southern Africa, where the dry bean seeds are used or the beans themselves are eaten as a vegetable.

To meet out continuously growing food requirements of increasing population, an indiscriminate use of chemical fertilizers has posed many problems to modern agriculture. The use of biofertilizers to replace chemical fertilizers is one of the most feasible solution to reclaim our agricultural land and to sustain required productivity. But the commercially available biofertilizers have not been found to show desired results in practice, may be because of the failure of bioinoculants in highly competitive soil ecosystem and its changing chemical composition. To short out the regional problems of bioinoculant failure we need to develop bioinoculants based on potential native strains. It will be economically more sound and environmentally more acceptable (Halliday, 1982).

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MATERIALS AND METHODS

Sample collection:

20 Samples of leguminous plants of mung bean (*Vigna radiata*) were collected from different fields of district Sagar (M.P.)

Isolation of bacteria:

The emerging healthy, pinkish nodules were harvested from healthy plants 30 days after sowing. Plant roots were rinsed in tap water to remove loosely adhering soil. four to five healthy nodules were removed from each plant with forceps and surface sterilized first with 95% ethanol and then with 0.1% mercuric chloride (Subba Rao, 1995). Individual nodules were crushed in sterilized water and the serial dilution of the suspension were streaked over yeast extact-mannitol agar (YEMA) congo red plates and incubated for 3 days at 35°C.

One single colony was taken from each nodule extract directly or after purification through subsequent streaking.

Slants of YEM medium were routinely used for maintenance of the parent cultures of rhizobia (Weaver and Graham, 1994). Bacterial cultures were incubated at 35°C. The purity of the bacteria was checked by repeat streaking as well as by microscopic examination with Gram staining.

Identification:

For this each bacterial isolates was grown on YEMA medium containing Congo red and after 3-4 days of growth

the colony characteristics including size, shape, pigmentation, elevation, margin of colony were examined. The colonies were also observed under transmitted and reflected light conditions to understand their optical properties. Bacteria were considered typical for *Rhizobium* as they showed 2-4 mm diameter, circular, colourless or same colour as the agar, pulvinate, raised, smooth margin, gram negative, translucent, opalescent, glistening.

Microscopic examination:

The bacteria were Gram stained and observe under light microscope. The shape of bacterial cells, *i.e.*, bacilli and coccobacilli in single, paired, chains and dense clusters that stained Gram negative were considered *Rhizobium*. Carbol fuchsin staining was carried out (Ehrlich, 1882) and the cells were examined for dark purple rounded bodies, the poly-â-hydroxy butyrate granules, inside the cells. Which were considered typical for *Rhizobium.*

Motility test:

Yeast extract mannitol (YEM medium) having 1% agar was used to test the motility of bacteria.

Biochemical characterization:

Lactose agar test:

Lactose utilization by test bacteria was tested using lactose agar medium containing lactose (10g/L). Isolated bacteria were grown on lactose agar plates for 2-3 days and the plates were then flooded with benedict's reagent. Benedict's reagent was prepared as follows: solution A.173 g sodium citrate and 100 g anhydrous sodium carbonate were dissolved in 66 ml distilled water; solution B. 17.3 g crystalline copper sulphate per 100 ml distilled water. Solutions A and B were mixed with constant stirring, the mixture was then filtered to remove any particulate matter and the volume was raised to 1000 ml with distilled water.

After the growth of test bacteria the plates were floded with the benedict reagent. Formation of yellow zone around the colony indicated the presence of *Agrobacterium,* where as the absence of yellow zone indicated the presence of *Rhizobium* (Subba Rao, 1993).

Growth on nitrogen free medium:

Jensen's medium having following composition was used to grow isolated bacteria. Growth on this medium indicated nitrogen fixing ability of test bacteria.

On this medium *Rhizobium* stands out as white, gummy, translucent, glistaining, elevated and comparatively smaller colonies with entire margin.

Phosphatase activity:

Phosphatase activity of each isolate was determined using Pkovskaya's medium (Pikovskaya, 1948). The bacteria were grown on this medium for 3 days at 35ºC and were observed for development of zone of clearance around the colony.

Physiological test for salt, acidity and temperature tolerance:

Differences in sodium chloride tolerance were tested in YEMA supplemented with NaCl at a concentration of 0.2%, 0.3%, 0.4%, 0.8%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, and 4.5% (wt/vol).

pH tolerance were tested in YEM agar adjusted at pH, 4.0, 4.3, 4.8 and 5.0 using sulfuric acid (1N) and pH 7.5, 8, 8.5, 9, 9.5, 10 using 0.5 molar Borax NaOH buffer. All the plates were incubated at 35ºC for 72 hours and plates containing basal YEM medium were used as controls.

Growth on different temperatures were investigated by incubation of bacterial cultures in YEM agar at 25°, 28°, 30°, 35°, 36°, 37°, 38°, 40°,45° and 47° C. Control plates were incubated at 35°C.

Utilization of different sugars:

Utilization of different sugars *i.e.* mannitol, glucose, fructose, xylose and sucrose.

Nitrate reduction test:

Nitrate broth (Himedia, mumbai) was used to determine the ability of an organism to reduce nitrate (NO_3) in to nitrite (NO_2) and test the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen using the enzyme nitrate reductase.

Incubation time – 24 hrs, chemical used – Alfa nepthile amine, sulphenilic acid and zinc dust.

Catalase test:

The catalase test identifies the organisms which produce the catalase enzyme, this enzyme converts hydrogen peroxide to water and oxygen gas, when organism is exposed to 3% hydrogen peroxide, the hydrogen peroxide was bubble.

Acid production:

Rhizobium acts on mannitol and produces an acid and in turn changes blue colour of the indicator to yellow and is thus acid production in medium observed.

Citrate utilization:

Koser's citrate was used to detect the utilization of

citrate as sole source of carbon .Colour changes from green to blue indicate positive test.

Starch hydrolysis (Amylase production):

Nutrient agar supplemented with 0.2% starch was used to detect amylase activity in all strains. The plates were inoculated by transferring bacteria and were incubated for 24 hrs at 35 ºC. The Petridishes were flooded with an iodine tincture solution a clear zone around the colony with dark blue background indicated starch degrading activity

RESULTS AND DISCUSSION

A total of 50 bacteria were isolated from mung bean (*Vigna radiate)*. All the isolates were examined for their primary identification based on the gram reaction and the presence of poly-β-hydroxy butyrate granules. Among 50 isolates, 23 have been found gram negative bacilli or coccobacilli and were found to possess PHB granules when observed under light microscope. All these isolates also showed negative test on lactose agar medium thus they have bean designated as *Rhizobium.*

Table 1 : Characteristics of *Rhizobium* isolated from mung bean (*Vigna radiata*)

Characteristics	M04	M09
Gram reaction	Gram -ve	Gram -ve
Presence of PHB granules	+	+
Gowth on N_2 free agar	+	-
Motility	+	+
Gowth on 4.5% NaCl conc.	-	+
Gowth at pH-4	+	+
Gowth at pH-10	+	+
Phosphate solubilizaion (zone of phosphatase activity)	+ 21mm	+ 24mm

Table 2 : Samples collected from different fields of district Sagar (M.P)

No. of plants	Place	Total isolates	*Rhizobium*	Other
4	Damoh road	13	9	4
8	Jat pathariya	15	10	5
3	Makroniya	10	7	3
5	Railway crossing road, Sagar	12	5	7
20	Total	50	31	19

Among 23 *Rhizobium* isolates only 9 strains were able to grow on nitrogen free Jensen's medium.

All the isolates grew well in medium containing 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4% NaCl, and in medium having pH 4.0 to pH 10.0. All the isolates grew well on different temperature *i.e*. 20ºC, 24ºC, 28ºC, 30ºC, 35ºC, 40ºC, 42ºC, 45ºC while few were able to grow at 47ºC thus showed salt, temperature and pH tolerance.

All the isolates were able to grow in the presence of KNo_3 and were able to utilize different sugars *i.e.* mannitol, glucose and sucrose.

13 isolates showed phosphate solubilization activity. Maximum phosphatase activity (phosphate solubilization) was noted in *Rhizobium* isolates of mung bean (*i.e.*, M04 and M09). These isolates showed 24 and 26 mm. zone of phosphatase activity. The characteristics of these isolates are given in Table 1.

19 isolates were able to reduce nitrate in to nitrite.

Rhizobium is well known to fix free atmospheric nitrogen and thus used as biofertilizers / bioinoculants. However, the field success of these inoculants depends on their ability to form effective nodule on the crop plant and also on the physical and chemical characteristics of soil.

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