Efficient Nitrogen Fixing Rhizobial Isolate Infecting Vigna radiata l.

1Neeraj, 1S.S. Gaurav, 2S.C. Chatterjee, 3Sachin and 1Mahesh Chandra
1Department of Biotechnology, C.C.S. University, Meerut, (U.P) India
2Division of Plant Pathology IARI, New Delhi, India
3Division-FLOF, Central Institute of Medicinal and Aromatic Plants, Lucknow, India

Corresponding Author: T. Neeraj, Department of Biotechnology, C.C.S. University, Meerut-250110 (U.P) India

Abstract: Among 972 rhizobia, isolated from nodules of different verities of Vigna radiata, 67 isolates were found to be potential ones on the basis of plant growth promotion/increased plant biomass. These isolates were further screened, where, only six potential isolates, showed higher plant biomass, maximum nodule numbers and higher nitrogenase activity, were selected for further studies. Maximum range of fresh weights of nodule per plant was shown again by isolate R0132 and noticed to be 261.37 mg nodule/plant. Nitrogenase activity of plant nodule inoculated with different rhizobial isolates varied from 2.56 to 3.21 μg/mg/nodule. When growth abilities of these isolates studied, it had been observed that with different nitrogen sources, isolate R0132 was found to be the best candidate as it utilized a wide range of nitrogen and carbon sources and was able to grow at slightly higher temperature i.e. 32°C and slightly acidic (pH 6.0) and alkaline (pH 8.0) conditions. Furthermore, this isolate demonstrated the ability to grow in presence 50 mM concentration of NaCl.

Keywords: Vigna radiata, nitrogen fixation, nitrogenase activity and rhizobial isolates

INTRODUCTION

Deficiency in mineral nitrogen often limits plant growth, and so symbiotic relationships have evolved between plants and a variety of nitrogen-fixing organisms (Estrada-De los, 2001). For more than 100 years, biological nitrogen fixation (BNF) has commanded the attention of scientists concerned with plant mineral nutrition, and it has been exploited extensively in agricultural practice (Burris, 1994). It is an essential step in the global nitrogen cycle and this reaction is necessary to sustain life on earth (Seefeldt, et al., 2004). However, international emphasis on environmentally sustainable development with the use of renewable resources is likely to focus attention on the potential role of BNF in supplying N for agriculture (Peoples, et al., 1995).

The establishment and maintenance of an effective symbiosis depends on several factors of which a favorable environment, that will allow maximum N$_2$ fixation, is extremely important (Singleton, et al., 1982). Several environmental factors such as soil pH, soil fertility, temperature extremes impose limitations on the symbiotic association between the host plant and microsymbiont (Van Wyk, 2003). Furthermore, the amount of nitrogen fixed by symbionts is variable; depending on the host legume, cultivar, presence of saturated or near-saturated soil water for movement, soil texture and composition, bacterial species and growing conditions - especially pH and the presence of soil nitrogen etc (Gardner, et al., 1985). In the present investigation different rhizobia were isolated from nodules of Vigna radiata for their ability to grow in various environmental conditions like pH, temperature, salt and other factors and further assessed for their efficiency to fix nitrogen in in-vitro condition. This study is not only useful in exploring the potential rhizobia but also in understanding of relation of natural rhizobial isolates to their environment.

MATERIALS AND METHODS

Plant varities and chemicals: Varities of Vigna radiata viz, PM-2, PDM-54, K-851 and PM-5 were taken from Indian Agricultural Research Institute (IARI), New Delhi. All the chemical for media YEMA were procured from Himedia India Pvt Ltd and for gas chromatography from Sigma Chemicals co. USA.

Isolation and identification of rhizobial isolates: Isolation of Rhizobium made by using followed usual techniques (3, 19). Yeast extract mannitol agar (YEMA) is the special medium used to grow Rhizobium. Colonies grown on a medium may not be only of rhizobium they can be even of Agrobacterium. For definite conclusion, one has to inoculate seeds of particular sequence and wait for formation of nodule on such plants. In order to confirm whether isolated colonies are of Rhizobium for this purpose one has to see that inoculated plants form effective nodule on roots. For further confirmation, isolates were grown on YEMA-congo red agar, glucose peptone broth, bromocresol purple agar medium and plant infection assay.
pH, temperature, N- source, C- source and salt tolerance: Effect of pH on growth of selected isolates was measured in liquid culture using YEM broth at different pH, ranging form 5.5 to 9.0. For temperature effect isolates were grown on YEMA agar media and incubated at different (22, 24, 26, 28, 30, 32, 34, 36 and 38 ºC) temperatures.

Ability of isolates utilizing different nitrogen sources like yeast extract, casein hydrolysate, ammonium chloride, and potassium nitrate were determined by measuring the growth in liquid medium with mannitol as carbon source. Four concentrations of each N source were taken and growth was determined by CFU counts after 72 hr of incubation at 200 rpm. Different carbon sources, viz., mannitol, sucrose, fructose and glucose were selected to observe the effect of C-source on growth. Growth profiles of rhizobial isolates were observed using yeast extract mannitol broth medium with these sugar’s supplements.

To study the effect of different salt concentrations on the growth of selected isolates of Rhizobium the isolates were incubated at 28 ºC for 6 days on surface agar and in liquid culture medium with YEM broth at 200 rpm for 72 h containing different concentrations of NaCl (Sodium chloride) ranged from 10 mM, 20 mM, 50 mM, 100 mM and 150 mM to 500 mM.

Nodulation assay: For vetch assays, seeds of Vigna radiata L. obtained, and sterilized in the same manner. Seeds then placed on sterile water-agar plates and TY plates; water-agar plates placed in the dark 24-36 hours at room temperature while TY plates were placed at 28 ºC to check for sterility. After germination on plates, seeds placed in modified jars containing sterile carrier material and 200 ml of plant medium. Three seeds placed in a pot. Overnight cultures of Rhizobium isolates used for inoculation. 1.0 ml of culture was added to 9.0 ml sterile H2O mixed gently, and the mixture added to one pot. Control pots had 10.0 ml of water added instead of inoculums.

Estimation of nitrogenase activity: Rhizobium cells cultured in YEM medium and harvested at exponential phase of growth. These cells were suspended in small amount of medium and again inoculated into fresh culture medium (10 ml of culture medium into a 100 ml bottles with serum rubber stopper) of (Keister, 1975) with some modification containing NaPO4 7.4 mM, MgSO4 0.8 mM, FeSO4 48 µm, NaMoO4 4 µm, mannitol 0.5 percent and sodium succinate 0.5 percent sodium glutamate 0.1 percent). The amount of other trace metals and vitamins were same as used in defined medium. The pH of medium adjusted to 6.8. After 96 hours of growth, the aerobic phase of bottles replaced by flushing several times with nitrogen. In final stages, 10 ml of acetylene as injected into the bottles at partial pressure. The bottles were then incubated up to 72 hours at 28 ºC on a rotary shaker at 200 rpm. The samples of gas phase 1.0 ml analyzed periodically after 18 hours for ethylene formation on sigma gas chromatography equipped with a Flame Ionization Detector and Dual Porpk N column of 2.0 M length.

The amount of ethylene produced through reduction of acetylene calculated from calibration curve. Nitrogenase activity expressed as the formation of n mol of ethylene/hour/mg protein.

RESULTS AND DISCUSSION

A total 972 isolates were collected from the nodules of different verities of Vigna radiata viz., PM-2, PDM-54, K-851 and PM-5. These isolates were designated as rhizobia on the basis of their colony characteristics and cell morphology and culture characteristics. Among these, only 67 isolates were short listed on the basis of plant growth promotion/ increased plant biomass (data not shown). These isolates were then assessed in pot experiments for their ability to increase plant biomass, maximum nodule numbers and higher nitrogenase activity, where only six potential isolates (Table 1) which were selected for further studies.

Although all the isolates showed optimum growth at 28 ºC but the isolate, designated as R0132, showed slightly higher temperature i.e. 32 ºC for its optimum growth (Fig. 1). Increased temperature optima of this isolate may be beneficial for its application in temperature stress condition as symbiotic performance of different rhizobial strains under temperature stress has been correlated with their ability to grow in pure culture at elevated temperatures (Hungria, 2000).

All the isolates showed their optimum growth at pH 7.2 but isolate, R0132 was able to tolerate slightly higher pH that is 8.0 (Fig. 2). Large variation in tolerance to pH of the culture medium has been found within Rhizobium species (Graham and Parker, 1964). Isolates to isolates differences are reported with regard to the growth in relation to pH of the culture medium observed by (Cooper, et al., 1988; Diat Loff, 1970).

No effect on growth of any isolate was observed in media containing up to 20 mM of sodium chloride. Excessive inhibitory effect of presence of NaCl on the growth of all the isolates was noticed by increasing the concentration above 50 mM except isolate R0132 which demonstrated good growth at this concentration of NaCl (Fig. 3). Salinity, a composite stress having both an ionic as well as osmotic component, can be extremely detrimental for the growth of soil-inhabiting bacteria like Rhizobium (Saxena, et al., 1996). With regard to the salinity stress it has been observed that a number of rhizobial isolates exhibit a large range of sensitiveness to salinity. Singleton et al. (Singleton, et al., 1982) showed that some isolates from the different rhizobial types failed to grow even at 0.6 percent NaCl while other show 3.7 fold range for insensitivity in term of fractional reduction in growth. These results are in accordance with Mansari et al. (2007) where Phaseolus vulgaris was able to grow at high salt concentration.

Table 1: Effects of inoculation of selected rhizobial isolates on nodule numbers, weight and texture.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Isolate Designation</th>
<th>Number of Nodule/plant</th>
<th>Fresh weight of Nodule (mg)</th>
<th>Dry weight of Nodule (mg)</th>
<th>Nodule texture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>12</td>
<td>1181.2</td>
<td>22.6</td>
<td>Pink color</td>
</tr>
<tr>
<td>2</td>
<td>R0034</td>
<td>32</td>
<td>226.3</td>
<td>31.9</td>
<td>Pink color</td>
</tr>
<tr>
<td>3</td>
<td>R0129</td>
<td>31</td>
<td>232.4</td>
<td>32.0</td>
<td>Pink color</td>
</tr>
<tr>
<td>4</td>
<td>R0132</td>
<td>37</td>
<td>261.3</td>
<td>35.4</td>
<td>Pink color</td>
</tr>
<tr>
<td>5</td>
<td>R0564</td>
<td>30</td>
<td>206.8</td>
<td>29.3</td>
<td>Pink color</td>
</tr>
<tr>
<td>6</td>
<td>R0789</td>
<td>32</td>
<td>212.3</td>
<td>30.6</td>
<td>Pink color</td>
</tr>
<tr>
<td>7</td>
<td>R1203</td>
<td>33</td>
<td>220.6</td>
<td>31.6</td>
<td>Pink color</td>
</tr>
</tbody>
</table>

Table 2: Nodulation efficiency parameter of selected rhizobial Isolates.

<table>
<thead>
<tr>
<th>S.N</th>
<th>Isolate Designation</th>
<th>Fresh weight of Nodule (mg)</th>
<th>Dry weight of Nodule (mg)</th>
<th>ARA (µmol h⁻¹ mg⁻¹ FW of nodules)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>226.3</td>
<td>31.9</td>
<td>2.56</td>
</tr>
<tr>
<td>2</td>
<td>R0034</td>
<td>226.3</td>
<td>31.9</td>
<td>2.35</td>
</tr>
<tr>
<td>3</td>
<td>R0129</td>
<td>232.4</td>
<td>32.0</td>
<td>2.90</td>
</tr>
<tr>
<td>4</td>
<td>R0132</td>
<td>261.3</td>
<td>35.4</td>
<td>3.21</td>
</tr>
<tr>
<td>5</td>
<td>R0564</td>
<td>206.8</td>
<td>29.3</td>
<td>2.73</td>
</tr>
<tr>
<td>6</td>
<td>R0789</td>
<td>212.3</td>
<td>30.6</td>
<td>2.94</td>
</tr>
<tr>
<td>7</td>
<td>R1203</td>
<td>220.6</td>
<td>31.6</td>
<td>2.81</td>
</tr>
</tbody>
</table>

Fig 1: Effect of temperature on growth of selected rhizobial isolated in broth culture

Sucrose and fructose could not prove to be an appropriate carbon source for growth of isolates. In case of all the isolates maximum growth was observed in R0132 on mannitol containing medium. In present investigation majority of the isolates showed their good growth on mannitol when was used as C-source (data not shown). It has been reported that slow growing isolates grow well on monosaccharides in comparison of other carbon sources.

The isolate were further assessed for the ability to produce nodule and N-fixation ability. The data presented in the Table 1, revealed that the ability to produce nodule by different isolates varied from 30-37. Higher number of nodule (37) were observed on the plant inoculated with R0132. Nodule fresh weight of plant inoculated by different rhizobial isolates also varied from 206.8 to 261.3 mg/plant. The lowest range was observed in R0564 (206 mg nodule per plant). Maximum range of fresh weights of nodule per plant was shown by isolate R0132 which was noticed 261.37 mg nodule/plant.

The measurement of nitrogenase activity was based on the reduction of acetylene to ethylene as quantities by gas chromatography. Nitrogenase activity of plant nodule inoculated with different rhizobial isolates varied from 2.56 to 3.21 µg/mg/nodule (Table 2). The maximum nitrogenase activity has been observed in isolates designated as R0132 while minimum nitrogenase activity has been recorded in isolates R0034.

It has been observed that growth of these isolates varied on different nitrogen sources. Yeast extract proved itself as a potential growth promoter at the concentration of 1.0 to 0.5 g/l. Growth of isolates on ammonium chloride were slightly low comparison of observed in case of yeast extract while casein hydrolysate and potassium nitrate were not supported (data not shown). When growth comparison of these isolates studied it has been observed that under different nitrogen sources isolates R0132 was found to be the best performer as it utilized a wide range of nitrogen sources.
However, Rhizobial isolates in present investigation with regard to their physical environment for example temperature; pH and salinity showed that some isolates may have significant value of tolerance in diverse condition. Efficiency of rhizobial isolates in pure culture at different temperature, pH and salinity has far reaching ecological significance in varied places (Duzan, et al., 2004; Howieson and Ballard, 2004).

Several environmental conditions are limiting factors to the growth and activity of the N₂-fixing plants. A principle of limiting factors states that "the level of crop production can be no higher than that allowed by the maximum limiting factor" (Brockwell, 1980). In the Rhizobium-legume symbiosis, which is a N₂-fixing system, the process of N₂ fixation is strongly related to the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors (e.g., salinity, unfavorable soil pH, nutrient deficiency and temperature extremes) impose limitations on the vigor of the host legume (Thies, et al., 1995). From the present investigation it may be concluded that the isolate R0132 which showed broad range of pH and temperature with minimum growth requirements, can fix high level of N-fixation in in-vitro condition as observed by ARA test. It is need less to state that such a candidate could be potential one, which can address the problem of nitrogen depletion.

ACKNOWLEDGEMENT

The authors are grateful to the Director, Indian Agriculture Research Institutes Pusa New Delhi, for providing facilities and encouragement.

REFERENCES


Seefeldt L.C., I.G. Dance and D.R. Dean, 2004. Substrate interactions with nitrogenase: Fe versus. Recent progress with organometallic model compounds, theoretical calculations, and biochemical, kinetic, and biophysical studies on nitrogenase are reviewed with a focus on substrate binding and activation at the Fe and Mo-sites of the FeMo-co. Biochem., 43:1401-1409.

