Exploring wild Rhizobium isolates for development of Rhizobium mutants suitable for blackgram under acid soil condition

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Abstract
Soil acidity is the major soil environmental stress factor limits Rhizobium survival and thereby reducing nodulation. In addition which results in deficiencies of Calcium, Magnesium, Potassium, Molybdenum and also cause aluminium toxicity which also affects nodulation and intum the productivity in pulses. Developing a mutant strain of Rhizobium to tolerate acidity from wild type available in the acid soils will survive in soil and enhance the nodulation thereby improve N2 fixation. In the present study the three Rhizobium mutants viz., VM1, VM2 and VM3 were developed using EMS mutagen for Chloramphenicol resistance from the native Rhizobium strains isolated from blackgram nodules grown under acid soils (pH 4.5 to 5.0) of NPRC, Vamban. Among the three, VM1 alone tolerated 200 ppm Chloramphenicol and others showed meagre growth. In the acid tolerant test VM1 Rhizobium mutant alone grew at both acidic pH levels (5.0 & 4.0) and confirmed in BOX PCR analysis also. The Rhizobium mutant (VM1) increased the ability of wild mutant to grow on acidic pH without causing major alteration in its DNA.

Keywords: Acidity, Rhizobium mutant, nodulation, acid tolerance

Introduction
The more common, and characterized, pH stress found in soil is acidity (Fujihara & Yoneyama, 1993) [3]. Soil acidity limits survival and persistence of Rhizobium and thereby reducing nodulation (Graham, 1992) [11]. Soil acidity is not only an increase in the concentration of protons, but also in the solubility of metals such as aluminium, which are toxic to root nodule bacteria. Acid condition of soil results in deficiencies of Calcium, Magnesium, Potassium and Molybdenum (Subba Rao, 1999) [12]. Hence, developing a mutant strain of Rhizobium to tolerate acidity from wild type available in acid soils will survive better and enhance nodulation and N2 fixation. Since, mutation could be used to add desirable characters to an already effective inoculant strain. Mutagenesis has been used to a greater extent to induce genetic variability in plant growth promoting bacterial like rhizobia (Engvild, 1987) [3] to achieve the desired genetic variability; this work also aims to solve soil acidity by developing mutants of Rhizobium that can enhance nodulation in blackgram grown in acid soils. Mutagens have proved to be wonderful tools in the hands of scientists to allow the tailoring of rhizobia. Efficient rhizobial mutants have been derived by using chemical mutagens such as N-methyl-N-nitro-N-nitrosoguanidine (NTG), ethyl methane sulphonate (EMS), etc, (Allan paau, 1989; Sidorova et al., 1995) [1, 10].

Blackgram (Vigna mungo (L)) is one of the major pulses grown normally as rainfed crop because of its wider adaptability and resistant to adverse climatic conditions and improves the soil fertility by fixing atmospheric nitrogen in the soil. In pulse growing areas normally due to more usage of ammonia cal fertilizers (DAP) for pod development causes acidity development in soil. Under acidic soil conditions, the availability of soil nutrient is less compared to normal soil condition which may be the reason for the reduced yield even with the improved varietal utilization. In Tamilnadu acid soils are prevalent in Kanyakumari, Thanjavur and parts of Pudukottai dts. Hence, the study was conducted to isolate Rhizobium from blackgram nodules grown in acid soils and to develop antibiotic mutants of Rhizobium from its native Rhizobium isolates for enhancing nodulation and nitrogen fixation for sustainable nutrient management and yield.

Materials and Methods
a. Isolation of native Rhizobium isolates
To isolate native acid tolerant Rhizobium, nodules were collected from blackgram grown under acid soils of National Pulses Research Centre, Vamban, Tamil Nadu.
The nodules were surface sterilized using 70 % ethanol and further washed in sterile distilled water for 5 times. After washing, nodule suspension was prepared by crushing them in sterile distilled water. A loopful of nodule suspension was streaked over the Congo red Yeast Extract Mannitol Agar (CRYEMA) medium already poured in the sterile Petriplates and incubated at 32°C in incubator for 48 hours. Single colonies were picked, purified and stored in YEMA slants for further studies. The native (wild) Rhizobium isolates were named as VW (Vamban wild).

b. Development of Rhizobium mutants by EMS induced mutation

The develop mutants from wild Rhizobium isolate, it was treated with EMS (Ethyl methane sulphonate) as follows: The purified Rhizobium isolate was grown in YEM broth for 2 days, centrifuged at 10,000 rpm for 5 minutes and the cell pellet was washed with phosphate buffer saline and suspended in sterile water. Cell suspension was dispensed into 1.5 ml eppendorf tubes and EMS solution was added at appropriate volume to get 0.4M and 0.5M EMS as final concentration. The cells were treated with EMS for 30, 90 and 120 minutes. The reaction was stopped at appropriate time interval by adding 1 ml of 6% sodium thiosulphate. The tubes were centrifuged and the pellet was washed twice with sterile water. The suspension was diluted and 10^-2 cells were spread plated on YEMA Plates containing antibiotics viz., Ampicillin, Chloramphenicol at 100, 150 and 200 ppm concentration along with wild strain. A spread plate of wild type Rhizobium was maintained as a control (without antibiotics). The Petriplates were incubated for 3 days in incubator at 32°C. After the incubation period the plates were examined for the growth of Rhizobium. Those Rhizobium colonies were picked from the antibiotic seeded YEMA plates treated as mutants. Totally three Rhizobium mutants were obtained and named as VM1, VM2 and VM3.

c. Acid tolerance test under in vitro

Based on the growth in antibiotic seeded YEMA plates, VM1 Rhizobium alone was selected and tested along with wild Rhizobium strain for their acid tolerance. For acid tolerance test YEMA broth was prepared adjusted to acidic pH levels from 6.0 to 4.5. The Rhizobium mutant (VM1) and the wild Rhizobium culture were inoculated to the YEM broth at the rate of 1% and kept for incubation at 32°C for 2-3 days. At the end of the incubation period the tubes were observed for the growth of Rhizobium. Based on the intensity of turbidity the acid tolerance of the Rhizobium was evaluated.

Results & discussion

Three Rhizobium mutants (VM1, VM2 and VM3) were obtained by EMS induced mutation. Of which, VM1 showed growth at 0.5 M EMS whereas, VM2 and VM3 tolerated up to 0.4M EMS. In the intrinsic antibiotic resistance (IAR), Rhizobium Mutant VM1 was resistant to 200 ppm chloramphenicol whereas wild was susceptible. Wild strain did not show growth in any of the concentrations of both antibiotics. Allan paau, 1989 [1]; Sidorova et al., 1995 [10] have also been derived efficient rhizobial mutants by using chemical mutagens such as N-methyl-N-nitro-N-nitrosoguanidine (NTG), ethyl methane sulphonate (EMS), etc.. In the present study also the EMS Rhizobium mutant performed better under acidic pH conditions. The study also revealed that Rhizobium mutant grew better at both acidic pH levels than the wild Rhizobium strain. Similar to this, some mutants of R. leguminosarum have been reported to be able to grow at a pH as low as 4.5 (Chen et al., 1993) [2], S. meliloti is viable only when the pH is down to 5.5 (Foster, 2000) [4].

Table 1: Growth of Rhizobium mutants on antibiotic seeded Yema plates

<table>
<thead>
<tr>
<th>S. No.</th>
<th>EMS Mutant</th>
<th>Growth of Rhizobium mutants</th>
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<td>Ampicillin (ppm)</td>
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<td>3</td>
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(++) good growth (+) Poor growth
(++) moderate growth (-) No growth
BOX PCR analysis

BOX PCR results revealed that similar banding pattern were observed in both wild and mutant strains when the amplified PCR products were documented and the gel image shows the wild and mutant shares same base pairs. Hence, it reveals that the mutation increased the ability of wild mutant to grow on acidic pH without causing major alteration in its DNA and the mutation may have resulted in point mutation and the genomic DNA of wild and mutant Rhizobium strains have got minor modifications. The mutation has positively improved its growth in acidic pH.

Polyacrylamide gel view of box PCR amplification products.

Many of the mutants performed better than their parents or wild. Mutants of Rhizobium trifolii 21M11B, produced more water soluble polysaccharide (752 mg/100 ml) than the parent (Ghai et al., 1985) also reported that isolation of Rhizobium mutants which produced curdlan type water-insoluble polysaccharides which could be used as gelling materials for many applications. Mutants of Rhizobium with altered symbiotic interaction have been reported (Kuykendall, 1981) whereas, in the present study from BOX PCR analysis mutation increased the ability of wild Rhizobium mutant to grow on acidic pH without causing major alteration in its DNA.

Conclusion

Mutation could be used to add desirable characters to an already effective inoculant strain sometimes it may cause alteration in the normal symbiotic relation between the Rhizobium and legume plant. In the present study, mutation the wild Rhizobium mutant developed performed better under acidic pH without causing major alteration in its DNA.

References