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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 inturn 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 N_2 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.

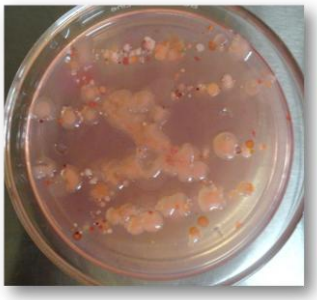
Keywords: Acidity, *Rhizobium* mutant, nodulation, acid tolerance

Introduction
The more common, and characterized, pH stress found in soil is acidity (Fujihara & Yoneyama, 1993) [5]. Soil acidity limits survival and persistence of *Rhizobium* and thereby reducing nodulation (Graham, 1992) [7]. 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) [11]. Hence, developing a mutant strain of *Rhizobium* to tolerate acidity from wild type available in acid soils will survive better and enhance nodulation and N_2 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-Nnitro-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 32ºC 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).

Blackgram nodules



***Rhizobium* on CRYEMA plate**

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 32ºC. 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 32ºC 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-Nnitro-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].

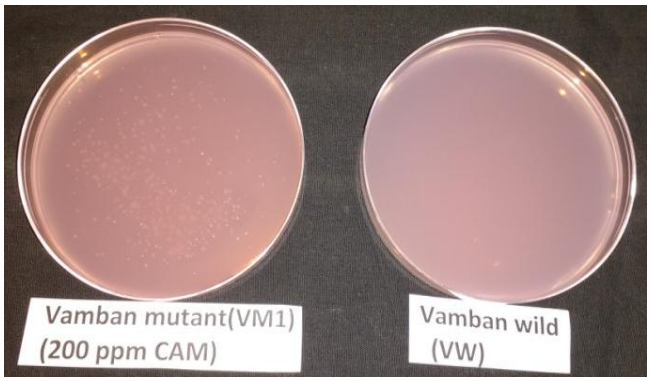


Table 1: Growth of *Rhizobium* mutants on antibiotic seeded Yema plates

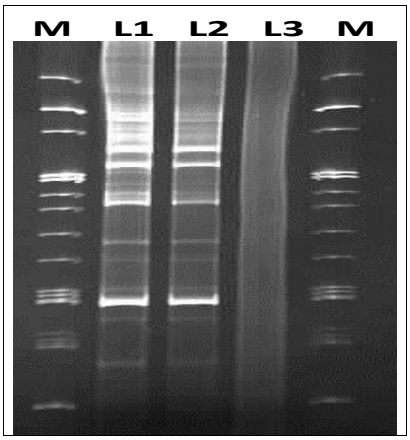
S. No.	EMS Mutant	Growth of *Rhizobium* mutants											
		Ampicillin (ppm)						Chloramphenicol (ppm)					
		100		150		200		100		150		200	
		0.4M	0.5M	0.4M	0.5M	0.4M	0.5M	0.4M	0.5M	0.4M	0.5M	0.4M	0.5M
1	30 min	++	++	++	+	++	+	+++	+++	+++	+++	+++	+++
2	90 min	+	----	+	----	+	----	++	-----	++	----	++	-----
3	120 min	---	---	----	----	----	----	----	----	----	----	------	-----

(+++) 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.



M- Marker, L1- *Rhizobium* (wild), L2- *Rhizobium* (mutant), L3- Negative

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) [6] 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) [9] 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.

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