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RESEARCH ARTICLE

Effect of physical and chemical mutagens on rhizobium and study of mutated *rhizobium* activity on seed germination and antibiotic sensitivity

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Abstract

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. Two bacterial isolates were successfully isolated from fresh nodules of groundnut and fenugreek. The isolated Rhizobium strains were allowed for UV irradiation for different time intervals to cause mutation and observed for their consequent action on the growth of groundnut and mung bean. The maximum growth with healthy appearance was resulted in the samples, treated with UV irradiation for 30 min. The isolated strains of rhizobium were also exposed to chemical mutagen (EtBr, Acrylamide and Tetra Methyl Ethylene Diamine - TEMED) to cause mutation and observed for their consequent action on the growth of groundnut and mung bean. When the chemical mutagen, EtBr was treated to groundnuts, there was no growth or sprouting observed in any of the seeds, where as in mung bean all the seeds showed higher growth rates. When the chemical mutagens, Acrylamide and TEMED were treated to groundnuts 50% of the seeds showed sprouting. The same mutagen when treated to mung bean, all the seeds showed higher growth rates. Antibiotic susceptibility and resistance of physically and chemically mutated rhizobium strains were studied tested by four different antibiotics namely, Penicillin-G, Kanamycine, Cefotaxime and Erythromycin. All the mutated strains showed resistance to Pencillin-G.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria, resides at the roots of plants and enhances the plant growth by different mechanisms. They help in increasing nitrogen fixation (legumes); promoting free-living nitrogen-fixing bacteria; supply of macronutrients; production of plant hormones. They also enhance the growth of other beneficial microbes and control the other biotic stress causing agents (Saharan and Nehra, 2011). *Rhizobia* reside in the soils and maintain symbiotic relation with the legumes. The general mechanism includes that, they inhibit root nodule formation and reduces the available atmospheric nitrogen and make it available to the concern plant (Sessitsch et al., 2002). In classical plant breeding, mutational studies are of much importance to enhance the physiological properties of any species. The previous studies showed that *rhizobium* can act better following mutational causes. Any genetic changes in *Rhizobium* may lead to increase or decrease their activity to fix nitrogen. The physical and chemical mutagens are capable of causing mutations at DNA level.

Siddiqui et al. (2011) studied the seeds of mung bean (*Vigna radiata* (L.) Wilczek and groundnut (*Arachis hypogaea* L.) and treated with ultra violet (UV-C) radiation. It was observed that both the plants showed increment in physiological characters and number of nodules. Bose and Venkataraman (1972) and Macgrego and Alexander (1971) isolated and characterized UV induced mutant of *rhizobia*.

Gunasekaran, A. and Pavadai, P. (2015) induced physical and chemical mutagenesis in groundnut (*Arachis hypogia*) and observed various results. Rathaur *et al.* (2012) studied the Effect of UV-B Tolerant Plant Growth Promoting Rhizobacteria (PGPR) on Seed Germination and Growth of *Withania somnifera.* Dadarwal et al. (1981) studied *In vitro* and *in vivo* nitrogenase activity of *Rhizobium* mutants and their symbiotic effectivity. The effect of plant growth promoting rhizobacteria on groundnut (*arachis hypogaea* l.) seed germination along with biochemical constituents were studied by Mathivanan *et al*. (2014). They also showed the role of chemical mutagens (Sodium azide and hydroxyl amine hydrochloride) in causing mutations leading to *rhizobia*l tolerance to acidic and alkaline soils.

Following objectives were focused in the current study: 1. to isolate and culture *Rhizobium* from fenugreek and groundnut roots; 2. to expose physical mutagen (UV light) to isolated *Rhizobium* and observe the corresponding response on groundnut and mung bean; 3. to expose chemical mutagen (Acrylamide, EtBr, and TEMED) to isolated *Rhizobium* and observe the corresponding response on groundnut and mung bean, and 4. to test the antibiotic resistance and sensitivity of *Rhizobium* towards various antibiotics following mutational changes.

Materials and Methods

Sample collection

The samples of *Rhizobium* were collected from agricultural farms that are located in the surroundings of P. V. P. College. The *Rhizobia* were collected from groundnuts and fenugreek roots. The *Rhizobia* were selected randomly for the mutational studies. The roots were washed and were stored in a dry environment. They were maintained under healthy conditions throughout the experimentation. The seeds and other necessary equipment for the experiment were maintained under aseptic conditions. The seeds were surface sterilized before treating with the desired *rhizobium*. The seed number per each test was maintained as five in number and the data of each sample was collected from the average of three replicates.

Isolation and culture of *rhizobium* strain from fresh nodules of groundnut and fenugreek

Groundnut and fenugreek plants were collected from farm of Loni and *rhizobium* was collected from root nodules. After surface sterilization with 95% alcohol and $HgCl_3$ followed by 70% alcohol, the nodules were ground in a sterile Petri dish using a sterile scalpel. The grounded nodule was allowed to grow on yeast extract-mannitol-agar (YEMA) solid medium and incubated at room temperature (Ngakou et al. 2009). The grown *rhizobia* were used as the samples in the experimental part.

Effect of UV irradiation on *Rhizobium*

The colonies of *rhizobium* were picked from the plate and transferred to YEM broth and incubated at 37^0C for overnight. The bacterial cultures were irradiated with UV light for 0min, 10min, 30min, 30min, 40min, 50min and 60min (Rathaur *et al*. 2012). Following UV treatment, the mutated *rhizobia* were allowed to act on groundnut and mung bean to observe the mutational effects on their germination.

Effect of Chemical mutagens (Acrylamide, EtBr and TEMED) on *Rhizobium*

The *Rhizobia* were inoculated into 50ml of filtered yeast extract-mannitol-broth containing 10µg/ml of acrylamide, ethidium bromide and TEMED, in separate flasks. The flasks were incubated on a shaker for 4 days at 38^0C (Dadarwal *et al.* 1981). The chemically mutated *rhizobia* were then allowed to act on groundnut and mung bean to observe the mutational effects on their germination.

Kirby-Bauer Test for Antibiotic Resistance and Susceptibility

The antibiotic sensitivity and resistance of the physically and chemically mutated *Rhizobia* were observed on comparing with the Kirby-Bauer's chart.

Results

Isolation of *rhizobium* from fresh nodules of groundnut and fenugreek

The *rhizobium* strains were isolated from the root nodules of groundnut and fenugreek roots. During the process, the nodules were crushed and spread on the nutrient agar plates and left for incubation. After 24 hours of incubation, the *rhizobium* growth was observed in the Petri plates (Fig. 1a & 1b).

Effect of UV irradiation on *Rhizobium* and consequent seed germination

The *Rhizobium* strains were exposed to UV irradiation for different time intervals (10, 20, 30, 40, 50 and 60 min.) (Fig.2a) to cause mutation and observed for their consequent action on the growth of groundnut and mung bean. In groundnuts, out of all the samples measured, the UV irradiation for 40 minutes has showed maximum growth (3 out of 5) and no growth resulted in 10 min. (0 out of 5).In the case of mung bean, all the samples exposed to UV irradiation have showed maximum sprouting, when compared with the control (Fig.2b). The maximum growth with healthy appearance was resulted in the samples, which were exposed to UV irradiation for 30 min. and 50 min. There was less growth observed in the last plate, i.e. 60 min.

Effect of Chemical mutagens on *Rhizobium* and consequent seed germination

The *rhizobium* strains were exposed to chemical mutagen (EtBr, Acrylamide and TEMED) to cause mutation and observed for their consequent action on the growth of groundnut and mung bean. The seeds were treated with a constant amount of 70 µL in all the cases.

When the chemical mutagen, EtBr is treated to groundnuts, there was no growth or sprouting observed in any of the seeds, where as in mung bean, all the seeds showed higher growth rates (Fig.3a & 3b). When the chemical mutagens, acrylamide and TEMED were treated to groundnuts 50% of the seeds showed sprouting (Fig.3c & 3d), whereas the same mutagens showed higher growth rates in mung beans (Fig.3e & 3f).

Antibiotic sensitivity and resistance of UV mutated *Rhizobia*:

Antibiotic susceptibility and resistance of UV mutated *Rhizobium* was studied against 4 different antibiotics namely, Penicillin-G, Kanamycine, Cefotaxime and Erythromycin (Fig.4, Table.I). The control showed that *rhizobium* was susceptible to all the antibiotics used. Differently exposed *Rhizobium* at 10, 20, 40, 50, 60 minutes was found to be mutated and showed resistance to Penicillin-G and Kanamycine. In contrast, mutated *rhizobia* were susceptible to Cefotaxime and Erythromycin. Based on Kirby-Bauer chart, these mutated *rhizobia* are showing intermediate susceptibility towards Cefotaxime and Erythromycin.

Antibiotic sensitivity and resistance of EtBr mutated *Rhizobia*:

EtBr mutated *rhizobium* (Fig.5, Table-II) was resistant to Penicillin-G and susceptible to Cefotaxime and Kanamycine, while intermediately susceptible to Erythromycin.

Antibiotic resistance and sensitivity of Acrylamide mutated *Rhizobia*:

Acrylamide mutated *rhizobium* (Fig.6, Table-III) was resistant to Penicillin-G, Erythromycin and Cefotaxime. Kanamycine was found to be intermediately susceptible antibiotic for Acrylamide mutated *rhizobium*. In the last plate with 80 µL of mutated *rhizobium*, Cefotaxime was found to be an intermediately susceptible antibiotic.

Antibiotic sensitivity and resistance of TEMED mutated *Rhizobia*:

TEMED exposed *rhizobium* (Fig.7, Table-IV) was resistant to Penicillin-G, and Erythromycin. Cefotaxime and Kanamycine were found to be intermediately susceptible antibiotic for TEMED mutated *rhizobium*.

Fig.1

***Rhizobium* strains isolated from the roots of groundnut (a) and fenugreek (b)**

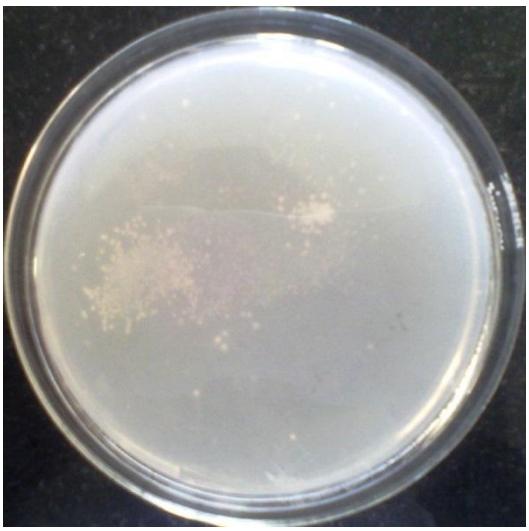
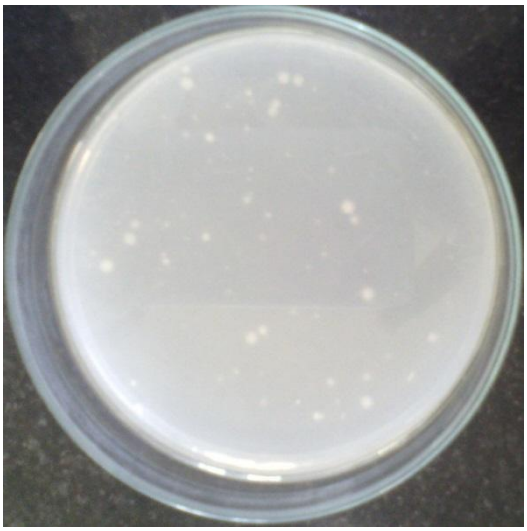
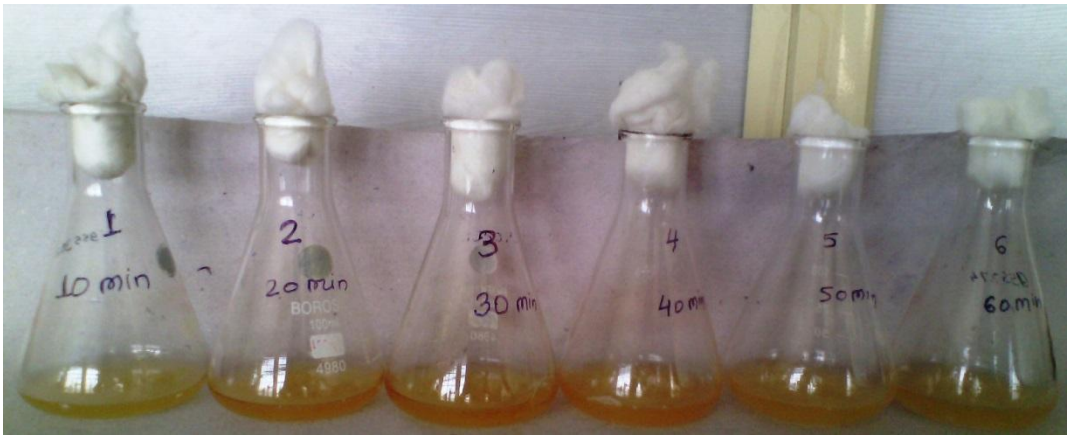
a)  **b)** 

Fig.2

(a) Exposure of UV irradiation for different time intervals on *Rhizobium* strain



(b) Effect of UV irradiated *Rhizobium* on mung bean

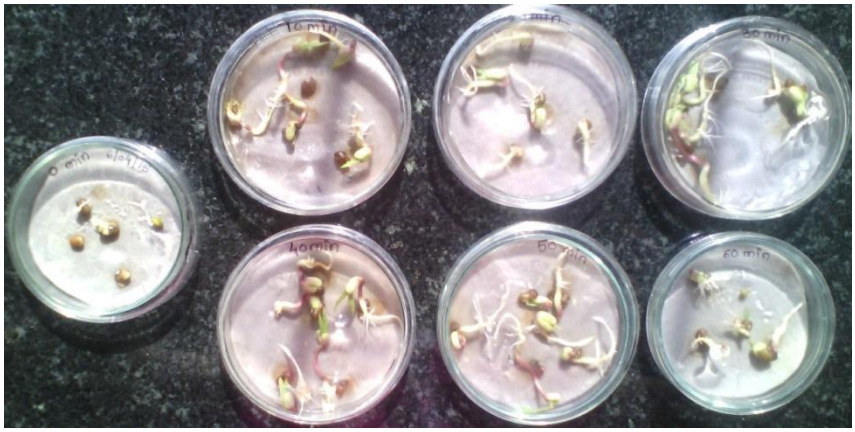
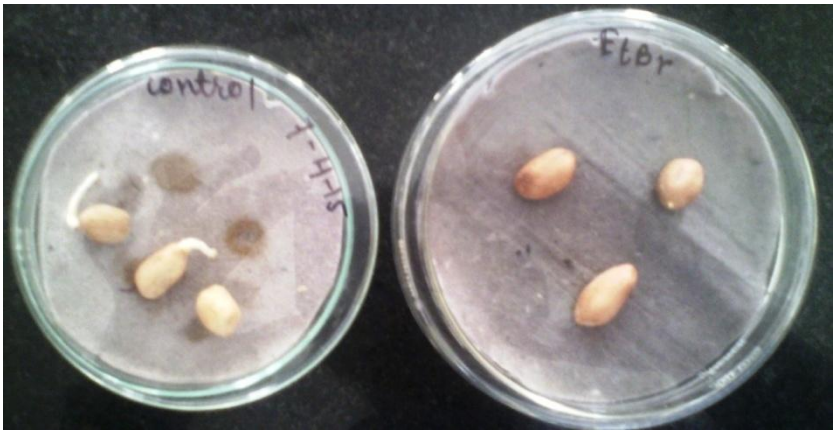
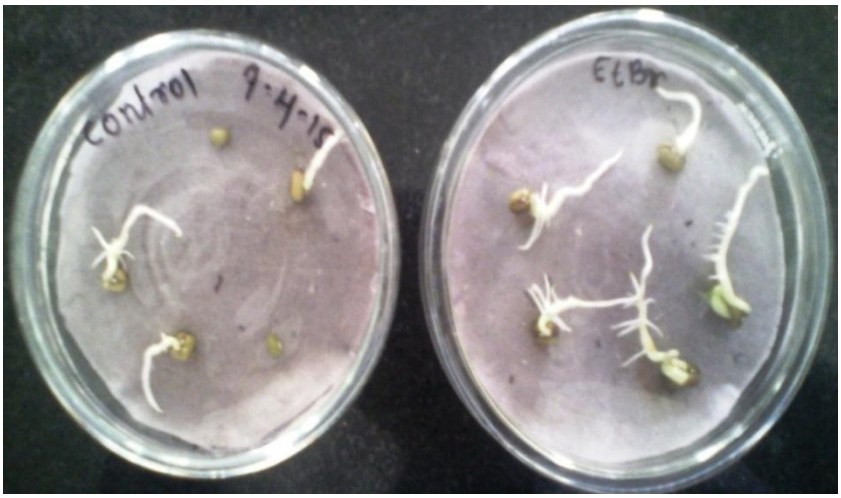


Fig.3

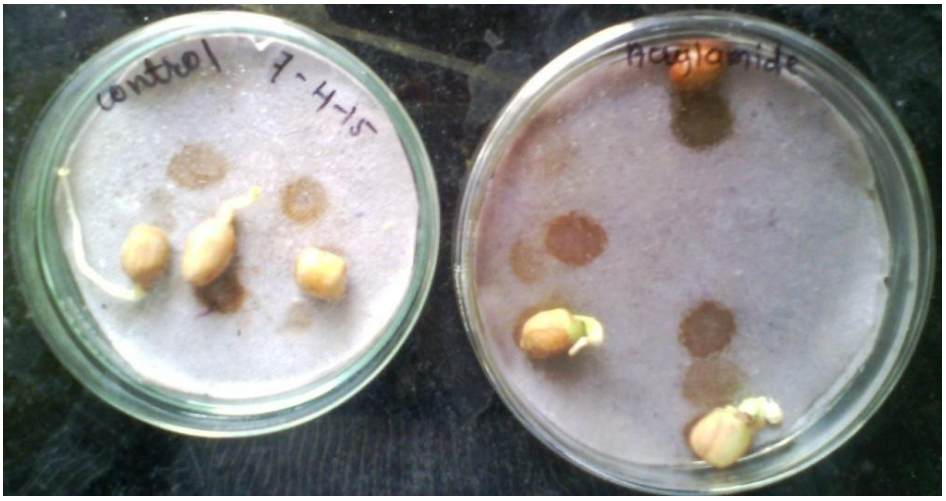
(a) Effect of EtBr treated *Rhizobium* on groundnut



(**b) Effect of EtBr treated *Rhizobium* on mung bean**

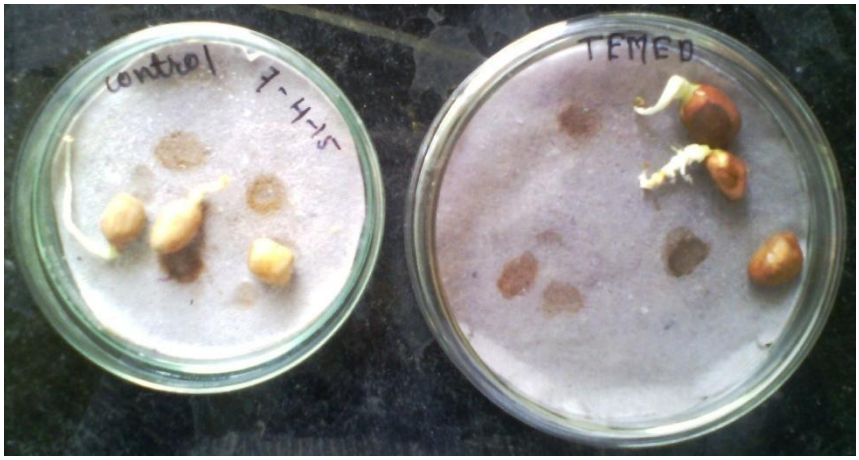


(c) Effect of acrylamide treated *Rhizobium* on groundnut



(d) Effect of acrylamide treated *Rhizobium* on mung bean

(e) Effect of TEMED treated *Rhizobium* on mung bean



(f) Effect of TEMED treated *Rhizobium* on mung bean

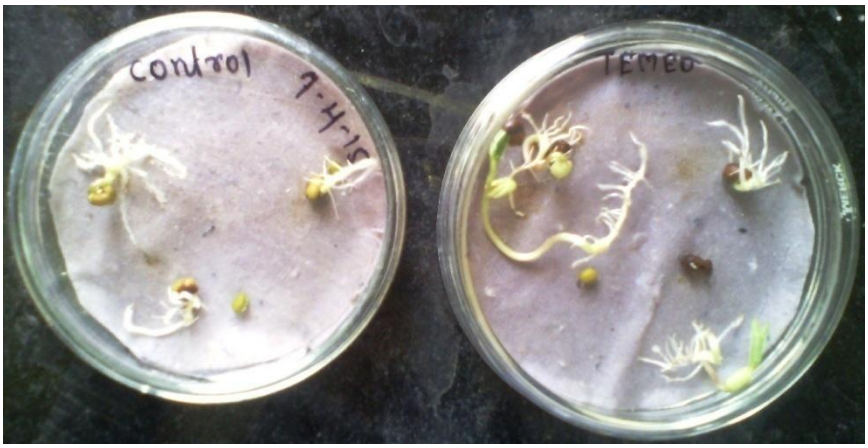


Fig.4

Antibiotic sensitivity and resistance test of UV mutated *Rhizobium*

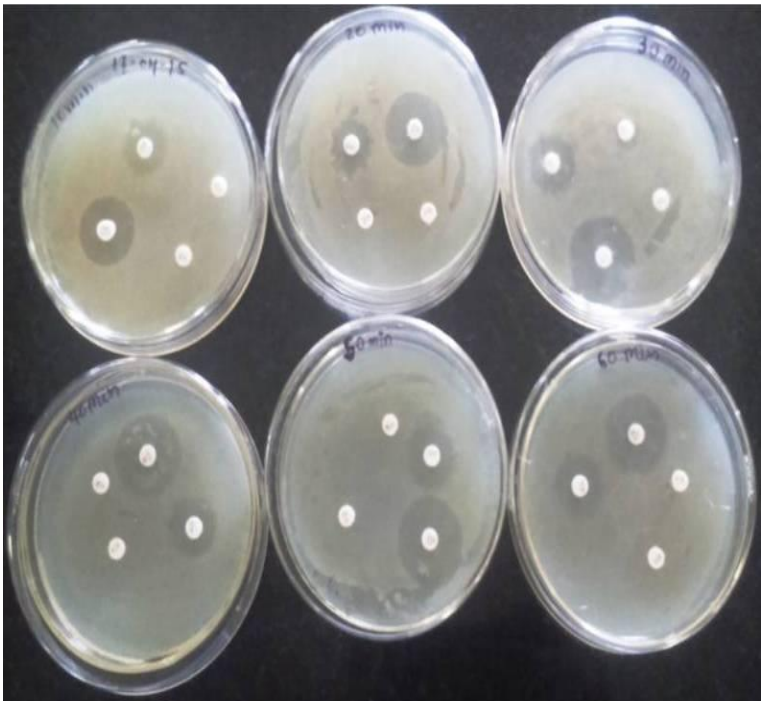


Fig.5

Antibiotic sensitivity and resistance test for EtBr mutated *rhizobium*

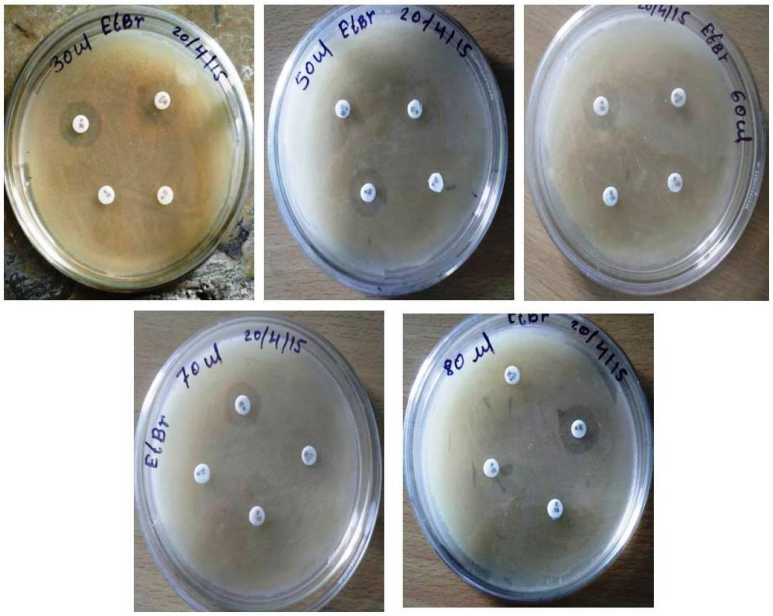


Fig.6

Antibiotic sensitivity and resistance test for Acrylamide mutated *rhizobium*

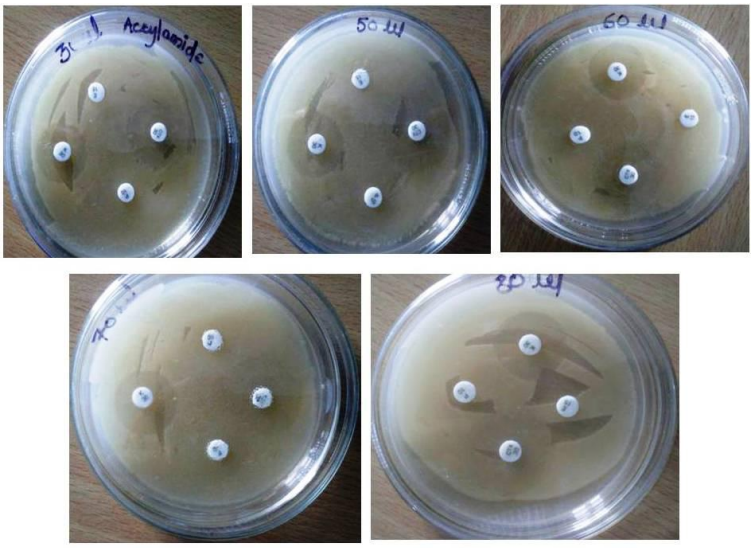


Fig.7

Antibiotic sensitivity and resistance test for TEMED mutated *rhizobium*

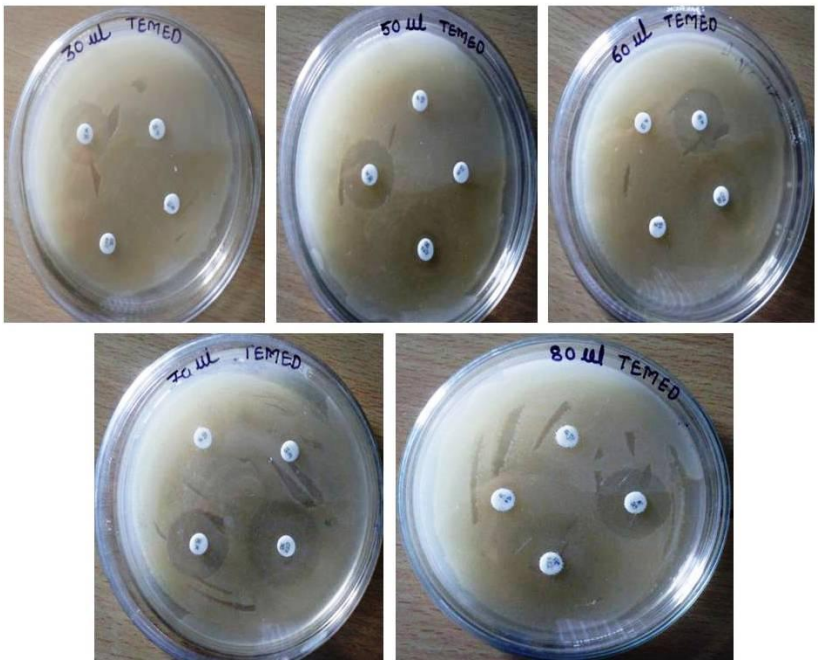


Table I

Time of UV exposure on *Rhizobium* & resulted zone of inhibition by antibiotics

Time of UV Exposure (Min)	Zone of Inhibition (mm)			
	Pencillin-G	Kanamycine	Cefotaxime	Erythromycin
10	-	19	20	-
20	-	-	25	20
30	-	-	15	10
40	-	-	25	2
50	-	19	27	-
60	-	15	20	-

Table II

EtBr exposure on *Rhizobium* & resulted zone of inhibition by antibiotics

EtBr Exposure (µL)	Zone of Inhibition (mm)			
	Pencillin-G	Kanamycine	Cefotaxime	Erythromycin
30	-	-	23	18
50	-	-	27	25

60	-	-	23	26
70	-	-	24	21
80	-	-	21	

Table III

Acrylamide exposure on *Rhizobium* & resulted zone of inhibition by antibiotics

Acrylamide Exposure (µL)	Zone of Inhibition (mm)			
	Pencillin-G	Kanamycine	Cefotaxime	Erythromycin
30	-	23	-	-
50	-	26	12	-
60	-	27	25	-
70	-	26	27	-
80	-	27	23	-

Table IV

TEMED exposure on *Rhizobium* & resulted zone of inhibition by antibiotics

TEMED Exposure (µL)	Zone of Inhibition (mm)			
	Pencillin-G	Kanamycine	Cefotaxime	Erythromycin
30	-	26	12	-
50	-	26	25	-
60	-	27	12	-
70	-	26	25	-
80	-	25	-	-

Discussion

The current work was mainly focused on the activity of *Rhizobium* with different mutagenic exposures. *Rhizobium* is the soil bacterium, which mainly helps in the process of nodulation in the plants. *Rhizobia* are responsible for nitrogen acquisition through symbiotic nitrogen fixation and are mainly observed in leguminous family members (Fujihara et al., 1994).

Mutations are the sudden, heritable changes that can cause damage or change the DNA sequence, which further result in the phenotypical modifications. Mutations can be caused by any physical mutagens (Ionizing radiations like Ultra violet rays, X-rays, gamma rays and alpha particles) or chemical mutagens (Sodium azide, bromine, alkaloids, benzene, etc.). These mutagens even in small quantities can determine differences at the cellular level as well as at the level of phenotypes.

In the current study, after collecting the *rhizobia* from groundnut and fenugreek roots, they were treated against various mutagens and observed for *rhizobial* response on seed growth, after the occurrence of mutations. The exact reasons may not be known, but the phenotypical variations resulted in the seed growth and antibiotic sensitivity and resistance were interpreted.

The physical mutagen, UV irradiation, when exposed to *rhizobia* at different time intervals, some changes were resulted in the seed germination, both in groundnut and mung bean. Similar results were observed in Siddiqui et al. (2011). The seeds of mung bean were exposed to ultra violet (UV-C) radiation for 0, 5, 10, 15, 20, 30 and 60 minutes for estimating growth parameters and root infecting fungi like *Fusarium spp*. UV-C exposure for 0-60 minutes showed reduction in root infecting fungi whereas UV-C exposure for 15, 20 and 30 minutes resulted significant increment of total chlorophyll and carbohydrate contents. Rathaur et al. (2012) showed that bacteria are particularly vulnerable to UV-B damage due to their effective cellular shading, small size limits, or protective pigmentation and their genetic material comprises a significant portion of their cellular volume. Results from field studies on Rhizobacteria indicates that exposure to natural solar UV-B radiation results in adecrease in total cell abundance, a reduction in amino acids uptake, a depression of the activity of degrading enzymes and a significant inhibition of protein and DNA synthesis.

Gunasekaran, A. and Pavadai, P. (2015), while studying groundnut (*Arachis hypogia*) var. VRI-2 treated with different concentration of physical and chemical mutagen namely gamma rays and Ethyl methane sulphonate (EMS). For inducing mutation various concentration of EMS such as 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 % for six hours were applied to 200 seed sample of each concentration and one respective control. The LD50 value was observed in 50% of gamma rays and 0.5 % of EMS. The mutagenized populations showed significantly higher variability in the M2 generation. In the current study, the groundnut and mung bean were treated with chemical mutagens. Dadarwal et al. (1980) showed that mutants with decreased and increased nitrogenase activity were derived from this strain by treatment with acridine orange and ethidium bromide. Even in the current study, it was found that acrylamide and ethidium bromide showed the similar results. The seeds treated with ethidium bromide resulted in blockage of the seed germination and never resulted any growth of any seed.

The antibiotics used in the current study are, Pencillin, kanamyxin, erythromycin and cefotaxime. In the current study, there is no action done by Pencillin against the mutated *rhizobium*. In the control, it is clearly showed that non-mutated *rhizobia* were susceptible to the action of pencillin. From the results, it is clear that, the mutations occurred due to the physical and chemical mutagens in the *rhizobia* are responsible for the development of resistance over pencillin. In respect to other antibiotics, *rhizobia* showed resistance in some cases and sensitivity in some cases. Erythromycin is able to show antibiotic action rarely. This result is supporting the work of Noor et al. (2012), but it is contrary to the work of Gauri et al. (2011), where erythromycin showed strong antibiotic activity. They showed that the wild type *rhizobia* with plasmid are capable of developing resistance against erythromycin. Out of all antibiotics, *rhizobia* showed sensitivity to mostly cefotaxime and followed by kanamycin.

The current study indicates that, the mutated *rhizobia* are capable of becoming resistant to some antibiotics, but not to all. The cell wall attacking antibiotics like Pencillin and cefotaxime are acting over mutated *rhizobia*. But, Pencillin is completely harmless to *rhizobia*, where as cefotaxime is highly degrading antibiotic. This area of research is of particular interest, as it is related to drug resistance.

The remaining two antibiotics, erythromycin and kanamycin in general, act on the protein synthesis machinery. But, here also, one antibiotic (kanamycin) is degrading the mutated *rhizobia* and the other is harming the mutated *rhizobia* rarely.

Conclusion

In the current study, *rhizobium* strains were isolated from groundnut and fenugreek root nodules and the *rhizobium* is used for the further experimentation. The *Rhizobia* were treated with physical mutagen (U.V. Irradiation) and chemical mutagens (Acrylamide, EtBr and TEMED) and these mutated *rhizobia* were used in nodule formation in groundnut and mung bean. Different results were observed as mentioned in the result section. Out of all, EtBr is found to be fatal to groundnut germination, where as the other mutagens showed normal growth.

The antibiotic sensitivity and resistance studies of mutated *rhizobium* are of great importance, as it is related to resistance development in the bacteria. In the current study, is is shown that the mutated *rhizobia* are capable of developing resistance against pencillin easily, when compared to the other antibiotics. The development of resistance in the mutated *rhizobium* can be given as: Cefotaxime < Kanamycin < Erythromycin < Pencillin.

Future aspects

The current study can give origin to the following future aspects: The molecular studies of nodulation process during the mutation can be studied; the antibiotic resistance studies of bacteria can be performed; the cell biology features of wild and mutated *Rhizobia* can be studied and the biological functions of wild type *Rhizobia* against the mutated *Rhizobia* can be studied.

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