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EFFECT OF MUTAGENIC TREATED *RHIZOBIUM* ON SEED GERMINATION, SEEDLING SURVIVAL, GROWTH AND NODULATION OF *TRIGONELLA FOENUM-GRAECUM* L.

NEETU MANGLANI
Department of Botany, Govt. College, M.D.S. University, Ajmer-305001, India.

Effect of alkylating agent Diethyl sulphate (DES mutagen) on *Rhizobium* isolate from the root nodules of *Trigonella foenum-graecum* L. to characterize and evaluate their effectiveness under sterile soil condition and its effect on seed germination, seedling survival, growth and nodulation of *Trigonella foenum-graecum* L. was studied. Isolate was used as control strain and pure culture was obtained in YEMA medium as white, milky, glistening colonies at 28±1°C. The *Rhizobium* isolate was rod shaped, Gram's negative and motile. It utilizes glucose and starch as sole carbon source, produce indole, ammonia and H_2S, show catalase and oxidase activity and reduce nitrate. Strain show sensitivity toward amphicilin, basitracin, erythromycin, neomycin, piperacillin, streptomycin, and sulphafurazole. To study the effect of mutagen, rhizobial strain was treated with 0.5% concentration of DES: The growth of seeds were assessed in term of germination index and seedling survival percentage after the inoculation of control and treated *Rhizobium* in the pots seeded with sterilized seeds of *Trigonella foenum-graecum* L. The data of root/shoot length, size and shape of nodules, number of nodules per plant were studied from uprooted plants. Seed germination was 100%, seedling survival 100%, maximum number of nodules and optimum growth in terms of root-shoot length of seedlings were found with control strain of *Rhizobium.* DES treated *Rhizobium* strain was not found to be effective for enhancing seed germination (78%), seedling survival (85%) and number of nodules. Treated *Rhizobium* strain also produced indole and H_2S, utilized more glucose and starch and showed high catalase and oxidase activity. The sensitivity of treated *Rhizobium* decreased towards amphicilin, pipercillin, streptomycin, sulphafurazole, and neomycin.

Keywords: Diethyl sulphate; *Rhizobium; Trigonella foenum-graecum* L.

Introduction

Alkylating agent (mutagen) *i.e.* Diethyl sulphate is most known chemical mutagen used for the study of different plant mutagenesis[1]. DES is grouped as mono functional alkylating agent and transfers its alkyl group to DNA. It directly alkylate DNA rather than other compounds[2]. The mutational observation and studies are more useful in genetic plant breeding for the improvement of crop economically and agronomically. This chemical mutagen also induces chromosome mutation, chromosome break, point mutation and breaking of sugar phosphate bond. *Rhizobium* of *Trigonella foenum-graecum* L. belongs to fast growing, motile, rod shaped Gram's negative, motile, acid and mucous producing bacteria[3]. It can grow in a wide range of carbohydrate but usually grow best on glucose, mannitol and sucrose. It is also an aerobic, mesophilic and chemoheterophilic soil inhabitant. *Rhizobium* through nodule formation infect underground roots of the legumes and fix atmospheric nitrogen through symbiotic process[4]. The beneficial effect of *Rhizobium* and *Bradyrhizobium* in legume in terms of biological nitrogen fixation has been a main focus in the recent past[5,6], as it is an important aspect of sustainable and environmental friendly food production and long term crop productivity. Inoculation with highly effective rhizobia is a common practice in agricultural[7], which increases the biomass of plant and seed production[8] and also requires survival and establishment of inoculated rhizobia in the soil environment[9].

In the present study, DES mutagen was used to treat *Rhizobium* isolate from the root nodules of *Trigonella foenum-graecum* L. to observe its effect on seed germination, seedling survival, growth and nodulation of *Trigonella foenum-graecum* L. The cultural and biochemical characteristics and the effect of several antibiotics on *Rhizobium* were also observed.

Material and Methods

Plant of *Trigonella foenum-graecum* L. was selected for study and rhizobial isolate treated with 0.5% concentration of Diethyl sulphate (mutagen).

Isolation of rhizobia from root nodules (Control Strain)- Fresh and standard seeds of fenugreek were grown in different pots. After 25 days, seedlings of *Trigonella foenum-graecum* L. were gently uprooted. Turgid, unbroken, healthy and pinkish root nodules were cut from thoroughly washed roots of plants. Root nodules were immersed in 0.1 % (w/v) mercuric chloride for surface sterilization and then repeatedly washed with sterile water. Nodules were crushed in sterilized water and 0.1 ml was spread on YEMA medium plates (Yeast extract,0.5g; Mannitol,10g; K_2HPO_4 0.5g; $MgSO_4.7H_2O$ 0.2g; NaCl 0.1g; Agar 20g/L, pH 7.0 with Congo red dye). Plates were incubated for 3 days at 28±1°C. After 3 days white, milky, glistening colonies of rhizobia appeared which was re-streaked to obtain pure discrete cells of *Rhizobium.* Isolates were stored in YEMA slant at 4°C for further study.

Treatment of Rhizobium isolate with Diethyl Sulphate (0.5% concentration)- YEM broth with 0.5% DES concentration was prepared, 1ml of rhizobial suspension was inoculated in YEM broth and incubated for 3-5 days at 28±1°C.

Soil Sterilization - Garden soil was taken from Botanical garden of Govt. College, Ajmer and sterilized at 121°C and 15 psi for 3 consecutive days.

Seed Sterilization- Dry pure viable seeds of *Trigonella foenum-graecum* L. were surface sterilized with 0.1 % (w/v) mercuric chloride solution for 3 min. The seeds were washed thoroughly with sterilized water. Surface sterilized seeds were than soaked in sterilized water for 6hr at room temperature.

Sowing of Seed - Surface sterilized and presoaked seeds of *Trigonella foenum-graecum* L. were transferred in pots containing sterilized soil (20 seeds in each pot).

Inoculation of Rhizobium (control and treated) - Incubated control and treated rhizobial growth was transferred in sterilized water separately and this rhizobial suspension was inoculated in equal amount *i.e.* 10ml of rhizobial suspension in all the pots. This process was repeated on every alternative day for 24 days.

Seed Germination- Seed germination was calculated in term of Germination index.

$$\text{Germination Index (GI)} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds sown}} \times 100$$

Seedling Survival Percentage - The growth of seeds was assessed in terms of seedling survival percentage (SSP).

$$\text{Seedling survival percentage (SSP)} = \frac{\text{No of seedling survived}}{\text{Total no. of seeds sown}} \times 100$$

Growth - Growth is assessed in terms of root/shoot length.

Nodulation - Induced plants from both control and treated *Rhizobium* were uprooted after 25 days of their emergence. The root system was washed with water subjected to nodulation studies. The data on root/shoot length, number of nodules per plant, size and shape of nodules were collected from uprooted plants.

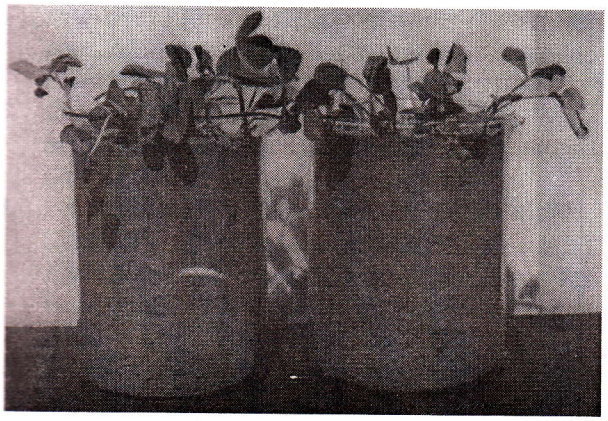
Morphological, Cultural and Biochemical Analysis - The morphological traits evaluated comprised colony morphology, and motility in growth medium. Mucous morphology analysis was based on type and appearance, while colony morphology parameters were diameter, form, transparency and color[10]. Gram staining reaction was performed to evaluate type of strain. For cultural analysis growth was evaluated in Hofer's alkaline and glucose peptone agar medium and the biochemical characteristics were observed by gelatin liquification, nitrate reduction, H_2S, indole and ammonia production, oxidase and catalase activity, and starch hydrolysis.

Antibiotic Sensitivity - Amphicilin, bacitracin, cefixine, erythromycin, pipercillin, penicilin, streptomycin, sulphafurazole and neomycin were used to evaluate the sensitivity of both strains. Rhizobial suspensions of the isolates were prepared in YEM broth with same optical density. 0.1ml of the suspension was evenly spread in plates containing YEMA medium. The commercially used antibiotic disc were aseptically placed (in triplicate) on the medium and incubated at 28±1°C for 3-5 days.

Results and Discussion

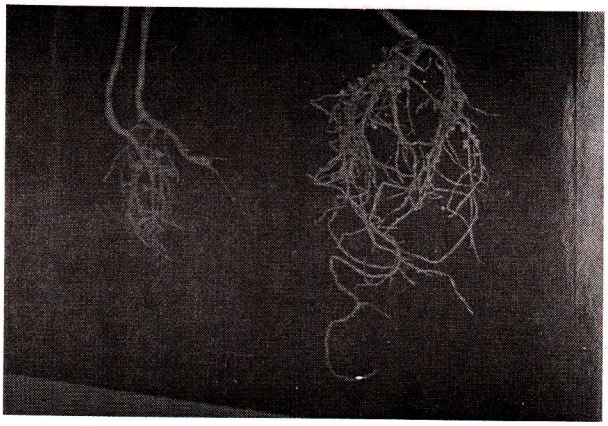
Rhizobial isolate was obtained from the root nodules of *Trigonella foenum-graecum* L. and characterized for their effectiveness under sterile soil condition for plant growth, seed germination, seedling survival and nodulation. *Rhizobium* was Gram's negative, fast growing, motile, grow on YEMA medium after 2-3 days of incubation. Colonies of *Rhizobium* were circular, glistening, diameter 5-7mm, creamish, white/light pinkish, translucent, small, smooth margin, convex and having mucilaginous appearance. Similar observations related to slow growing genus *Breadyrhizobium,* isolated from root nodules of *Glycine max* L. and *Glomus manihotis* plant were given earlier[11,12]. *Rhizobia* were identified on the basis of relative growth[13] and also on the basis of morphological features, growth requirement, physiological and biochemical activities[14].

The data obtained from the uprooted *Trigonella*



Control Treated

Fig.1. Pots showing seedlings after the inoculation control and treated rhizobial suspension.



By treated strain By control strain

Fig.2. Nodule size and shape and Root/ Shoot length of *Trigonella* grown with treated and control rhizobial strain.

Table 1. Effect of treated *Rhizobium* on seed germination, seedling survival and nodulation of *Trigonella foenum-graecum* L. (Average of 30 pots)

Rhizobium	Germination Index %	Seedling Survival %			Growth		Nodules per plant
		No. of seed sown	No. of seedling survived	SSP (%)	Root Length (cm)	Shoot Length (cm)	
Control	100	20	20	100	16.5±0.18	31.5±0.5	27±2
Treated	78	20	17	85	15.6±0.10	29.7±0.10	25±2

plant showed that the treated *Rhizobium* reduces the germination index (Table 1). The recorded observation revealed that growth index in control was 100% while it decreased 22% by using treated strain. It was also observed that germination of seeds was delayed by treated strain. Change in morphological characters in *Trigonella foenum-graecum* L. due to chemical mutagen were studied byJain and Agarwal[15] and growth analysis of plant were given by Anand and Sharma[16]. There was reduction in seedling survival % in plants induced by treated strain. The toxic effect of mutagen on seed germination and seedling growth were observed by Motto *et al.*[17] in *Phaseolus vulgaris* and by Khan *et al.*[18] in *Phaseolus aureus* Roxb. (mungbean). Treated *Rhizobium* also reduced the effectivity of plant[19].

The data collected for root/shoot length and nodulation indicate that the treated strain retard the length of root/shoot and size of nodules (Fig. 2). In Euphorbiaceous plant the effect of alkylating agent on germination, branching and plant height were given by Chauhan and Kumar[20]. Treatment of *Rhizobium* with DES mutagen reduces the number of nodules in *Trigonella* plant (Table 1). Influence of physical and chemical mutagen on nodule characters was also reported earlier[21-23]. The effect of strong irradiation with gamma rays on *Rhizobium* isolated from *Pisum sativum,* was recorded by Homatava[24] which leads to poor nodulation in plants. Effect of induced mutant sp. of *Rhizobium* in *Vigna aconitifolia* L. and *Trigonella corniculata* L. were studied by Bhalla[25].

Rhizobium was motile in peptone agar medium, show cloudiness around stab. Studies on cultural and biochemical characteristic of *Rhizobium* were also done by Gaur[26] with the same observation. Identification, authentication and efficiency of bradyrhizobial strain were given by Mahna *et al.*[11]. Similar characteristics of *Rhizobium* isolated from *Trigonella corniculata* L. and *Vigna mungo* L. was given by Raisinghani[27]. Both control and treated strain were able to grow on gelatin peptone agar and Hofer's alkaline broth (pH=10)[28]. Strains also showed positive reaction towards gelatin liquification, nitrate reduction, H_2S and indole production (Table 2). Control and treated strains were positive towards catalase and oxidase activity. Strains easily hydrolyzed starch and

Table2. Morphological, cultural and Biochemical characteristics of *Rhizobium* under control and treated condition.

S. No	*Rhizobium* Isolates Tests	Control	Treated
1	Gram's Reaction	-ve	-ve
2	Motility Test	++	+++
3	Growth on YEMA medium	++	+++
4	Growth on CYEMA medium	++	+++
5	Hofer's Alkaline medium test	++	+++
6	Glucose Peptone Test	++	+++
7	Gelatin liquification	++	++
8	Catalase Activity	++	++
9	Hydrolysis of Starch	++ (0.1)	+++ (0.3)
1 0	Nitrate Reduction	++	++
11	H_2S Production	++	+++
12	Indole Production	++	+++
13	Oxidase Activity	++	+++
14	Ammonia Production	++	++

Table 3. Sensitivity of *Rhizobium* isolates against various antibiotics.

Rhizobium / Antibiotic	Control	Treated
	Size of Inhibition zone(cm) around disc containing 10µg of antibiotic	
Amphicilin	0.2	0.1
Bacitracin	0.3	0.3
Cefixine	R	R
Erythromycin	0.2	0.2
Pipercillin	0.7	0.6
Penicilin	R	R
Streptomycin	1.3	1.0
Sulphafurazole	1.6	1.2
Neomycin	0.5	0.4

R= Resistant toward antibiotics

the colourless zone observed around the colony was 0.1mm for control and 0.3mm for treated. Ammonia was produced by both the strains. The growth pattern, acid production and carbohydrate utilization by *Rhizobium* was also studied earlier[29].

Both treated and control rhizobial isolates were resistant toward penicillin and cefixine. Control strain was more sensitive toward streptomycin, sulphafurazole and less toward bacitracin, pipercillin, and erythromycin (Table 3). Sensitivity of *Cicer arietinum* (chickpea) *Rhizobium* towards tetracycline, penicillin, vinomycin, and streptomycin were studied by Verma and Dadarwal[30]. Streptomycin resistant *Rhizobium* sp. was isolated from *Braziliaz carrador* by Scotti and Doberiner[31]. The sensitivity of treated *Rhizobium* decreased towards amphicilin, pipercillin, streptomycin, sulphafurazole, and neomycin. Sensitivity of the bacterial isolates towards a specific antibiotic was studied by Baur[32] and determined the zone size on the basis of PSADST[33].

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