Executive summary
UGC Minor Research Project
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Title: -"Exploitation and Potential of *Rhizobium japonicum* for Soybean growth promotion"

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The Soybean (*Glycine max*) is commonly called "Cinderella" crop or "King of Legumes". *Rhizobium japonicum* are the symbiotic microorganisms which form root nodules on the roots of Soybean and fix the chemically locked atmospheric nitrogen in these root nodules and provide to the plants. Farmers use chemical fertilizers to promote the growth of soybean, which are costlier and also affecting the natural flora of soil. So to reduce the use of chemical fertilizer, it is necessary to exploit and screen the potential of the naturally occurring microorganism, *Rhizobium japonicum* for soybean growth promotion. The commonly cultivated soybean variety selected for the investigation was JS 335.

The first part is includes: Isolation of *Rhizobium japonicum* and screening for the potential of growth promoting abilities of *Rhizobium japonicum.*

R. japonicum is isolated from fresh, healthy, unbroken and pink nodules. For identification of Rhizobium spp. CRYEMA Test, Microscopic Observations. Glucose peptone agar test (GPA test), Salt tolerance test and Lactose test. On the CRYEMA medium, white coloured colonies of *Rhizobium spp.* were isolated. Gram negative bacteria were observed. The cells showed the stained PHB (poly β-hydroxylbutyrate) granules when stained with carbol fuschin. *Rhizobium spp.* is unable to grow on GPA medium. There is no growth of *Rhizobium spp.*on the YEMA medium containing 2% NaCl. *Rhizobium spp.* does not grow on the lactose agar.

Rhizobium japonicum screened for the plant growth promoting activity by assaying attributes like Indole Acetic Acid (IAA)), Phosphate solubilization and ammonia production.

Isolates were inoculated in nutrient medium supplemented with 500 µg/ml of tryptophan, a precursor of indol-3-acetic acid (IAA). Culture was incubated on rotary shaker at 28°C for 7 days. IAA was detected by a method described previously (Gordon and Weber, 1951). Bacterial cells were removed from the culture medium by centrifugation (10,000 rpm, 15 min).

A 2-ml aliquot of the supernatant was acidified with two drops of ortho-Phosphoric acid and mixed with 4 ml of Salkowski's reagent (50 ml, 35% Perchloric acid +1 ml 0.5% $FeCl_3$) and allowed to stand at room temperature for 20 min. After 20 minutes, pink colour was observed which indicated that *Rhizobium japonicum* was producing IAA.

Isolates were spot inoculated on Pikovskaya Agar (Pikovskaya, 1948) for detection of their phosphate solubilizing ability and incubated at 37°C for 48 hours. There was development of clear zone around the growth of *Rhizobium japonicum* on Pikovskaya Agar This indicates that

Rhizobium japonicum is having the ability to secrete the Phosphatase enzyme. This enzyme solubilizes the phosphate around itself and provide to the plant. That is by dissolving the phosphate; it supports the growth of the host soybean plant.

Rhizobium is a genus of Gram negative bacteria that fixes atmospheric Nitrogen symbiotically i.e. in association with the soybean plant root. *Rhizobium* forms an endosymbiotic nitrogen fixing association with roots of legumes. *Rhizobia* colonize plant cells within root nodules; here the *Rhizobium spp.* Convert atmospheric Nitrogen to ammonia and provide organic nitrogenous compounds such as glutamine or ureides to the plant. The plant provides the bacteria with organic compounds made by photosynthesis. *Rhizobium Spp.* fixes atmospheric Nitrogen from the air into ammonia which acts as a natural fertilizer for the plants. This increases the plant productivity without using chemical fertilizers. For testing the ammonia production, two sterile tubes containing 10 ml of peptone water were taken, one acts control and other acts as test. In test, *Rhizobium spp.* culture was inoculated. The tubes were kept at 37 °C for 48 hours for incubation. After incubation there was appearance of yellow colour after addition of Nessler's reagent in the test as shown in photo plate 3. This indicated that *Rhizobium spp.* is having the ability to produce the ammonia. This production of ammonia ability is the plant growth promoting character of *Rhizobium sp.*

1: Isolation of *Rhizobium*

2: Phosphate Solubilization Testing

3: Ammonia Production Testing

Photo Plates

The second part includes the experimental work: Screening for the biocontrol abilities of *Rhizobium japonicum* and growth promotion of soybean by using *Rhizobium japonicum.*

The biocontrol testing exerts a protective effect on roots through antagonism towards rhizospheric fungi as well as promotive effects of *Rhizobium japonicum* towards the soybean plant. These Biocontrol characteristics include: HCN detection, testing of antifungal activity, Siderophore. Testing of growth promotion ability done by the pot assay.

This HCN production ability of *Rhizobium japonicum* is the biocontrol mechanism. This HCN production inhibits the host pathogen and protects the host. Hydrogen cyanide (HCN) is known to effectively block the Cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. HCN forms stable complexes with several essential divalent metal ions Culture was grown at room temperature (28^0C) on a rotary shaker in Kings B broth.Whatmann Filter paper No.1 was cut into uniform strips of 10cm long and 0.5cm wide saturated with alkaline picrate solution and placed inside the conical flask in a hanging position. After incubation at 28^0C for 48 hours, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. Hydrogen cyanide (HCN) is known to effectively block the Cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. HCN forms stable complexes with several essential divalent metal ions Culture was grown at room temperature (28^0C) on a rotary shaker in Kings B broth. Whatmann Filter paper No.1 was cut into uniform strips of 10cm long and 0.5cm wide saturated with alkaline picrate solution and placed inside the conical flask in a hanging position. After incubation at 28^0C for 48 hours, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to to the amount of hydrocyanic acid evolved. After incubation, the Whitman Filter paper No.1 strips saturated with alkaline picrate solution show the weak reaction (yellow to light red) red due to less hydrocyanic acid evolution The *Rhizobium japonicum* was less efficient in HCN production. Due to this HCN production ability, Rhizobium is used in agriculture as a component of bioinoculants in the form of plant growth promoting rhizobacteria (PGPR).

For the testing of *Rhizobium japonicum against* the fungi**,** different fungi were isolated from the rhizospheric region of soybean root nodule. Fungal cultures were identified on the basis of cultural characteristics by mounting with cotton blue. The various fungi observed in the rhizospheric region of soybean were the species of *Alternaria, Aspergillus, Curvularia, Helminthosporium*, *Mucor*, *Penicillium*. *Periconia* and *Trichoderma.* Testing of *Rhizobium japonicum against* the fungi isolated was done by dual culture technique on king's B agar. The percent inhibition of fungal pathogen growth in presence and absence of bacterial *Rhizobium japonicum* was noted down. The percent inhibition was calculated using the following formula:

Percent inhibition= (C-T) X 100/C

C=Radial growth of fungus in mm in control plate and

T= Radial growth of fungus in mm on plate inoculated with *Rhizobium japonicum.*

The results were expressed as in the table No.1. *Rhizobium japonicum* was causing maximum percent inhibition in *Cladosporium spp.*and minimum inhibition in the *Periconia spp*.

Table 1: Dual Culture Technique:

S/No	Name of the Fungus		Radial growth of fungus in mm											Percent inhibition
			Days of incubation											
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean**	
1	*Alternaria spp.*	C	11	15	25	30	32	34	36	38	41	44	30.4	21.0526
		T	7	12	15	24	26	27	29	31	33	36	24.0	
2	*Aspergillus spp*.	C	11	15	22	28	32	35	37	42	48	56	32.6	11.6564
		T	9	14	19	25	29	34	36	38	41	43	28.8	
3	*Cladosporium spp.*	C	12	17	19	38	48	59	74	85	90	90	54.2	69.5572
		T	8	9	10	11	12	18	22	26	28	31	16.5	
4	*Curvularia spp*.	C	12	22	33	39	41	43	45	48	51	55	38.9	21.5938
		T	10	12	20	24	33	36	38	41	44	47	30.5	
5	*Helminthosporium spp.*	C	10	14	25	29	34	39	41	43	46	49	32.0	22.1875
		T	9	21	22	23	24	25	27	29	33	36	24.9	
6	*Mucor spp.*	C	10	16	23	34	39	49	54	65	78	90	45.8	44.7598
		T	9	10	11	18	23	27	34	37	37	37	25.3	
7	*Penicillium spp*.	C	9	12	16	18	21	24	27	29	33	36	22.5	16.6078
		T	8	10	14	16	17	19	21	23	24	26	17.8	
8	*Periconia spp*.	C	9	9	10	10	11	12	12	13	15	16	11.7	9.4017
		T	8	9	9	10	10	11	11	12	13	13	10.6	

Rhizobium spp was isolated and tested for the production of siderophore.Siderophores are low molecular weight high-affinity ferric iron chelators,are synthesized and secreted by many microorganisms in response to iron deprivation. These compounds solubilise and bind iron and transport it back into microbial cell, usually through specific membrane receptors. Siderophore production was studied using modified succinate medium consisting (gm/l) Succinic acid(4),K_2HPO_4(3),NH_4SO_4(1),$MgSO_4$(0.2)and pH(7.0).0.1ml of inoculum was separately inoculated in 250ml Erlenmeyer flask containing succinate medium and then incubated on rotary shaker incubator for 48 hrs at 28^0C.After incubation, supernatant was harvested by centrifugation of the culture at 10000 rpm in cooling centrifuge at 4^0C for 10 minutes. The supernatant was used for the qualitative detection of siderophore as: 0.5ml culture supernatant was mixed with 2.5 ml Ferric perchlorate reagent. Orange to purple complexes will appear. Orange to purple complexes will appear if the test is positive. There is no colour change. That means the test is negative. The *Rhizobium* species is not having the Siderophore production ability.

Production of extracellular protease was tested by streaking *Rhizobium japonicum* isolate on Skim milk agar plate. Skim milk agar plate was spot inoculated by *Rhizobium japonicum* and incubated overnight for production of proteases. There was production of a halo zone around the colony .This shows that *R.japonicum* was protease positive. These organisms are having the ability to produce a proteolytic exoenzymes, called protease .This enzyme breaks the peptide bond CO-NH by introducing water into the molecule, liberating smaller chains of amino acids called peptides, which are later broken down into free amino acids . This protease production is the antagonistic activity of *R.japonicum*.

In the sulphate reduction test, hydrogen sulphide production is detected. Hydrogen sulphide acts as an electron doner for CO_2 reduction in photosynthesis. Photosynthetic bacteria oxidise hydrogen sulphide to elemental sulphur.Organic compounds are dehydrogenated and hydrogen is used for the reduction of sulphate to H_2S. A loopful of culture in a cysteine broth tubes containing 100 ml peptone water with 0.1gm cysteine was inoculated. A lead acetate filter paper strip was aseptically inserted in the cysteine broth. The tubes were incubated at 37^0C for 24 hours. In the control tube, the culture was not inoculated. After incubation blackening of lead acetate filter paper strip indicated liberation of H_2S due to reduction of Sulphate. This indicates that Rhizobium species are having the role in sulphate reduction. Cysteine by the activity of desulfurase enzyme secreted by *R.japonicum* liberates H_2S gas. This H_2S reacts with lead acetate to form black colour.

Photoplate No.4

Growth promotion of soybean by using *Rhizobium japonicum* was tested by Pot Assay. Pot experiment was performed to evaluate the effect of inoculation of *Rhizobium japonicum* strain on biomass and crop yield of soybean. Sterilized garden soil and sterilized sand in the ratio of 2:1 was used for the experiment. The different variety seeds were treated with respective *Rhizobium japonicum* isolated. This is done by mixing isolated rhizobia culture in 10 percent sugar and 40 percent gum arabic to form a slurry. In this seeds were added. With the result, a uniform coat of the *R japonicum* is formed around the seeds. The treated seeds were dried in shade and sown immediately .In the test pot five coated seeds were sown. In the control pot the uncoated five seeds were sown. The root nodules, roots and shoots were harvested on 60^{th} day after soybean sowing and dry weight was recorded. The roots were washed with water to remove the soil particles attached. Nodules formed, Shoot length as well as root length was recorded in cm and the dry weight was measured in grams.

Table2: The effect of *Rhizobium japonicum* on soybean

S/No.	Soybean Variety		Length in cm.		Dry weight in grams		Number of root Nodules
			Shoot	Root	Shoot	Root	
1	JS 71-5	Control	22.8272	10.8454	0.1625	0.0325	23
		Test	26.6167	14.0000	0.17625	0.0533	34
2	JS 335	Control	23.6572	11.1214	0.17225	0.0421	45
		Test	27.4521	16.2345	0.1843	0.0682	59
3	JS93-05	Control	21.9740	8.4200	0.1475	0.0315	12
		Test	22.1200	7.0000	0.1120	0.0240	23

Data obtained showed that soybean variety JS 335 showing more shoot length, root length, dry weight and also more root nodule number as compared to JS 71-5 and JS93-05 varieties of soybean.

CONTROL *TEST*

SOYBEAN VAREITY – JS 71-5

Photoplate No.5

CONTROL *TEST*

SOYBEAN VAREITY – JS335

Photo plate No.6