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Isolation and Characterization of Rhizobium form Legume Plant

Dr. C. Vinothini[1], Dr. Kadhar Nivas[2]

[1]*Assistant Professor, Department of Microbiology, E.G.S. Pillay Arts and Science college, Nagapatinam.*
[2]*Assistant Professor, Head, P.G. Research Department of Biotechnology Department of Microbiology, E.G.S. Pillay Arts and Science college, Nagapatinam*

***Abstract*-- *Rhizobium* plays an important role in agriculture by including Nitrogen-fixing nodules on the roots of legume plants. The present study describes the physiological and biochemical characterization of *Rhizobium* species isolated from root nodules**

The *Rhizhobium* sp. were rod shaped, gram negative acid and mucous producing. *Rhizobium* isolated from root nodules of leguminous plants were characterized for the presence of Nif genes. Three separate experiments were conducted to study the effect of Rhizobium using slurry soil as a growing medium. First experiment was conducted using seeds of *Vigno mungo* coated with slurry by paper sheet experiment. Second was done using *Arachis hypogeae* using slurry soil from *Vigno mungo* and third was conducted using *Citrus aurantifolia* which was amended with slurry soil from *Arachis hypogeae*. In all these three experiments the growth of plants such as leaves, roots and shoot length were significantly increased when compared to unamended soil. Genomic DNA from *Rhizobium* was isolated from all these soil samples and it was amplified by PCR to analyse the presence of Nif gene.. *Rhizobium* sp. was unable to

hydrolysis the gelatin and showed sensitivity against different antibiotics such as tetracycline, kanamycin and streptomycin. The isolates were also showed demonstrable antibacterial activity against *Streptococcus* sp., *E.coli* and

***Pseudomonas* sp. The 16S rRNA gene is widely used for bacterial identification and phylogenetic comparison. A 16S rRNA result should be based on actual sequence data, quality checking, comparison against a curated database, and transparent reporting of sequence length, identity, coverage, and accession information. Detection of a nif PCR band alone does not prove gene expression or quantify nitrogen-fixation activity.**

***Keywords*-- Rhizobium, Nif genes, Genomic DNA, PCR**

I. INTRODUCTION

Nitrogen is available in atmosphere in high amount (78%) in the form of gas. It is converted into high organic compounds by some prokaryotic microorganism through biological reactions. The phenomenon of fixation of atmospheric nitrogen by biological reactions, means is known as biological nitrogen fixation and these prokaryotes are known as diazotrophs or nitrogen fixers.

Beijerinck (1888) isolated *Rhizobium* from root nodules of leguminous plants. *Rhizobium* is a symbiont, gram negative, non sporulating, aerobic, motile rod shaped bacteria which resides in soil and is capable of infection, nodulation, establishing symbiosis and nitrogen fixation. *Rhizobium* is more prominent in the rhizosphere of leguminous plants. *Rhizobium* induces nodules in roots of leguminous crops that fix nitrogen from atmosphere into a form of nitrogen that the plants can use. This conversion is called nitrogen fixation (Amijee *et al.,* 1998).

Seed inoculation of *Rhizobium* is done by mixing its culture with 10% sugar and 40% gum Arabic to form slurry to which seeds are added. As a result a uniform coat of *Rhizobium* culture is formed around the seeds. These seeds are immediately dried in shade and sown there after. It is used as biofertilizers to reduce the need of expensive chemical fertilizers. Growth promoting diazotrophs can enhance the growth and development of associated crops by transferring fixed nitrogen or by improving nutrient uptake through nodulation of hormone linked phenomena in inoculated plants. Certain strains of *Rhizobium* can promote plant growth and yield most likely through mechanisms that involve changes in growth physiology or root morphology rather than biological nitrogen fixation (Ladha *et al.,* 1998).

The complex of genes in nitrogen fixing bacteria that code for the proteins required for nitrogen fixation, particularly nitrogenase. Nif gene is the specific activator for nitrogen fixation. Nif gene of *Rhizobium* consists of 17 identified genes arranged in eight. 15 of these genes are involved in the formation of an active complex of four proteins, capable of reducing atmospheric nitrogen to ammonia. The expression of these genes is activated by the nif A gene product (Dixon *et al.,* 1980) and the function of this activator is repressed by nif L protein (Merrick *et al.,* 2004). In the present study, *Rhizobium* isolated from root nodules of leguminous plants were characterized for the presence of Nif genes.

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II. Review Of Literature

2.1 Sample collection: Soil samples and root nodules were collected from different area like Chrompet, Tambaram, Vadapalani, T. Nagar, Pallavaram, Avadi, Guindy, Kodampakam, Nungambakkam, and Anna Nagar from Chennai, Tamil Nadu, India.

2.2. Isolation of bacterial isolates: Root nodules collected were washed with sterile distilled water to remove soil particles. Nodules were externally sterilized by mercuric chloride and then crushed using sterile distilled water and suspension was streaked on Petri plates containing yeast extract mannitol agar medium (YEMA). Isolation and enumeration of bacteria from root surface soil were performed by standard plate count technique using YEMA medium (Amarger *et al.,* 1994). All the plates were incubated for 3 – 4 days at 28 - 30 °C. After incubation white, translucent, glistering and elevated with entire margin colonies appear on the surface of medium were selected and purified for further studies (Vincent, 1970).

2.3. Identification of bacterial isolates: Suspected colonies of *Rhizobium* grown on YEMA medium were identified by Congo Red Yeast extract mannitol agar (CRYEMA) media. The isolated bacteria were identified by biochemical methods which include indole production test, MR – VP, citrate utilization test and catalase test. The organisms were identified as *Rhizobium.* Then they were sub cultured in CRYEMA and incubated at 28 - 30°C for 3 – 4 days. Pure cultures were stored at 4°C until use.

2.4. Biochemical characterization of Isolates:

The biochemical traits evaluated comprised change in pH of medium during growth and growth rates, temperature etc.

2.5. pH variation Assay: To analyzed the effect of pH variation on the growth of microorganism, media were prepared with pH 4.0 and 9.0. After incubation of 48 hours growth was determined by measuring the optical density at 620 nm.

2.6. Temperature Tolerance: Tolerance to high temperatures was tested by typing on YEM broth and incubating at 37, 40, 42, and 45ºC.

2.7. NaCl Variation Assay: Rhizobium cultures were grown on YEMA medium having different concentrations of sodium chloride ranging from 0.4 to 0.6%. Growth was determined by measuring the optical density at 620nm after 48 hours of incubation.

2.8. Gelatin Hydrolysis: The test was performed to determine capability of *Rhizobium* to produce gelatinase enzyme as use gelatin as media source. Degradation of gelatin Available online at http://www.urpjournals.com

2.9. Soil analysis: Collect control and amended soil using the same sampling depth and handling procedure, with independent replicates. A suitable analysis plan may include soil pH, electrical conductivity (EC), organic carbon/organic matter, available nitrogen, available phosphorus, exchangeable potassium, and moisture; add texture or micronutrients if relevant to the study question. Use validated laboratory methods and report the method, units, sample number, mean ± standard deviation, and quality-control procedure for each parameter. No numerical soil chemistry values were present in the supplied source, so no soil results can be reported here.

2.10. Seed inoculation with Rhizobium: Rhizobium culture was inoculated in CRYEMA medium and incubated at 28°C for 7 days. Using a needle the growth of the bacterium is scrapped and it was suspended in water. 50 g of sugar solution is taken and dissolved in 500 ml of distilled water. The solution was heated for 15 min. 200 g of gum Arabic was added to this solution. This was allowed to cool at room temperature and the inoculum was mixed to make slurry (Killpack *et al.,* 1993). *Vigno mungo* seeds were added to the slurry till it gets coated completely. The seeds were then transferred on to a paper sheet containing soil and it was dried in shade. The seed was then sowed in field and nodulation was observed (Dazzo *et al.,* 1995). Then the same slurry soil (*Vigno mungo* growing soil) was spread on the field and *Arachis hypogaea* was grown on that field as well as the same *Arachis hypogaea* grown on normal field. The seed was sowed in the field and observed for nodulation. The same slurry soil (after *Arachis hypogaea* growing soil) was transferred to the paper sheet and the *Citrus aurantifolia* was grown as well as *Citrus aurantifolia* grown on normal soil. The seed was sowed in the field and observed for growth and nodulation.

2.11. Isolation of genomic DNA: Rhizobium was isolated from all these soil samples. The isolated cultures were inoculated in the fresh CRYEMA broth which provides the rich turbidity growth. The inoculated broth was incubated at 28 – 30°C for 3 – 4 days. After sufficient growth was achieved genomic DNA was extracted from *Rhizobium* (Rajagopal, 2005) it was amplified by PCR to analyse the presence of Nif gene (Pillai *et al.,* 1991).

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The source reports growth of isolates in CRYEMA broth, genomic DNA extraction, and PCR screening for nif, with an approximately 450-bp band described. A complete molecular methods section should specify isolate IDs, DNA extraction kit/protocol, primer sequences, reaction mixture, cycling conditions, positive and no-template controls, DNA ladder, gel concentration, stain, and imaging system. The expected amplicon size should be tied to the actual primer pair. A band of the expected size is preliminary evidence only; sequence confirmation or another validated assay is recommended.

2.12 16S rRNA gene sequencing and analysis: For each selected pure isolate, amplify an appropriate 16S rRNA gene region using a documented primer pair and controls, verify the amplicon, and obtain Sanger sequence reads or validated sequencing output. Inspect chromatograms, trim low-quality ends, assemble forward/reverse reads when available, and record the final sequence length and quality. Compare the sequence with a curated reference database such as NCBI BLAST, report the closest matches with percent identity and query coverage, and use a phylogenetic analysis if justified. Deposit the sequence in a public repository if required and report the accession number. No 16S sequence, chromatogram, BLAST output, or accession number was supplied; therefore, taxonomic identity from 16S cannot yet be stated.

2.13 Statistical analysis: Use the individual replicate observations, not only the group averages. Summarize continuous outcomes as mean ± SD (or median and interquartile range if assumptions are unsuitable). For two independent groups, use an independent-samples t-test when assumptions are reasonably met; use Welch's t-test when group variances may differ. Use a paired t-test only when each treated value is naturally matched to a control value. For three or more independent groups, one-way ANOVA may be used for one factor, followed by an appropriate multiple-comparison procedure if the omnibus test is significant.

A factorial ANOVA may be appropriate when plant species and treatment are both factors and the design supports it. Check residual normality and variance assumptions, report test statistic, degrees of freedom, exact p-value, effect size, confidence interval, and biological replicate number. Define alpha in advance, commonly 0.05. Do not infer statistical significance from means alone.

III. Results And Discussion

The Rhizobium was isolated from different root nodules of leguminous plants. It grows in YEMA medium at 28 - 30°C. The colonies were identified as convex and creamy (Fig. 1). Similar results were also reported by Atlas et al. (1998). The Rhizobium isolates utilize the Congo red and show growth in CRYEMA medium (Fig. 2). From fig. 2.1 it is evident that Rhizobium is positive for indole production test, catalase test and citrate utilization test and negative for MR – VP test (Table 1).

Fig. 3 and Table 2 shows that Rhizobium induce the growth of Vigno mungo with well detection of root, shoots and leaves but without Rhizobium it shows slow growth of root, shoot and leaves. Fig. 4 and Table 3 reveal that slurry soil from Vigno mungo spread on the field to Arachis hypogaea shows the well growth of root, shoot and leaves when compared to unamended soil. Fig. 5 and Table 4 shows that slurry soil from Arachis hypogaea spread on the field to Citrus aurantifolia shows the well growth of root, shoot and leaves. But without the slurry soil it shows slow growth. Our results also correlated to Ladha et al. (1998).

From fig. 6 it is evident that genomic DNA was isolated and the bands were observed on agarose gel electrophoresis. The bands of genomic DNA were subjected to the analysis of PCR amplification. Nif genes of 450 bp were observed that there was no remarkable change in base pairs. This was also similar to the report of George and Borthakur (1994) and Nuti et al. (1999)

3.1 Biochemical characterization

Test	Reported result
Indole	Positive
Methyl red	Negative
Voges–Proskauer	Negative
Citrate utilization	Positive
Catalase	Positive

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These are the qualitative results reported in the supplied project. They are consistent with the report's presumptive identification, but confirmatory identification should use additional validated methods.

3.1.1Antibiotic Resistance Test: Antibiotic resistance of the *Rhizobium* isolates was tested against Kanamycin, streptromycin, and tetracycline using Disk Diffusion method (National Committee for Clinical Laboratory Standards, 1999) [6].

3.1.2. Antimicrobial Activity Test: Antimicrobial activity of the *Rhizobium* isolates against potential pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus* and *Pseudomonas* was tested by Agar well assay technique (Schillinger and Lucke, 1989) [7].

3.2 Plant growth comparison

Plant species	Parameter	Control mean	Test mean	Change (test − control)	Relative change
Vigna mungo	Leaves (number)	10.2	15.4	5.2	51.0%
Vigna mungo	Root length (cm)	1.9	4.7	2.8	147.4%
Vigna mungo	Shoot length (cm)	11.6	15.1	3.5	30.2%
Arachis hypogaea	Leaves (number)	10.3	13.3	3.0	29.1%
Arachis hypogaea	Root length (cm)	1.7	3.7	2.0	117.6%
Arachis hypogaea	Shoot length (cm)	11.4	13.1	1.7	14.9%
Citrus aurantifolia	Leaves (number)	9.1	11.48	2.38	26.2%
Citrus aurantifolia	Root length (cm)	1.4	2.9	1.5	107.1%
Citrus aurantifolia	Shoot length (cm)	10.1	12.1	2.0	19.8%

All nine reported test-group averages exceed their corresponding control averages. These are descriptive comparisons only. Because replicate counts and individual observations are absent, standard deviations, confidence intervals, t-test p-values, and ANOVA p-values cannot be calculated reliably from these means.

3.3 Plant growth graph

The accompanying graph plots the control and test averages reported in the source. It is a descriptive figure and does not display error bars because variability data were not supplied.

3.4 Soil analysis

Parameter	Control soil (mean ± SD)	Amended soil (mean ± SD)	Unit/method	Statistical test
pH	To be measured	To be measured	pH units; calibrated pH meter	Welch t-test or design-appropriate model
Electrical conductivity	To be measured	To be measured	dS/m; EC meter	Welch t-test or design-appropriate model
Organic carbon	To be measured	To be measured	%; validated laboratory method	Welch t-test or design-appropriate model
Available nitrogen	To be measured	To be measured	mg/kg; specified method	Welch t-test or design-appropriate model
Available phosphorus	To be measured	To be measured	mg/kg; specified method	Welch t-test or design-appropriate model
Available potassium	To be measured	To be measured	mg/kg; specified method	Welch t-test or design-appropriate model

No measured values for these soil parameters were provided. Complete this table only after laboratory analysis.

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3.5 nif PCR and 16S rRNA sequencing

Molecular analysis	Information in supplied report	What is still required
nif PCR	An approximately 450-bp band was reported	Gel image, primer sequences, controls, isolate IDs, replicate details, sequence confirmation if available
16S rRNA sequencing	No sequence result supplied	Raw sequence/chromatogram, trimmed sequence, sequence length, database matches, identity, coverage, accession number

3.6 Statistical results table template

Outcome	Comparison/model	n per group	Test statistic (df)	p-value	Effect size / 95% CI	Conclusion
Each plant growth outcome	Control vs test: Welch t-test	Enter n	Calculate from raw data	Not available	Not available	Cannot conclude significance from means alone
Each outcome across ≥3 groups	One-way ANOVA if design is appropriate	Enter n/group	F(df1, df2)	Not available	η^2 or ω^2; CI if available	Requires replicate-level data
Soil parameters	Control vs amended soil	Enter n	Calculate from raw data	Not available	Mean difference and 95% CI	Requires measured soil values

Table 1. Biochemical characterization of *Rhizobium*

S.NO	CHARACTERISTICS	RESULT
1	Indole test	Positive
2	Methyl red test	Negative
3	Voges Proskauer test	Negative
4	Citrate utilization test	Positive
5	Catalase test	Positive

Table 2. Effect of *Rhizobium* on the growth of *Vigno mungo*

S.NO	GROWTH PARAMETER	CONTROL	TEST
1	Number of leaves	10.2	15.4
2	Root length (cm)	1.9	4.7
3	Shoot length (cm)	11.6	15.1

Control – normal growth plant

Test – Rhizobium inoculated plant

Table 3. Effect of *Rhizobium* on the growth of *Arachis hypogaea*

S.NO	GROWTH PARAMETER	CONTROL	TEST
1	Number of leaves	10.3	13.3
2	Root length (cm)	1.7	3.7
3	Shoot length (cm)	11.4	

Control – normal growth plant

Test – Rhizobium inoculated plant

Table 4. Effect of *Rhizobium* on the growth of *Citrus aurantifolia*

S.NO	GROWTH PARAMETER	CONTROL	TEST
1	Number of leaves	9.1	11.48
2	Root length (cm)	1.4	2.9
3	Shoot length (cm)	10.1	12.1

Control – normal growth plant

Test – Rhizobium inoculated plant

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FIGURE-1 RHIZOBIUM SAMPLE



FIGURE-2 SOIL SAMPLE

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SOIL SAMPLE

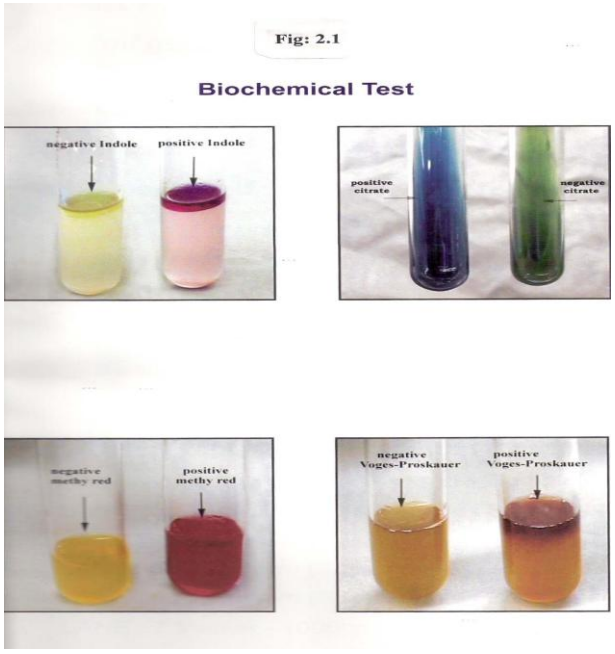


FIGURE-3 BIOCHEMICAL TEST



Fig.1 Root of *Pisum sativum* showing nodules developed by symbiotic bacterium *Rhizobium*

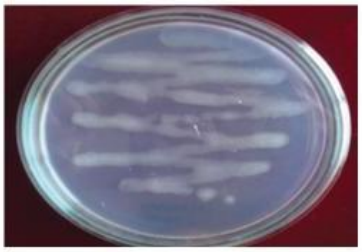


Fig.2 Pure culture of *Rhizobium* on Yeast Extract Mannitol Agar media



Fig.3 Glucose Pentose Assay of *Rhizobium* isolates

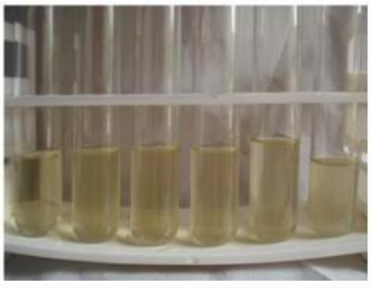


Fig.4 *Rhizobium* isolates showing Gelatin Hydrolysis

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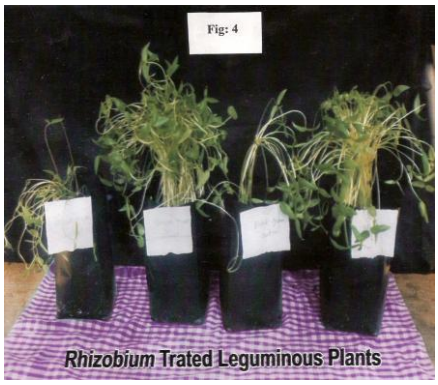


FIGURE-4 Effect of *Rhizobium* on the growth of *Vigno mungo*

EFFECT OF RHIZOBIUM ON THE GROWTH OF ARABIS HYPOGIYA

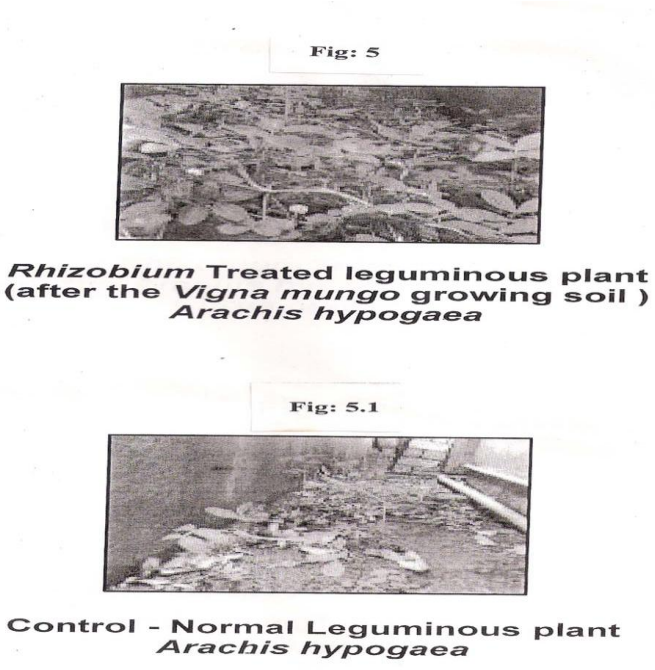
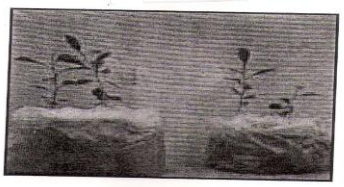


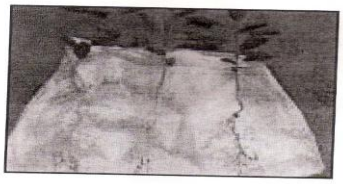
FIGURE-5

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Fig: 6



Rhizobium Treated plant
(after the *Arachis hypogaea* growing soil)
Citrus aurantifolia



Rhizobium Treated plant
(after the *Arachis hypogaea* growing soil)
Citrus aurantifolia Root

FIGURE-6 EFFECT RHIZOBIUM ON THE GROWTH OF CISTRAS aurantifilia

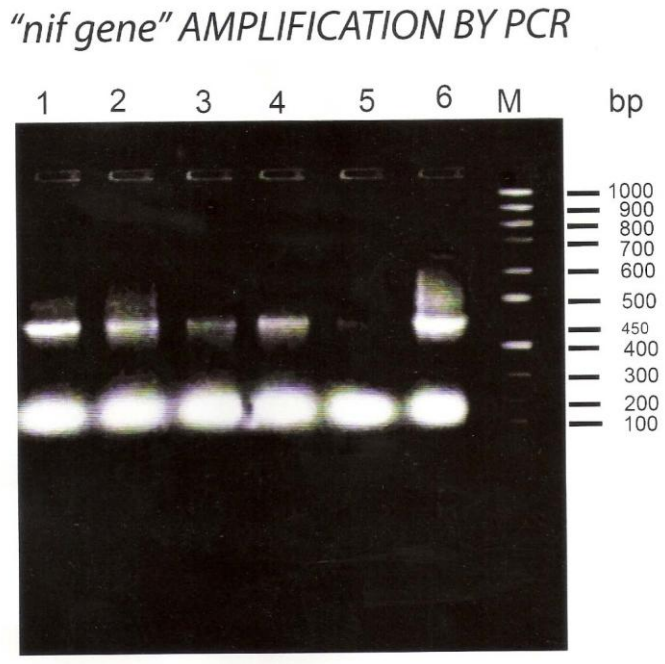


FIGURE-7

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Effect of nitrogen-fixing bacteria on leaf number

Comparison of control and test-group mean leaf numbers.

Arachis hypogaea

Control mean10.3

Test mean13.3

Control mean

Test mean

05101520Vigna mungoArachis hypogaeaCitrus aurantifolia

Comparison of root length

Mean root length in control and test groups.

Arachis hypogaea

Control mean1.7 cm

Test mean3.7 cm

Control mean

Test mean

0 cm2 cm4 cm6 cmVigna mungoArachis hypogaeaCitrus aurantifolia

Graph 2: Root length (cm)

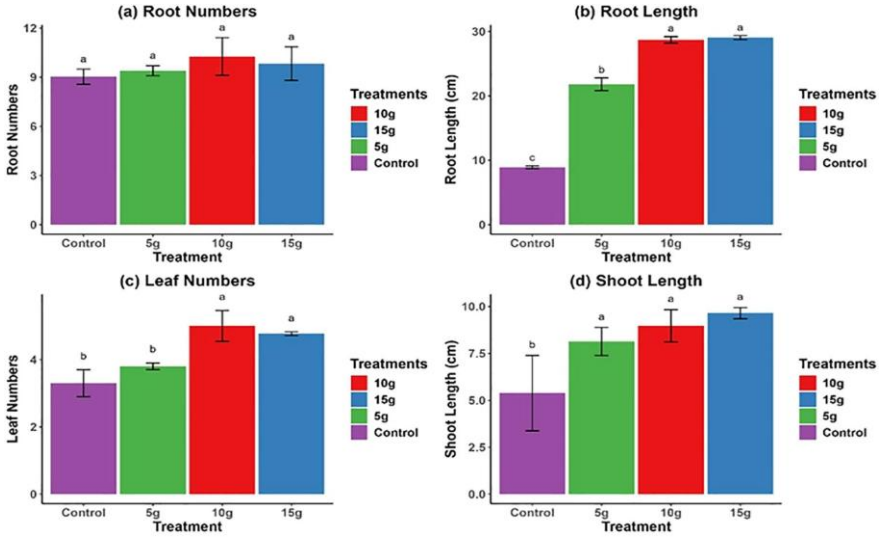


FIGURE.9

Colonies of *Rhizobium* sp. were obtained on YEMA medium after incubation at 30ºC for two days. General microscopic view of the isolates showed them to be rod cells and germ negative in nature. It is known that salt140 International Journal of Agricultural and Food Science 2013, 3(4): 138-141 stress significantly reduces nitrogen fixation and nodulation in legumens. Hashem *et al.,* (1998) [8] have proposed that salt stress may decrease the efficiency of the Rhizobium Legume symbiosis by reducing plant growth and photosynthesis survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function. Some rhizobial isolates have been shown to grow under high salt conditions 4-5% (Kucuk *et al.*, 2006) [9]. Results indicated that cells were able to grow on 1% NaCl containing medium but unabled to grow on higher concentrations, showing that the isolate was sensitive to salt.

pH is an important parameter for the growth of the organism. Slight variations in pH of medium might have enormous effect on the on the growth of organism. Superior growth of *Rhizobium* has been reported at neutral pH. Results showed that cells were able to grow only at pH 7.0 at 30ºC temperature. No growth was observed in medium with pH 4.0 and 9.0. Similar observation were made by Deora *et al.*, 2010 [10]; Baoling *et al.*, 2007 [11]. Rhizobial cells were able to grow on the GPA media showing the utilization of glucose as the carbon source by the *Rhizobium* (Fig.3). It is a confirmatory test for *Rhizobium* and these are able to utilize glucose as carbon source (Deora *et al.*, 2010). It was observed that rhizobial cells do not produce gelatinase enzymes as medium containing gelatin solidified when kept at 4ºC for 30 as well as 60 min.(Fig.4). Nagative gelatinase activity is also a feature of *Rhizobium* (Hunter *et al.*, 2007) [12]. Positive results were obtained from the starch hydrolysis assay.

On subjecting inculate plates to iodine test, clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas, blue colour appears on no growth areas (Fig.6). This indicates that the isolates have the potential to hydrolyze starch present in the medium. De Oliveira *et al.*, (2007) [3] also observed that *Rhizobium* strains can utilize starch obtained from different sources. Yellow slants ans red butt (Fig.5) was obtained showing the utilization of glucose and sucrose in the triple sugar ion agar medium (Hajnaa, 1945) [5]. No such studies have been conducted on *Rhizobium* strains. *Rhizobium* isolates were showed antibacterial activity against *Streptococcus* and *E.coli* whereas it did not showed antibacterial activity against pseudomonas (Fig.8). *Rhizobium* isolates found to be the most potent strain and showed maximum diameter of inhibition zone ranges from 15 mm to 13 mm against *E.coli* and *Streptococcus*, respectively. The *Rhizobium* isolates were antibiotics (Fig.7) the diameter of zone of inhibition of bacterial growth was found to be 3.0, 1.9 and 2.0 cm for tetracycline, kanamycin and streptomycin, respectively. There are 3 known determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge and amount of the antibiotic (Deora *et al.* 2010) [10] and the *Rhizobium* that showed a high level of resistance did not take up the antibiotics.

IV. Conclusion

The supplied report describes presumptive Rhizobium isolates, a qualitative biochemical profile, higher reported plant-growth averages in test groups, and an approximately 450-bp nif-associated PCR band. These findings are preliminary and descriptive. Soil-analysis results, statistical significance, and 16S rRNA-based identification remain unverified because the necessary raw measurements and sequence data were not supplied. The project can be finalized scientifically once replicate-level plant data, soil laboratory results, and molecular sequence outputs are added.

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