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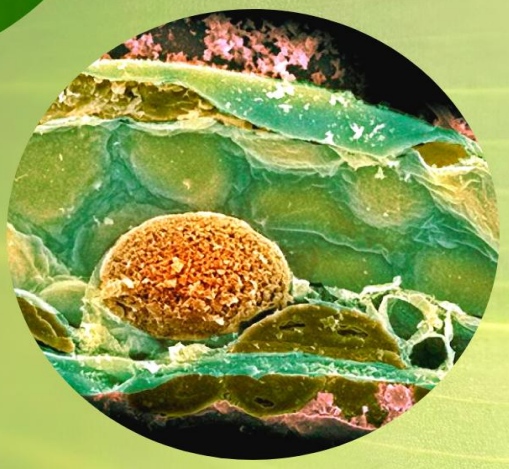
RESEARCH INTERVENTIONS AND ADVANCEMENTS IN PLANT SCIENCES



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ISOLATION AND IDENTIFICATION OF RHIZOBIUM FROM ROOT NODULES OF FENUGREEK PLANT COLLECTED FROM VILLAGE VANGAON AND TO STUDY ITS EFFECT ON SOIL FERTILITY AND PLANT GROWTH

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Abstract:

As the population of the world is increasing day by day, the demand for food is also increasing. Increase in population and increased food demand, impart stress on agriculture. To increase the productivity of crops, farmers prefer to use chemical fertilizers. But these chemical fertilizers are responsible for various types of pollution. To avoid pollutionand to prevent environmental damage, now there is a need of biofertilizers. One of the biofertilizers that can be used is rhizobium which is symbiotic nitrogen-fixing bacteria. In the present study, isolation and identification of rhizobium is done by using the isolation technique and by using biochemical tests. Isolated and identified culture is used to inoculate soil, then the nitrate content of the soil is determined by the colorimetric method and also its effect is checked on plant growth by measuring its height. It was found that rhizobium shows an increase in soil nitrate content as well as plant growth. So it can be used alternative to chemical fertilizers.

Keywords: Rhizobium, biofertilizers, Isolation.

Introduction:

The population of the world is increasing drastically; therefore there is a demand for food. To satisfy that need agricultural productivity should get increased. For that purpose, farmers prefer to use chemical fertilizers. In agriculture, for better product yield, soil fertility plays an important role. Basic requirements for soil fertility include nitrogen, phosphorus, sulfur, etc. The concentration of atmospheric nitrogen is nearly 71% but unfortunatelyplantsas well as animals can’t use it directly. To convert this atmospheric nitrogen into a usable form of nitrogen,

certain microorganisms play an important role. One of such microorganisms is Nitrogen-fixing bacteria. These nitrogen-fixing bacteria have one enzyme called Nitrogenase which will convert atmospheric nitrogen into a usable form of nitrogen by plants like nitrate, nitrite, ammonium salts, etc. Leguminous plants include many important species that are used as food and fodder crop throughout the world. They can provide their nitrogen requirements through nitrogen fixation in symbiosis with soil bacteria collectively known as rhizobia. These bacteria form root nodules on leguminous plants and convert atmospheric N2 into a form usable by plants. Application of effective rhizobial strains as biofertilizers to improve legume production is an important approach in sustainable agriculture (Olivera, 2011). Nitrogen-fixing bacteria are of two types- symbiotic nitrogen-fixing bacteria and non-symbiotic nitrogen-fixing bacteria. Symbiotic nitrogen-fixing bacteria are those who live in symbiotic association with leguminous plants. The symbiotic association is also called mutualism because both the member in the association get benefited from this association.Fenugreek is an important legume crop for consumers as a popular spice in Indian cuisine and has long been used in both Ayurvedic and traditional medicine to induce labor and lactation. It aids in digestion and as a general health and wellness tonic (Basch, 2003). Its crop contributes an important nutrient N for the soil. Fenugreek is also a good soil renovator and widely used as green manure. Fenugreek was reported to fix 48% of its total N_2during the growing season. It is also a good source of atmospheric nitrogen fixation by *Rhizobium* present in its root nodules(Desperrier,1985).In the present study, bacteria present in root nodules of fenugreek plants collected from different localities of Vangaon were collected, isolated and then biochemically identified. The effect of these isolates was tested for plant growth and soil fertility.

Materials and Methods:

Study Area:

Vangaon is a village in the Palghar district of Maharashtra, India. It is located in the Dahanu taluka. Its Latitude is 19° 51' 59.99" N and Longitude is 72° 44' 59.99" E. Fenugreek plants (*Trigonella foenum-graecum*) were collected from three different locations nearby to Vangaon village that is Kotim, Kolavali, Bavada respectively having healthy root nodules. Then these samples are brought to the laboratory in clean, polythene bags.

Methods:

Surface sterilization and isolation of Rhizobium:

Roots of Fenugreek plants were washed with tap water to remove soil from it. Then pink, healthy root nodules were removed carefully by using sterile forceps. Root nodules were treated with 20% tween 20 detergents, followed by washing with sterile distilled water. Next root nodules were washed with 0.1% mercuric chloride for 3 minutes. Then washed with sterile distilled water for 3 times. Then root nodules were washed with 75% ethanol for 3 minutes. Finally, nodules were washed with sterile distilled water for 8 times. Nodules were taken on slide and teased with sterile forceps. White exudates from nodules were streaked on sterile CRYEMA plate (Congo red Yeast extract Mannitol agar pH 6.8 ± 0.2) by using a flame sterilized nichrome loop. The inoculated plate is then incubated at 37^{o} C for 3- 4 days. Rhizobium will form white-colored colonies while other organisms will give red colonies. Rhizobium does not allow Congo red inside its cell andtherefore give white colonies. White translucent colonies developed on the plate were then morphologically observed for size, shape, color, opacity, elevation. Marginand then gram staining was carried out.

Gram staining:

Colony obtained after incubation was then subjected for gram staining. Smear was prepared on a microscopic slide. Then smear was treated with 1% crystal violet for 1 minute. Wash smear with distilled water. After that, 1% of Gram's iodine was addedand kept for 1 minute. The slide was washed with distilled water followed with washing with decolorizer that is 75% Alcohol for 30 seconds. The slide was counterstained with 1% Safranine for 1 minute. The slide was washed with distilled water. The slide was air-dried. One drop of cedarwood oil (immersion oil) was added on the smear. The slide was observed under the microscope (100X). Gram nature and shape was noted.

2. Biochemical tests:

i) Sugar fermentation test:

To find out types of sugar utilized by rhizobium, a sugar fermentation test was carried out. Loopful of culture was inoculated in sterile test tubes having 1% Glucose, 1% lactose, 1% mannitol, 1% maltose. Test tubes were incubated 37^{0} C for 24 hours – 48 hours. Change in color of media and gas production in Durham's tube was noted.

ii) IMViC TEST:

a) **Indole test:** To determine the ability of the organism to convert tryptophan into indole due to the presence of enzyme"tryptophanase"; an indole test was done. Loopful of culture was inoculated in sterile 2% tryptone water and tube was incubated at 37^{o} C for 24 hours. 2 to 4 drops of Kovac's reagent was added in inoculated, incubated tube. The Colour of the ring was noted.

b) **Methyl Red test:** To find out acid production ability of microorganisms,Loopful of culture was inoculated in sterile 1% Glucose phosphate broth. The tube was incubated at 37^{o} C for 24 hours. 5 drops of methyl red reagent were added after incubation. A Colour change was noted.

c) **Voges Proskauer Test:** To determine whether an organism can synthesize acetoin or not, Loopful of culture was inoculated in sterile 1% Glucose phosphate broth. The tube was incubated at 37^{o} C for 24 hours.4 drops of omera's reagent was added. The result was noted.

d) **Simmon Citrate agar Test:** To find out whether an organism can use citrate or not, loopful of culture was streaked on the surface of sterile Simmon citrate agar slant and slant was incubated at 37^{o} C for 24 hours. Change in color was noted.

iii) Triple sugar ion test:

Loopful of culture was taken on sterile straight loop and then it was stabbed in sterile TSI slant incubated at 37^{0} C for 24 hours. The change in color was recorded. This test will help to find out which sugars were utilized by bacteria.

iv) Urease test:

To determine whether isolated bacteria is urease producer or not, loopful of culture was inoculated in sterile Christensen's urea broth and incubated at 37^{o} C for 24 hours and change in color was noted.

v) Catalase test:

One colony was picked from the plate and kept on the slide. Then 2 drops of hydrogen peroxide were added on it. The presence or absence of effervesces was noted. The Presence of effervesces indicates an organism is a catalase synthesizing organism.

vi) Starch hydrolysis test:

Sterile 1% starch agar plate was taken. One loopful of culture was streaked on the plate. The plate was incubated at 37^{o} C for 24 hours. After incubation, few drops ofLugol's iodine was added on a plate. Change in color due to the presence or absence of starch was recorded. If the clear zone was observed around a colony, indicate, bacteria can produce an amylase enzyme.

vii) Glucose peptone agar test:

Loopful of culture was streaked on sterile Glucose peptone agar plate and incubated at 37^{o} C for 24 hours. The presence or absence of colonies after incubation was recorded. Rhizobium cannot grow on this media because this organism cannot utilize nutrients available in media.

Effect of rhizobium on soil nitrate content and plant growth:

Sterile soil was taken. It was divided into two equal half. One was inoculated with 100 ml of rhizobium suspension having density $1*10^{9}$cells/ ml while second was uninoculated and used as control.

i. The nitrate content of soil:

1gm of soil was taken and it was mixed with 10 ml of water. Then it was mixed properly and allowed it to settle down. The supernatant was transferred to the new test tube. One pinch of zinc dust was added and allowed to stand for a few minutes.Then the supernatant was mixed with 0.5ml of NEDD (*N*-(1-Naphthyl) ethylenediamine) and0.5 ml of sulphanilamide reagent. O.D. was measured by colorimeter at 540 nm. The same procedure was repeated for uninoculated soil also. Soil nitrate content was measured daily for 10 days. Standard reading was taken by using standard nitrite solution with a concentration of 100 ug/ml. Reading was taken at 540nm using colorimeter.The nitrate content of the soil was calculated using the following formula:

$$\text{The nitrate content of soil } (\mu g\ NO_3\text{-}N/lit) = \frac{\Delta \text{ O.D. of sample}}{\Delta \text{O.D. of a standard}} \times \frac{\text{concentration of standard}}{\text{volume of sample}} \times 1000$$

ii. **Plant growth**:

25 healthy surface-sterilized fenugreek seeds were added in both inoculated and uninoculated soil. Both inoculated and uninoculated soil with seeds was incubated in light for 16hours and in dark for 8 hours. Growth germination and height of plant was measured for 10 Days.

Results:

Isolation and Gram Staining:

After 48 hours of incubation,

Sample 1: Fenugreek plants collected from Kotim showed two white, translucent colonies.

Sample 2: Fenugreek plants collected from Kolavali showed three white, translucent colonies

Sample 3: Fenugreek plants collected from Bavada showed two white, translucent colonies.

Colony characteristics: All colonies were white, translucent, having entire margin, convex elevation, 3mm in size, consistency was smooth. Gram nature observed was gram-negative rods.

Table 1: Biochemical tests

Name of the test	Kotim Isolate	Kolavali Isolate	Bavada Isolate
i)Sugar fermentation test:			
1% Glucose	+	+	+
1% Lactose	+	+	+
1% Maltose	+	+	+
1% Mannitol	+	+	+
ii)Indole test	-	+	+
iii)Methyl red test	+	+	+
iv)Voges Proskauer test	-	+	+
v) Simmon citrate test	+	+	+
vi) Triple sugar ion test	+	+	+
vii) Urease test	+	+	+
viii) Catalase test	+	+	+
ix)Starch hydrolysis test	+	+	+
x) Glucose Peptone Agar test	-	-	-

Key: + = Growth, - = No growth

Effect of rhizobium on nitrate content of soil:

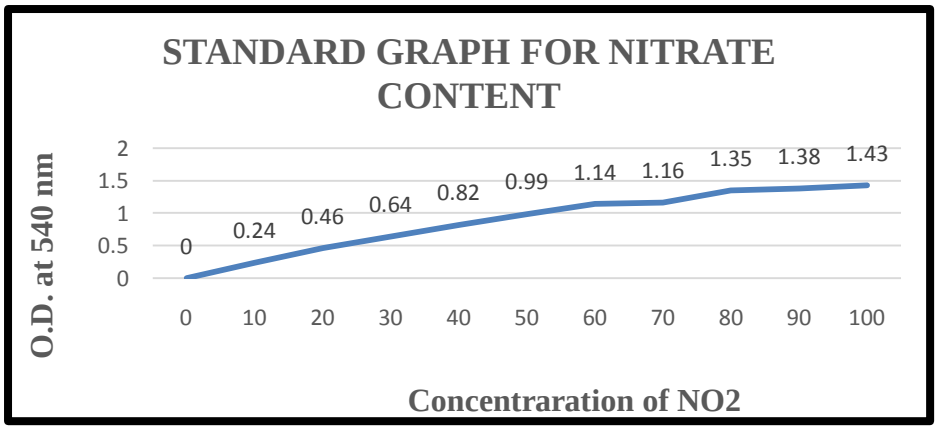

Figure 1: Standard graph for the Nitrate Content

Table 2: The nitrate content of the soil was calculated and it was as follows

Day	Kotim soil Nitrate content (µg NO3-N/lit)		Kolavali Soil Nitrate content (µg NO3-N/lit)		Bavada Soil Nitrate content (µg NO3-N/lit)	
	Inoculated soil	Uninoculated soil	Inoculated soil	Uninoculated soil	Inoculated soil	Uninoculated soil
1	260	253	198	196	169	160
2	457	242	406	180	384	158
3	856	221	752	175	679	155
4	1986	180	1647	156	1456	148
5	2797	160	2013	151	1987	146
6	2450	152	1856	132	1723	145
7	1932	139	1665	119	1470	132
8	1761	127	1400	98	1198	114
9	1340	126	1240	97	887	99
10	945	107	1188	97	800	88

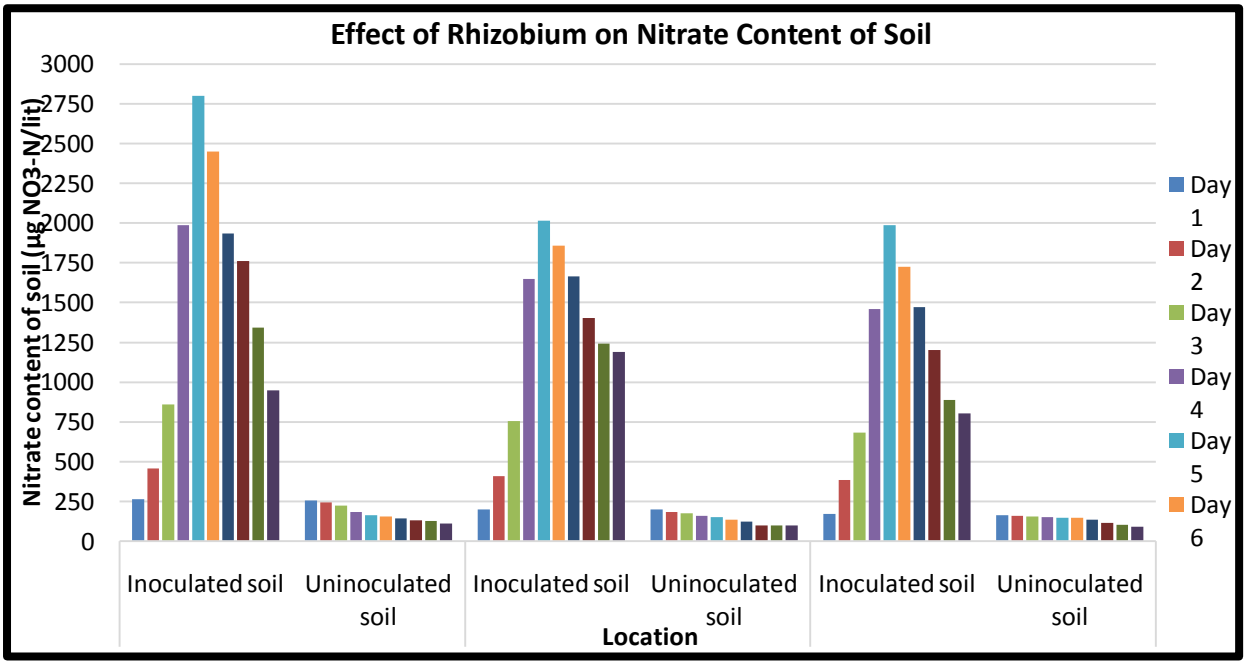

Figure 2: Effect of Rhizobium on the soil nitrate content

Table 3: Effect of Rhizobium on plant growth

Day	Kotim soil Height of shoot in cm		Kolavali Soil Height of shoot in cm		Bavada Soil Height of shoot in cm	
	Inoculated soil	Uninoculated soil	Inoculated soil	Uninoculated soil	Inoculated soil	Uninoculated soil
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0.5	0	0.4	0	0.3	0
5	2	0.2	0.8	0.1	0.5	0.1
6	3.5	0.8	3	0.3	1	0.3
7	5	1.9	5	0.8	1.8	0.7
8	8	4	6	2	2	1
9	10	6	8	4	3.6	2
10	10	7	9	6	5	4

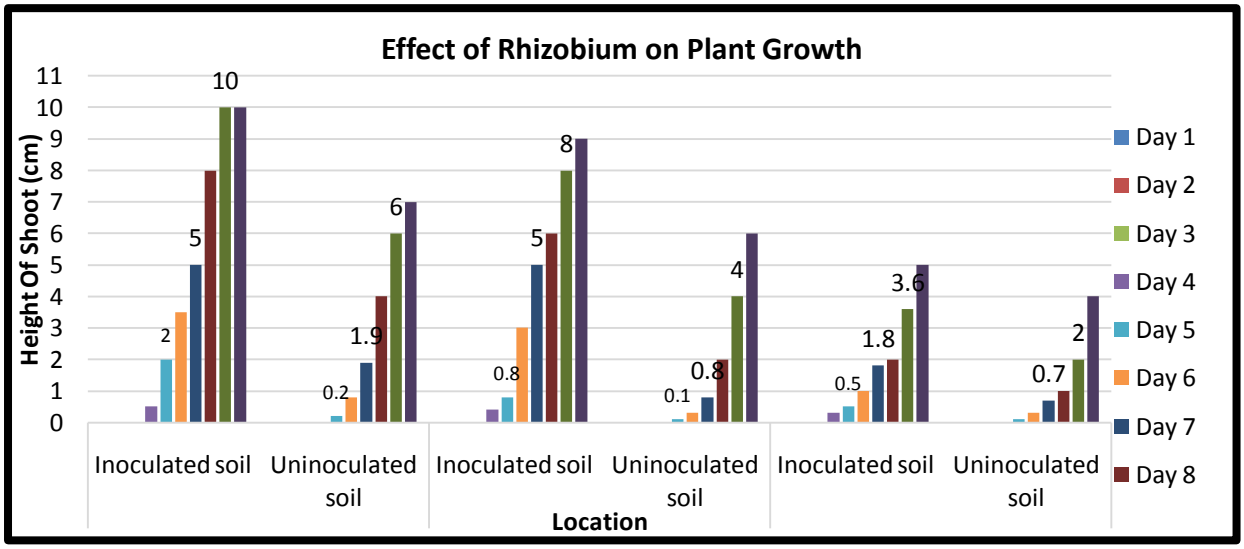

Figure 3: Effect of Rhizobium on the plant growth

Conclusion:

As after incubation there was the presence of white, translucent colonies on sterile CRYEMA plate but same isolates could not grow on Sterile Glucose peptone agar plate,thereforethere was the presence of Rhizobium in the root nodules of fenugreek plants which

were collected from three locations that were Kotim, Kolavali, Bavada nearby to the Vangaon village. The gram nature of isolated colonies was Gram-negative rods.

Rhizobium isolated from Kotim fenugreek sample wasable to use 1% glucose, 1% lactose, 1% maltose, 1% Mannitol as a source of energy. But this isolate did not have tryptophanase enzyme and also could not synthesize acetoin. This isolate was acid and it could utilize citrate also. This isolate was amylase, urease and catalase-positive organism. While isolate observed from KolavaliandBavada fenugreek plant sample was able to use 1% glucose, 1% lactose, 1% maltose, 1% Mannitol, citrate as a source of energy. The isolate was able to synthesize tryptophanase and acetoin. These isolates were acid producer, amylase, urease and catalase-positive organisms.

In all three soil samples that are Kotim, KolavaliandBavada, Nitrate content of inoculated soil was found to be much higher than uninoculated soil. The nitrate content of Kotim soil was found to be much higher than the Kolavali soil sample as compared to the Bavada soil sample. In all three soil samples, nitrate content was initially increased as number incubation days increased, on the fifth day of incubation, nitrate content was found to be maximum which on further incubation decreased due to utilization of soil nitrate for plant growth. From that, it was concluded that Rhizobium inoculation in the soil will increase the nitrate content of the soil.

From results obtained for the shoot, height showed that rhizobium inoculated soil show faster plant growth as compared to uninoculated soil. Shoot height of plant grown in inoculated soil was more as compared to shoot height of plant grown in uninoculated soil. This indicates that rhizobium will show a positive effect on plant growth.

Future Prospectus:

In the future, isolatedand identified strain will be subjected to DNA sequencing.The growth curve has also to be studied to determine whether it is a fast or slow grower. Invasiveness (nodule formation capacity) of strain, Competitiveness of organism, the stability of organisms in carrier moleculeshas to be determined. To use it as biofertilizer.

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