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EFFECT OF RHIZOBIUM AS A BIOFERTILIZER ON GROUND NUT AND BLACK GRAM

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ABSTRACT

The main sources of bio- fertilizers are *bacteria, fungi, cyanobacteria* etc. Such bio-fertilizers are cultured and are used for inoculating seed or soil or both under ideal conditions to increase the availability of plant nutrients. Biofertilizer promotes plant growth and productivity has internationally been accepted as alternative sources of chemical fertilizer. *Rhizobacteria* effectively colonize plant root and increases plant growth by production of various plant growth hormones, P- solubilizing activity, nitrogen fixation and biological control activity (Deshwal et al., 2011).*Rhizobacteria* stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into soil by interacting symbiotically with leguminous plants, using the nitrogenase enzyme complex (Kiers et al., 2003).Rhizobium is the soil microorganism that can survive in the soil or forms a symbiotic association with the host legume. The present study aims to harness the potential of symbiotic nitrogen-fixing Rhizobium bacteria as a sustainable biofertilizer. By focusing on isolation Characterization, mass production, and practical application, this research seeks to contribute significantly to sustainable agriculture, enhance soil health, and improve crop productivity while minimizing environmental impacts.

INTRODUCTION

The symbiotic fixation of nitrogen is of extreme importance for the maintenance of soil fertility and, in agricultural practices, it is utilized to increase crop yields. Nitrogen fixation is ecologically important because some of the fixed nitrogen is likely to become available to plants. The best characterized symbiotic association involving nodules occurring on the roots of leguminous plants. Members of the family Leguminosae (Fabaceae) are the only plants capable of forming nitrogen- fixing nodules with rhizobia, with the single nodule exception of the non legumes genus parasponia a tropical tree (Atlas and Bartha, 1987). The largest contribution of combined nitrogen in terrestrial habitats is due to the symbiotic nitrogen-fixation by rhizobia.

Biofertilizer promotes plant growth and productivity has internationally been accepted as an alternative sources of chemical fertilizer. *Rhizobacteria* effectively colonize plant root and increases plant growth by production of various plant growth hormones p-solubilizing activity, nitrogen fixation and biological control activity (Deshwal et al., 2011).

In this study various species of rhizobia were isolated from leguminous plants grown at the different region of Nanded. Soil contains many types of microorganisms such as bacteria, actinomycetes, fungi and algae, which are important because they affect the physical, chemical, and biological properties of soil. Systems of agriculture foremost among such alternatives is biological nitrogen fixation (BNF) which has the potential to reduce and/or replace the continuous use of nitrogen fertilizer, which is very often the nutrient limiting the productivity of several annual crops. As there are many leguminous crops that in symbiosis with root nodule bacteria are capable of BNF, this area of study has become critical for their utilization in agriculture.

The present study aims to harness the potential of symbiotic nitrogen-fixing Rhizobium bacteria as a sustainable bio fertilizer. By focusing on isolation characterization, mass production, and practical application of this research seeks to contribute significantly to sustainable agriculture, enhance soil health, and improve crop productivity while minimizing environmental impacts.

MATERIALS AND METHODS

1. Chemical and media

Yeast Exatract Manitol Agar (YEMA) are used for the isolation rhizobium bacteria. where all the microbiological media were steam sterilized by autoclaving at 15 psi at 121°C for 15min. Solvents and other chemicals were purchased from Himedia laboratories Pvt. Ltd. (Mumbai, India), all the solution were freshly prepared.

2. Media Composition

Yeast Extract Mannitol Agar media

Table: A chemical composition.

S/No.	Chemicals	Grams/ml
01	Mannitol	10gm
02	K2HPO4	0.5gm
03	Mgso4,7H2O	0.2gm
04	Nacl	0.1gm
05	Yeast extract	0.5gm
06	Distilled water	1000ml
07	Agar	15gm

1. Collection of sample

Collected the different plants root nodules of leguminous plants. Which are big sized and pink coloured nodule are selected from the leguminous plants like ground nuts, green gram etc from the different region of Kalaburgi for the isolation of nitrogen fixing rhizobium strains.

2. Isolation of nitrogen fixing rhizobium bacteria

Collected rhizosphere soil sample and different root nodules for the Isolation of rhizobium bacteria Soil sample are serially diluted and further inoculated on to the yeast extract mannitol agar media for the study of cultural and colony characteristics and samples of root nodules are washed in 95% of ethanol to remove contamination. Than seven times indirectly wash the root nodules with the help of sterile water. After those nodules are crushed in test tubes which contain 5-10ml of sterile distilled water make them milky colour. Then solution is subjected serial dilution are inoculated into the YEMA media (with Congo Red Indicator) is used for the growth of rhizobium bacteria (Incubation period is 5- 7days, at 37°C).

3. Screening of Nitrogen fixing bacteria

Isolated colonies on YEMA media further used for the morphological characterizationYEMA media is suitable for growth and identification of nitrogen fixing rhizobium bacteria. Those colonies on the YEMA media streaked on the CYEMA medium and that streaked plates are incubated at 37°C for 5-7 days.

4. Growth on Bromothymol blue (BTB)

Colonies conformation test by using BTB on the YAME media (BYEMA). The positive sample show moist gammy colonies after incubation 37°C for 48hrs.Colonies turns the whitish pink to yellow due to acid production by same rhizobium strains. Those colonies which turn the whitish pink to yellow 20 colonies show the rhizobium strains are selected & named as B1 GN4 Ma6 S12.

5. Characterization of nitrogen fixing bacteria

Morphological Characterization

Prepare a smear by heat fixing of isolated culture on a clean and glass slide flood the smears with crystal violet and leave for 30 seconds. Rinse with water for 5 seconds, then add gram's iodine wait for 1 minute. Rinse with tap water and decolorize with 70% ethanol for15-30 sec, and then rinse with water. Counter stain with saffranine for about 1 min. Rinse with tap water for 5sec, then blot dry with filter paper and examine under 100X using oil immersion.

6. Biochemical Test

Indole test

Tryptophan broth was prepared and sterilized at 121°C for 15 min and inoculated with test organism, Incubated the medium at 37°C for 24 hours. Then add 1 ml of kovacs reagent to test tubes including control. Shake and observed the test tubes for presence of red rings.

Methyl Red test

Prepared MR broth in two flasks, inoculate the broth with the test organism and incubated for 24 hours at 37^0C. After 24 hours of incubation transferred 5 ml of broth into the test tubes. To the each broth culture added 5 drops MR indicator into the test tubes and shake them. Examine the colour of the each test tubes.

Vosges – Prosakauer Test

Prepared VP broth in flask, inoculated the broth with test organism and incubated for 24 hours, Prepare barrette regent A and B added to the test tubes after 24 hours of incubation 0.5 ml of reagent A and 0.2 ml of reagent B was added to the broth and observe for color change.

Citrate Utilization Test

Prepared citrate agar slant and inoculated each of the test organism into labelled test tubes. The slant was left inoculated that serve as control, incubated for 24 hours at 37°C. After 24 hours all agar slant were examined for the presence of growth and coloration of the medium.

Catalase Test

The test organisms are inoculated into nutrient broth along with control and incubated at 37°C for 24 hours. After incubation take a drop of culture on clean slide and then add 3% H_2O_2 on to the slide and see the bubbles formation within 20-30 minutes, it indicates Catalase positive, if no bubbles are formed it is termed as Catalase negative.

7. Mass production of rhizobium bacteria

Preparation of pre inoculum

In this cultivation we prepared large amount of YEMA broth prepared in separate flasks and inoculum from respective mother culture is transferred to flasks. The culture is grown under shaking condition at 37°C as submerged culture. The culture is incubated until maximum cell population of 1010 to 1011 cfu (colony forming units) per ml is produced.

Mass production of rhizobium bacteria

A pure sterilized YEMA broth is prepared and yellow zone colonies are inoculated in the YEMA broth for mass production of *rhizobium* bacteria for under optimum condition. This population level could be attained within 4 to 7 days of incubation. The culture obtained in the flask is called starter culture. This starter culture slurry, media is mixed with soil and seed are coated with the rhizobium bacteria.

Field trials of Rhizobium bacteria (Application)

Different types of leguminous seeds are selected such as groundnut, green gram. Simultaneously seed coated with the *rhizobium* bacteria and without seed coated, are grown and measured the growth of both plants as comparison to both coated and controlled seed coated seed have more growth and yield.

RESULTS AND DISCUSSION

1. Collection of Sample

Soil sample of the different rhizospheres soil and different plants root nodules of legume plants from the different area of Kalaburagi region" were collected for the isolation nitrogen fixing bacteria rhizobium and collected into sterile polyelylene bags, then stored at 4°C.

2. Isolation screening and production of symbiotic nitrogen fixing rhizobium bacteria

Rhizobium bacteria species were isolated from leguminous plants from Kalaburagi region. They were gram negative rod, curved or straight under aerobic condition. They grow on YEMA agar plates containing Congo red indictor and the colonies in (BTB) bromothymal blue the indicator to yellow. Although it provides a useful indicator of relatively small changes in acidity most isolates of rhizobia lack ability to absorb Congo red from a yeast extract mannitol salt medium containing 0.025% final concentration of this dye, resulted in colorless or pink colonies. Where contaminant colonies absorb the dye and become a deep red, it indicated non rhizobium strains, Most of the rhizobial isolates were able to grow. Rapidly on minimal salts agar containing yeast extract and fructose, glucose, ribose, arabinse, xylose, and mannose as the sole carbon source Similar results to that obtained in Graham and Parker (1964) and Glenn and Dilworth (198 l) in addition they grew on lactose, maltose and sucrose as presented in table-2 The carbon sources utilized by rhizobium have been reviewed (Elkan 1981) and studies of 20 starins of rhizobium in different species showed fat 20 out of 4 utilized lactose, maltose, and sucrose, While mannitol is the carbon source routinely used for in vitro cultivation of rhizobium a number of strains do not utilize mannitol.

3. Sub culturing on C- YEMA media

Based on the morphological and microscopic observation, it was identified as rhizobium bacteria. Isolated colonies were sub cultured on YEMA media to get the pure rhizobium bacteria species.

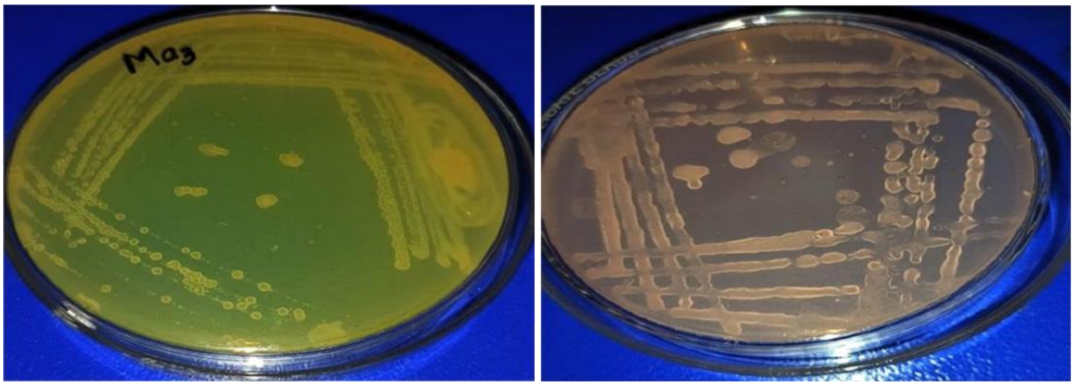
Fig: 3 Conformation sub cultured species (BTB Plates).

4. Conformation Sub Cultured Species

The YEMA medium was enriched with BTB 13'(25ug/ml). All the samples were subjected to grow on BTB added medium. The positive sample show moist and gummy colonies after incubation for 48 hours at 37°C and surrounding medium plates were yellow due to acid production by some rhizobium species.

5. Mass cultivation of R hizobium bacteria

Isolated rhizobium bacteria are used as a inoculated separately in the YEMA broth (250ml) and inoculated until the number of cells or sufficient and incubated for 4-7 days at 30°C temperature The culture obtained in the flask is called starter culture(fig: 11). This Starter culture, slurry media is mixed with the sterilized soil and seed are coated with the rhizobium bacteria.



6.Mass Cultivation

Compost soil was sterilized in hot air oven for 100°C for four hours, at this extreme heat and pressure compost soil get sterilized. Sterilized compost soil mixed with starter culture prepared for seed coating of 4 different seed in aseptic condition

Measurements of both the pots were recorded for 13 days. At last the graphical representation was done by

7. Ground Nut Seed Growth Observation

Mass cultivation of Rhizobium was done and mixed with sterilized soil. Then the seed was coated with the soil containing Rhizobium culture. The sil coated seeds are sowed in pot containing moistured soil. Two pots are taken one for control and the other for coated. The control pot contains only those seeds which are not coated. Whereas, control pot contains seeds coated with Rhizobium Sowing was done on first day.

On the 5th day of sowing, seedlings were observed only in coated poi. And on the next day seedlings were observed in control pot. Day to day measurements of both the pots were recorded for 13 days. At last the graphical representation was done by comparing both control pot seedling and coated pot seedlings.

Table 1: Ground Nut Plant Growth Measurement.

DAY	CONTROL	COATED
1		
2		
3		
4		Seed Germination
5	Seed Germination	1cm
6	lcm	2.5cm
7	2.3cm	3.8cm
8	3.5cm	5cm
9	4.6cm	6.8cm
10	5.9cm	8cm
11	7cm	9.5cm
12	8.3cm	11 cm
13	10 cm	12.5cm

Mass cultivation of Rhizobium was done and mixed with sterilized soil. Then the seed was coated with the soil containing Rhizobium culture. Then the coated seeds are sowed in pot containing moistured soil. Two pots are taken one for control and the other for coated. The control pot contains only those seeds which are not coated. Where as, coated poi contains seeds coated with Rhizobium Sowing was done.

After 24 hours, seedlings were observed in bo1 the pots and the coated seeds were slightly longer compare to control pot. Day to day measurements of both the pot were recorded for 13 days. At last graphical representation was done by comparing both control pot seedlings and coated pot seedlings.

Table 2: Black Gram Plant Growth Measurement.

DAY	CONTROL	COATED
1		
2	l.2cm	2.8cm
3	3cm	5cm
4	4.4cm	7cm
5	6cm	8.8cm
6	8cm	llcm
7	l0cm	l4cm
8	l2cm	l6cm
9	14.2cm	18.2cm
10	15.2cm	19.3cm
11	17cm	20cm
12	18.2cm	2lcm
13	l9cm	22.5cm

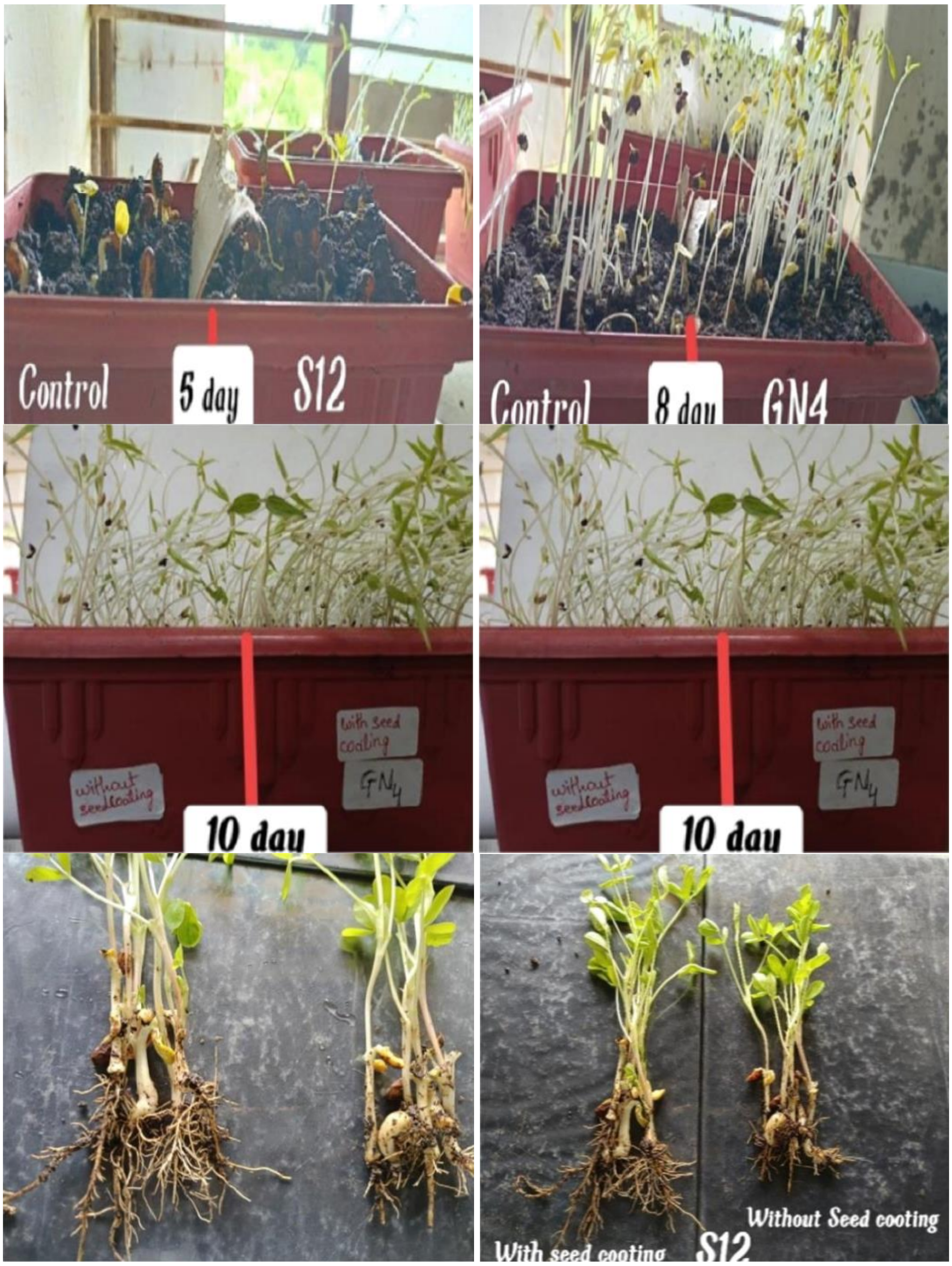


Fig. 2: Roots observation of black gram.

Table 5: Black Gram Plant Growth Measurement.

DATE	DAY	CONTROL	COATED
24\7\24	1		
25\7\24	2	l.2cm	2.8cm
26\7\24	3	3cm	5cm
27\7\24	4	4.4cm	7cm
28 7\24	5	6cm	8.8cm
29\7\24	6	8cm	llcm
30\7\24	7	l0cm	l4cm
31\7\24	8	l2cm	l6cm
1\8\24	9	14.2cm	18.2cm
2\8\24	10	15.2cm	19.3cm
3\8\24	11	17cm	20cm
4\8\24	12	18.2cm	2lcm
5\8\24	13	l9cm	22.5cm

Mass cultivation of Rhizobium was done and mixed with sterilized soil. Then in seed was coated with the soil containing Rhizobium culture. Then the coated seeds are sowed in pot containing moistured soil. Two pots are taken one for control and the one for coated. The control pot contains only those seeds which are not coated. Whereas, coated pot contains seeds coated with Rhizobium Sowing was done. After 24 hours, seedlings were observed in both the pots and the coated seeds were slightly longer comparing to control pot. Day to day measurements of both the pots was recorded for 13 days.

Root observation of ground nut

After 13 days of measurement seedling from both the pots were plucked with the root. Different pots seedlings of a same seeds were kept side by side and root observation wax done. (Fig.17) Coated seed seedling has more roots and nodules compare to the control seed seedlings.

After 13 days of measurement seedling from both the pots were plucked with the root. Different pot seedlings of a same seeds were kept side by side and root observation was done coated seed.

Seedling has more roots and nodules compare to the control seed seedlings. After 13 days of measurement seedling from both the pots were plucked with the root. Different pot seedlings of a same seeds were kept side by side and root observation was done. Coated seed seedling has more roots and nodules compare to the control seed seedlings.

SUMMARY AND CONCLUSION

Goal of the project is to discover new and better strain for use in legume inoculants. The collection of isolates, strain characterization of symbiotic capacity and comparison to strains currently included within inoculants. It can be concluded that the selection of highly performed isolates using different consecutive steps of identification and proving of further investigation of field try for rhizobiological science. Symbiotic performance is a key but the ability of rhizobia to survive stress condition or to utilize less expensive growth media are also important consideration. The process of rhizobium examination and characterization is somewhat difficult, and efforts must remain focused up on relatively few legumes of interest are the strains emerging from a work must be recognizably superior. Rhizobium generally does not absorb Congo red when plates incubated in the dark, but it absorbs this dye in light condition.

Rhizobium is symbiotically associated with in leguminous plant nodules. Rhizobium is genus of gram negative soil bacteria that fix nitrogen. Rhizobium species from an endosymbiotic nitrogen fixing association with roots of leguminous plants, fixation of nitrogen cannot be done independently, that is by rhizobium require plant host they convert di nitrogen into ammonia and ammonia compounds. Nitrogen fixation helps in increase in soil fertility & Rhizobium infects the leguminous plant they usually found in soil and produce nodules after infecting the roots of leguminous plants. As a result nitrogen gas is fixed from atmosphere. Specific strain of rhizobium is required to make the nodules functional in order to carry out in process in increases the yield of crops Legume inoculation has a constantly improved over a time due this purpose Rhizobium is used as bio fertilizer.

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