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ISOLATION OF *RHIZOBIUM* FROM ROOT NODULES OF *PISUM SATIVUM* AND ITS USE AS BIOFERTILIZER

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Abstract

Biofertilizers are substances which contain living microorganisms which when applied to a soil promote the growth by increasing the supply or availability of primary nutrients to the host plant. Biofertilizers are usually prepared as a carrier based inoculants containing effective microorganism which would show a good relationship with the host plant. This makes it easy to handle with wide range of acceptance by the host. Rhizobia are a special type of bacteria which live in root nodules in symbiotic association and fix atmospheric free nitrogen and make it available to the plants. In this study *Rhizobium* is isolated and cultured from the root nodules of *Pisum sativum* (pea plant) in a selective media i.e.YEMA (Yeast Extract Mannitol Agar medium with Congo Red). The isolate was found with poor absorption of congo red and appeared as whitish gummy colony which was biochemically tested. The isolated strain was then mixed with carrier and applied on pea. Uninoculated soil was used as control. The growth of plants was observed at regular time intervals. The growth parameters observed were germination of seed, shoot initiation, root initiation, root length and shoot length. The seed started to germinate by day 2. After 20 days the plant with the biofertilizer showed a shoot length of 11.5cm and root length of 18cm while control showed 7cm and 9cm respectively. This report showed that application of isolated rhizobial strain enhanced the growth of plant. The isolated strain can be used as biofertilizer.

Keywords: *Rhizobium*, biofertilizers, carrier

INTRODUCTION

Nitrogen is an essential but limiting nutrient for growth and yield of crops. It is predominatly found in the gaseous form which is unavailable to plants and animal. Plants usually depend upon combined, or fixed, forms of nitrogen, such as ammonia and nitrate. Much of the nitrogen is provided

to the plants in the form of industrially produced nitrogen fertilizers, synthesised from atmospheric dinitrogen (N_2).This has caused extensive loss to soil quality and fertility (Dobereiner and Baldani 1998; Okon and Vanderlegden 1998 Victoria et al 1992). Soil contains many types of bacteria such as algae, fungi, actinomycetes which play an essential role in maintaining the soil quality. Nutrient enrichment of soil by symbiotic nitrogen fixing bacteria has been known for centuries. The bacteria present in the root nodule of leguminous plant are responsible for fixing atmospheric nitrogen (Zsbrau, 1999). Amongst the soil bacteria rhizobia has a beneficial effect on growth of the plant (Shahzad *et al*, 2012). Rhizobia is a root nodulating bacteria which reside inside the root nodule of leguminous plants and fix the atmospheric nitrogen. The soluble form of nitrate and nitrite can be assimilated by the plant root and utilized in protein and nuleic acid. This form of nitrogen can be converted to ammonia by plants, animals and micro organism. *Rhizobium* has two different life styles one as free living or as a nitrogen fixing symbiotic bacteria in root nodules of the host plant. Low soil pH does not allow the survival of *Rhizobium* in the free living state (Tsegaye *et al*, 2015).. Hence it becomes inevitable to inoculate the crop in *Rhizobium*. The *Rhizobium* strain secrete growth hormones like indole acetic acid (IAA) which shows a good influence on plant growth and also play a role in development and formation of the root nodules. Screening an effective *Rhizobium* strain and enhancement of their quality is very much important for sufficient nitrogen fixation (Deka and Azad, 2006). The main purpose of this study was to isolate an efficient strain of *Rhizobium* and its immobilization with carrier and application in the soil as biofertilizer. *Pisum sativum* was used as experimental plant.

MATERIALS AND METHOD

Isolation of *Rhizobium* from the root nodules

For the following study nodules were collected from the roots of Pea Plant (*Pisum sativum*). The nodules were surface sterilized with 0.1% $HgCl_2$ and 70% ethanol and distilled water several times. These surface sterilized nodules were crushed in a large drop of sterile water and the nodule suspension, so obtained was streaked on YEMA (Yeast Extract Mannitol Agar) plates containing congo red (Bala *et al*, 2011 and Gwyn *et al*, 1989) It was incubated at 28^0C for 48 hrs. Well isolated typical single colonies were restreaked on freshly prepared YEMA plates in order to obtain pure cultures. Pure isolates were used for further biochemical analysis and all tests were performed in triplicates.

Morphological Characteristics: Circular, raised, sticky and gummy colonies with smooth edges were observed under low power microscope. Using Gram staining technique pink colored Gram negative rods were observed (Aneja, 2003).

Biochemical Tests: All the collected samples were processed through different biochemical tests viz, Catalase Test, Indole Production Test, Methyl Red Test, Voges Proskauer Test, Citrate Utilization Test, Carbohydrate fermentation test (Aneja, 2003).

Inoculum Preparation About 100 ml of broth was taken in a 250 ml Erlenmeyer flask and 1 ml of pure suspension culture containing 6×10^{-7} cells was inoculated. It was incubated in a rotary shaker at 28 ± 2°C for 4 to 6 days. The population of the test isolate was determined by dilution plate method (Singh *et al*, 2008). After the quantitative determination of population in the inoculum suspension, the broth culture was mixed with sterilized carrier for seed inoculation.

Biofertilizer Preparation The *Rhizobium* was immobilized onto charcoal used as a carrier which was applied as a biofertilizer in the soil (Jakhar *et al*., 2011). The carrier (charcoal) was powdered and dried in sun to get 5% moisture level. Then it was screened through mesh sieves and sterilized by autoclaving. Rhizobial culture was then mixed with carrier and kept in trays or tubes. The moisture content was maintained to about 40%. After proper mixing, it was left for curing for 2-10 days by covering the trays with polythene at 22-24°C, wherein *Rhizobium* cells multiplied (Einarsson, *et al,* 1993)

Pot Inoculation The isolated *Rhizobium* strain was applied as a biofertilizer on Pea plant to estimate a better growth effect. The rhizobial inoculant as a biofertilizer was applied on to a Pea seed. Activated charcoal was mixed with soil in the ratio of 1:3. No biofertilizer was used in control .This experiment was performed in triplicates. All the pots were keenly observed for the parameters such as shoot initiation, root initiation, shoot length, and root length. The data was recorded day by day (Kukkamalla & Vishnu, 2016).

RESULTS AND DISCUSSION

Colonies of *Rhizobium* was obtained on YEMA medium after incubation at 28°C for two days. The colonies were white or creamy white with sticky and gummy appearance. They did not absorb congo red dye. These were re-streaked to obtain a pure culture. The strains were maintained on YEMA plates and also on Nutrient agar slant. The obtained strain was biochemically tested.

General microscopic view of the isolate showed it to be rod cells and gram negative in nature. Bacteria were found to be positive for Catalase, Indole Methyl Red (MR), Citrate utilization and carbohydrate utilization and negative for Voges- Proskeaur (VP) tests. Table-1 shows the result of all biochemical tests.

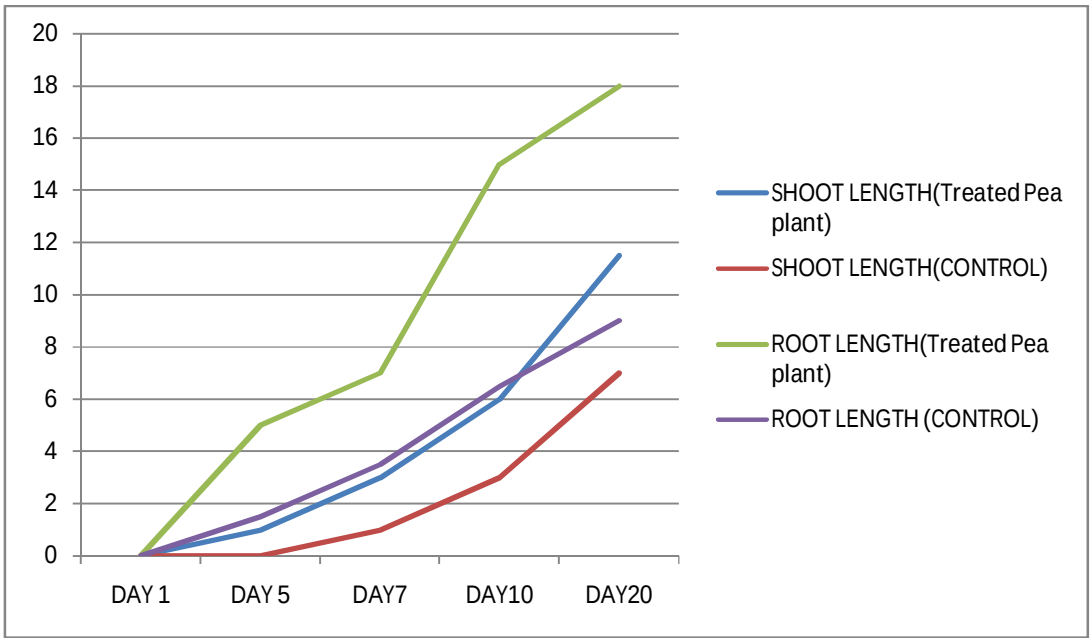
Table 1: Biochemical characteristics of isolated *Rhizobium*

S. No.	Biochemical test	Result
1	Gram staining	Gram negative
2	Catalase	Positive
3	Indole production test	Positive
4	Methyl Red	Positive
5	Voges –Proskeaur	Negative
6	Carbohydrate utilization test	Positive
6	Citrate utilization test	Positive

The rhizobial strain was immobilized onto a carrier (charcoal) in a ratio of 2:3. Two sets of pea plants were established in pots. The *Rhizobium*-charcoal carrier was used as a biofertilizer in the soil for potting in one set. The rhizobial biofertilizer was mixed with the autoclaved soil. The soil with no biofertilizer was used as a control. The pots were observed regularly for the parameters such as root initiation, shoot initiation, root length, shoot length, and the data was recorded after definite time intervals. The pot with the biofertilizer of rhizobial inoculant showed a good root and shoot growth when compared to the control pot. In pea plant with the biofertilizer in it the seed started to germinate by day 2, by day 5 rooting started to appear, by day 10 the shoot length observed was 6cm and root length as 15cm and in control 3cm and 6.5cm respectively and by day 20 the shoot length observed was 11.5cm and root length as 18cm, where as the control shows shoot length as 7cm and root length as 9cm.The recorded data is depicted in Table 2 and represented graphically in Graph 1.

Table 2: Growth parameters of pea plant in presence of biofertilizer (*Rhizobium*)

	Shoot Length (Pea) (cm)	Shoot Length (Control) (cm)	Root Length (Pea) (cm)	Root Length (Control) (cm)
Day 1	--	--	--	--
Day 5	Shoot Appears	--	Root Appears	--
Day 7	3cm	1cm	7cm	3.5cm
Day 10	6cm	3cm	15cm	6.5cm
Day 20	11.5cm	7cm	18cm	9cm



Graph 1: Graphical representation of Growth parameters of pea plant in presence of biofertilizer (*Rhizobium*) compared with control.

CONCLUSION

The pea seed with the biofertilizer shows a better growth enhancement than the control. Hence the isolated *Rhizobium* strain functions effectively as a biofertilizer and enhances the growth of the plant. The Rhizobial inoculant was found to good, effective, growth enhancer, easy and safe to be used as a biofertilizer .

REFERENCES

1. Bala, A., Abaidoo, R. and Woomer, P. (2011). *Rhizobia* Strain Isolation andCharacterisation Protocol, Milestone reference number 3.1.1
2. Aneja, K. R. (2003). Experiments in Microbiology Plant Pathology and Biotechnology.4th edition, New Delhi, India New Age International Publishers, 6: 571-585.
3. Kukkamalla, A., Vardhan, Z.V. (2016). IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT) e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 10, Issue 11 Ver. I , PP 106-111
4. Deka, A. K and P. Azad (2006). Isolation of Rhizobial strains cultural and Biocemical Characteristics. Legume. Res. 29 (3):209-212
5. Dobereiner, J. and V.L.D., Baldani, 1998. *Biotechnol. Sci. Dev. Bras.*, 1: 16- 17.
6. Shahzad, F., Shafee, M.,. Abbas, F., Babar, S.,. Tariq, M. M and Ahmad, Z. (2012). Isolation and Biochemical characterization of *Rhizobium meliloti* from root nodules of alfalfa (Medico Sativa). The Journal of Animal & Plant Sciences, 22(2): Page: 522-524 ISSN: 1018-7081
7. Gwyn A., B e a t t i e and Jo Handelsmann(1989). A rapid method for the isolation and identification of *Rhizobium* from root nodules Journal of Microbiological Methods 9 29-33 29
8. Jakhar,M., Purbia,U., Singhal, K. and Deora, G.S. (2011). Isolation, Characterization and Preparation of Biofertilizer using *Azospirillum* and its *in-vivo* effect on crop plants, Asian Journal of Biochemical and Pharmaceutical Research, Issue2 (Vol.1) 16-26
10. Tsegaye, M., Assefa, F., Zeleke1, J. (2015). Symbiotic and phenotypic characterization of *Rhizobium* isolates nodulating fenugreek (Trigonellafoenum-graecum L.) from North and East Shewa,

Ethiopia, International Journal of Agronomy and Agricultural Research (IJAAR), Vol. 7, No. 1, p. 93-104

9. Okon, Y. and Vanderlayden, J., 1998. *Features*. 63: 366-370.
10. Einarsson,S.L., Gudmundsson, J., Sverrisson,H., Jakob,K. and Runolfsson,S. (1993) Applied and Environmental Microbiology, Vol. 59, p. 3666-3668
11. Singh, B., K. Ravneet and S. Kashmir (2008).Characterization of Rhizobium strain isolated from the roots of *Trigonella foenumgraecum* (fenugreek) Afri. J. Biotech.7 (20): 3671–3676
12. Victoria, R.L., Picolo, M.C. and Vargas, A.A.T., 1992. Microbiology of the ground campinas, *Brazilian science of the ground.* The cycle of Nitrogen. pp: 105-117.
13. Zsbrau HH (1999). *Rhizobium*-Legume symbiosis and nitrogen fixation under severe conditions and in arid climate. Microbiol. Mol. Biol. Rev.63: 968-989.