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Exogenously Supplied Rhizobium Induced Nodulation In Vigna Unguiculata

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Abstract: ***Nitrogen plays an important role in plant growth; metabolism and biological nitrogen fixation in legumes through diazotrophs is well documented. The endosymbiotic interaction of bacterial strains with the roots of plants forming nodules is one way to meet the requirement of nitrogen supply to the plant. The present work focused on Invitro Rhizobium inoculation in leguminous plant Vigna unguiculata grown in sterile soil and in vermiculite. Artificial Rhizobium inoculation in legumes and the frequency of their nodulation was studied in 1) Control plants grown in distilled water only, 2) Plants grown with rhizobium + Knop solution, 3) Plants grown with Rhizobium + water, 4) Plants grown with Knop solution only. Plants were grown under lab conditions from seed stage until complete growth (buds and pods). The results obtained indicated interaction of Rhizobium with legumes irrespective of inoculation even in control plants. However artificial inoculation with Rhizobium culture, enhanced nodulation frequency. Our results also indicate that efficient nitrogen fixation and nodulation can be induced in legume species through Rhizobium coated biofertilizer sources like charcoal and saw dust. The concept of using Rhizobium inoculated biofertilisers can be a new approach for induction of nodulation in non-legume plants and may provide insight into understanding of the interaction of artificial Rhizobium inoculation and nodule formation.***

Keywords: ***Nitrogen fixation, Rhizobium, Inoculation, Leguminous plants, Nodulation, Endosymbiosis***

I. INTRODUCTION

Nitrogen is an essential nutrient and is universally occurring element in all the living beings (Xinning & Darcy, 2016). It is also a constituent element of many important organic compounds like proteins and nucleic acids (Adamczyk et al., 2010), chlorophylls, cytochromes, alkaloids, many vitamins (Satyanarayana & Chakrapani, 2006). Nitrogen thus plays a very important functional and fundamental role in metabolism, growth, reproduction and heredity (Karatkar, 2016). Plant cells are unique in having ability to convert inorganic nitrogen into organic nitrogen (Harper, 1984). Unavailability or lack of nitrogen results in deficiency symptoms like reduced cell division and dormant lateral buds; delayed maturity, chlorosis in the older leaves and appearance of purple colouration in shoot axis, petioles and on the underside of the leaves (Saxena, 2009 & Snyder, 2011).

Biological nitrogen fixation by the metalloenzyme nitrogenases is the main route for natural entry of fixed nitrogen to earth's ecosystems (Gruber & Galloway, 2008). In legumes, it occurs within the bacteroids; the reaction requiring hydrogen and energy in the form of ATP. Rhizobia are Gram-negative free living soil bacteria in the vicinity of legumes. However, they cannot fix atmospheric nitrogen until they invade the roots of the legumes (Zahran, 1999). These free living bacteria are motile and feed on the remains of dead organisms (Dubey & Maheshwari, 2014). Nodulation is hence an important process in leguminous plants helping in enriching and improving the standards of nitrogen-fixation. Rhizobia also induce disease resistance, reduce heavy metal in

the soil, facilitate bioavailability of soil iron and is environment friendly (Namkeleja, Mtei, & Ndakidemi, 2016). Application of biological nitrogen fixation (BNF) through rhizobial inoculums is highly promoted as a solution to solve the problem of poor soil fertility. Since the time, symbiotic relationship between rhizobium and the roots of the leguminous plants was studied; attempts were made to improve the standard of nitrogen fixation through natural and artificial methods of nodule formation in these plants. Simple techniques like natural nodule formation by inoculation, or through complex methods of gene targeting, have been attempted by researchers, to understand and improve nitrogen fixation thus improving the quality of the plants (Stambulska & Lushchak, 2015). However, use of nitrogenous fertilizers has resulted in increased water pollution through eutrophication of lakes and rivers and hence in current scenario biological nitrogen fixation is gaining practical importance (Ahlgren, Bernesson, Nordberg, & Hansson, 2010).

The entire focus of world today is on environmentally sustainable development through efficient use of biological nitrogen fixation for crop improvement (Dixon, R. O. D., and C. T. Wheeler. 1986; Peoples, M. B., et.al.1995). Considering the importance of biological nitrogen fixation in crop improvement, the present study was undertaken with an objective to understand the nature of rhizobial interaction and nodulation by exogenously supplied rhizobium inoculation in *Vigna unguiculata*.

II. MATERIALS AND METHODS

Plant Materials and growth conditions: Seeds of cowpea (*Vigna unguiculata*) were purchased from local market and surface sterilized with 0.1%$HgCl_2$, washed with distilled water repeatedly and left to soak in water overnight. The seeds were then tied in a muslin cloth to initiate germination. Plastic pots containing sterile vermiculite and sand (3:1) were used for sowing the seeds. Seedlings were grown under laboratory conditions with 12 hours of light and dark photoperiod at temperature 25°C±2°C with 500 µmol $m^{-2}s^{-1}$ PAR light intensity for 3 weeks. They were watered regularly with distilled water during growth conditions. Three weeks old seedlings (4 in number) were then transplanted in fresh pots containing sterile vermiculite and sand (3:1) for further treatment.

SOURCE OF RHIZOBIUM CULTURE

Fresh plantlets of (*Mimosa pudica*) were uprooted from soil, roots were washed with water to remove all the soil particles and nodules from the roots were collected. The nodules were further washed thoroughly to get rid of the soil particles if any and were used for rhizobium cultures.

PREPARATION OF LB MEDIA & GROWTH OF RHIZOBIUM CULTURES IN LB MEDIA

LB medium was prepared according to the method of (Miller, 1972) containing beef extract, peptone and water adjusted to pH 7.2. The media was autoclaved at 120 lb pressure for 20 minutes, cooled and stored in refrigerator until use. Under sterilized conditions using laminar air flow, the root nodules were crushed and put in conical flask containing 25 ml liquid broth (LB) and was placed on a rotator shaker maintained at 90 rpm for 24 hours.

PREPARATION OF PDA PLATES & INOCULATION OF RHIZOBIUM CULTURE ON PDA PLATES

Plates were prepared using disposable sterile petriplates from High media with 20gms PDA/L. The media was then autoclaved at 120 lb pressure for 20 minutes, cooled to about 50°C. Under sterile conditions, 10-15 ml of warm media was then poured in the petriplates and left to dry. About 100µl of rhizobium culture was inoculated on PDA plates and was spread using a sterile spreader. Plates were dried, sealed with paraffin and left in an inverted position in an incubator at 37^{o}C for 48 hours.

PREPARATION OF RHIZOBIUM CULTURE FROM RHIZOBIA GROWN ON PDA

Autoclaved 25 ml of LB medium was taken in a sterile conical flask and was inoculated with loop full of rhizobium culture grown on PDA plate. The flask was placed on a rotator shaker maintained at 90 rpm for 24 hours. This culture was used for treatment of the plants.

TREATMENT PARAMETERS: The treatment to the seedlings was started after 4 days of transplantation. Four sets of plants were maintained as follows:

- ✓ Control
- ✓ Rhizobium + Knop solution
- ✓ Rhizobium + Distilled water
- ✓ Knop solution only

Plants were supplied with 2ml of rhizobium culture on 5th day of transplantation and again a second inoculation a week after the same. Plants were regularly watered with standard knops solution (Wilhelm Knop) while control plants were watered with distilled water alone. Plants were allowed to grow under above treatment conditions for nearly 6 weeks after transplantation at 27°C ± 2°C with light intensity of 500µmol PAR in laboratory. Growth of plants was monitored regularly.

ANALYSIS OF NODULATION

Six weeks old plants from respective treatment were uprooted carefully by loosening the soil/ vermiculite, washed with tap water to get rid of vermiculite and soil. The growth of roots was observed, nodules were located and photographed (Fig 1.1 and 1.2 resp.).

PREPARATION OF BIOFERTILIZER

Rhizobium inoculated biofertiliser was prepared by coating the inert materials like charcoal and sawdust with the rhizobium cultures (Fig 2.1). These materials were then mixed with vermiculite and used to grow the plants (Singh, Kaur, & Singh, 2008).

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III. RESULTS

Our results showing inoculation of rhizobium and root nodulation in plants grown in vermiculite and soil are shown in table number 1 A, 1B; Fig 1.1 and 1.2.

Plants grown with vermiculite under control condition (distilled water only) showed healthy growth with broad leaves and proportionate growth with fibrous roots. Nodules were seen to be large in size but of low frequency. Under experimental condition, plants grown along with rhizobium and knop solution showed only vertical growth with less fibrous root. Nodules were very small, spherical and higher in frequency. Normal growth with medium sized, round nodules of low frequency were observed in plants grown with rhizobium and distilled water. However, plants grown with nutrient solution alone, showed proportionately healthy growth with small sized, round, white nodules of higher frequency (Fig 1.1).

Our results with plants grown with soil under control condition (distilled water only) showed healthy, proportionate growth. Nodules were large with an area of about 0.18 cm^2, blackish, spherical with low frequency (Table No 1A). Plants grown under experimental condition of rhizobium and knop solution showed normal growth with pods. Nodules were very small, oval to round, with very high frequency of nodulation. In our study plants grown with rhizobium and distilled water showed healthy growth with broad leaves. Nodules were large in size, round, white with low nodulation frequency in comparison to plants grown with nutrient solution alone which showed healthy growth with less fibrous root (Fig 1.2) (Table 1B).

Our result with rhizobium inoculated biofertiliser sources (charcoal and saw dust) are shown in Fig No. 2. Plants grown using charcoal and sawdust as biofertiliser source were efficient in their growth. Clustered nodule formation was seen in both the plants. Nodules were also significantly bigger in size in comparison to that of the nodules grown in above mentioned experimental conditions.

SR. NO	GROWTH CONDITIONS	OVERALL PLANT HEALTH	SIZE OF NODULES	SHAPE AND COLOUR	FREQUENCY OF NODULES
1	CONTROL (only distilled water)	Healthy growth with broad leaves Plant growth proportionate Roots fibrous Pods were seen	Large in size	Round Red	Low frequency solitary nodules
2	RHIZOBIUM + KNOP SOLUTION	Only vertical growth was seen Roots less fibrous Pod were not seen	Very small	Spherical White	Higher frequency of nodulation Nodules appeared in row
3	RHIZOBIUM + DISTILLED WATER	Normal growth Roots very fibrous in nature	Medium sized	Round White	Low frequency
4	KNOP SOLUTION ONLY	Healthy growth with broad leaves Plant growth proportionate Roots less fibrous	Small in size	Round White	Higher frequency

Table 1 A: Comparative account of root nodulation characteristics in plants grown in vermiculite

SR. NO	GROWTH CONDITIONS	OVERALL PLANT HEALTH	SIZE OF NODULES	SHAPE AND COLOUR	FREQUENCY OF NODULES
1	CONTROL (only distilled water)	Healthy growth with broad leaves Plant growth proportionate Pods were seen	Large in size Area of the nodule 0.18cm^2	Spherical in shape Blackish	Low frequency of nodules
2	RHIZOBIUM + KNOP SOLUTION	Normal growth Plant Growth proportionate Pods were seen	Very small nodules	Small oval to round nodules Black to brown in colour	Very high frequency of nodulation
3	RHIZOBIUM + DISTILLED WATER	Healthy growth with broad leaves Roots very fibrous	Large sized Area of the nodule: 0.15cm^2	Round White	Few nodules appeared
4	KNOP SOLUTION ONLY	Healthy growth with broad leaves Roots less fibrous Plant growth proportionate	Small in size	Round White	Higher frequency of nodulation

Table 1 B: Comparative account of root nodulation characteristics in plants grown in soil

Figure 1.1: Root Nodulation of plants in vermiculite

Figure 1.2: Root Nodulation of plants in soil

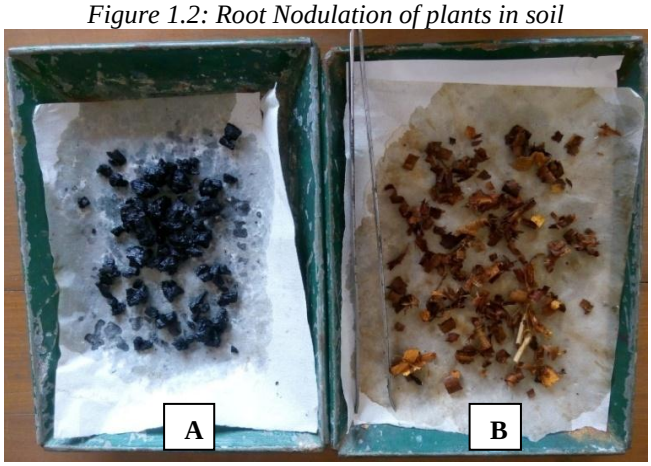

Figure 2: Rhizobium inoculated Biofertilisers: A) Charcoal B) Sawdust

IV. DISCUSSION

Biological nitrogen fixation is one of the most important processes for ecosystem to access available nitrogen in all the living organisms. Enrichment of soil nutrients by nitrogen fixing symbiotic bacteria present in legumes is known since many years. Annual rate of natural nitrogen fixation is estimated about 232 x 106 t, and the 97% depends on biological nitrogen fixation (Bloom, 2011). Zahran (1999) scientifically demonstrated that bacteria in root nodules of legumes are responsible for fixing atmospheric nitrogen. Role of rhizobia in nitrogen fixation is of great importance in agriculture in several ways. Legumes such as peas, beans, lentils, soybeans, alfalfa and clover are the food crops to animals as well as to humans. Crop yields are greatly improved in nodulated plants; legumes can also grow well in poor soils where there is not enough fixed nitrogen to support other types of plants (Araujo, Figueiredo, & Monteiro, 2008).

Nitrogen fixation by natural means significantly cuts down on the use of artificial fertilizers. This not only saves money but helps to prevent the many problems brought about by excessive use of commercial nitrogen and ammonia fertilizers such as eutrophication of rivers and lakes, generation of acid rain, and overgrowth of agricultural land by non-food crops (Moran.R., 1997). Since fixed nitrogen is often the limiting factor for plant growth in all environments, with suitable climate and availability of water supporting life, lot of research is being carried out to find ways of improving the amounts available to the plants. This includes not only enhancing the efficiency of rhizobia as nitrogen fixers in legumes, but also using genetic engineering to bring about nitrogen fixation in non legume crops. Legumes have evolved with altered phenotypic responses to internal nitrogen compared with non nodulating plant species due to the presence of root nodules as endogenous nitrogen source (Goh, Nicotra, & Mathesius, 2016).

In our study we have observed that artificial inoculation leads to more frequency of nodulation in legumes. This could possibly be because of the increased internal nitrogen level. We have also observed that supply of nitrogen externally through mineral nutrition such as Knop solution significantly reduces the level of nodulation which may possibly be due to already available nitrogen in the Knop solution. The results are in correlation to Carroll & Gresshoff (1983), Walch-Liu et al (2006) who have reported lower number of nodule formation through artificial supply of nitrogen. Through the research work by Harper & Gibson (1984), Gibson & Harper (1985), Davidson & Robson (1986) it is well understood that heavy supply of nitrogen fertilizer often causes the inhibition of nodulation and nitrogen fixation (Goh, Nicotra, & Mathesius, 2016). We also reported similar results.

The inhibitory effects of externally supplied N especially NO_3^- have been reviewed (Streeter, 1988). The nitrate inhibition is a complex process and cannot be explained by a single mechanism (Harper J. , 1987). It has been suggested that there are multiple effects of nitrate inhibition, such as the decrease in nodule number, nodule mass, N_2 fixation activity, as well as the acceleration of nodule senescence or disintegration (Streeter, 1988). Nitrate inhibition is primarily host plant dependent and it is independent of nitrate metabolism of rhizobia (Gibson & Harper, 1985) (Carroll & Mathews, 1990). Many hypotheses are proposed for the cause of nitrate inhibition of nodulation and N_2 fixation, i.e. carbohydrate deprivation in nodules (Vessey & Waterer, 1992), feedback inhibition by a product of nitrate metabolism such as glutamine (Neo & Layzell, 1997), asparagine (Bacanamwo & Harper, 1997) and decreased O_2 diffusion into nodules which restricts the respiration of bacteroids (Schuller *et al.*, 1988).

Hashem *et.al*. (1998) have proposed that salt stress may reduce the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis and hence nitrogen demand. This may be by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, that directly interfere with root nodule functioning (Hashem *et.al*. 1998).

In our study we have also taken an approach for immobilization of rhizobium using charcoal and saw dust as an alternative method of artificial inoculation in plants. Earlier AbdelGadir and Alexander (1997) reported that rhizobial cells can be immobilized on calcium alginate beads; their work was mainly focused on isolation of heat tolerant strains of rhizobia and not on production of useful enzymes. The use of immobilized cells as industrial catalysts can be advantageous compared to batch fermentation process. Whole cell immobilization has been a better choice over enzyme immobilization (Adinarayana *et al.*, 2005). Biomass yield was found to be high in immobilized cells as compared to normal YEM broth culture.

Short-term local effect of nitrate supply on nodule formation and nitrogen fixation was evaluated using hydroponically grown soybean plants (cultivar Williams), which were inoculated with *Bradyrhizobium japonicum*, (*strain USDA110*) (Fujikake *et al*. 2002). In recent years, new insights into rhizobium–legume, rhizobium–Parasponia, actinorhizal and AM symbioses led to renewed interest in the possibility of transferring nitrogen- fixing ability to non-legume crops.

V. CONCLUSION

In this work we focused on effect of artificial inoculation of rhizobium in *Vigna unguiculata*. From our results we conclude that rhizobium and leguminous plants showed positive symbiotic relationship enhancing the nodulation frequency, hence more nitrogen assimilation. This is absent in the presence of any kind of external nitrogen source given to the plants in the form of nutrient medium like Knop or biofertilisers. One of the new methods of nitrogen inoculation can be biofertilizer based immobilisation of rhizobium using inert materials like charcoal, dry plant seeds and saw dust. Thus immobilisation technique can certainly be a possible new approach for nitrogen fixation for non leguminous plants.

SIGNIFICANCE OF THE WORK: This work is of significant nature as it may provide insight into understanding of the interaction of exogenous rhizobium inoculation and nodule formation. The concept of using rhizobium inoculated biofertilisers can also be a new approach for induction of

nodulation in non-legume plants and need to be further studied.

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