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A Study on Effective and Ineffective Root Nodules of Trigonella Foenum Graecum Elicited by Bradyrhizobium

Aruna Kukkamalla[1] , Dr.Z.Vishnu Vardhan[2]
[1]*Dept. Of Environmental Science, Acharya Nagarjuna University, Guntur,Andhra Pradesh,India*
[2]*Dept.of Botany,Acharya Nagarjuna University, Guntur,Andhra Pradesh,India*

Abstract: *Microscopic studies indicate that the lateral roots of all Acacia&Prospois species examined have root hairs. Only very young seeding has hairs on the top roots. Nodules are classified as effective and ineffective strains of rhizobia form ineffective nodules .Which are generally small and contain poorly developed bacterial tissue showing accumulation of starch in host cells .Which do not contain rhizobium and dextrin in host cells infected by rhizobium .On the other hand effective nodules formed by effective strains are well developed, pink in colour due to leghemoglobin and the bacterial tissue is well organized with plenty of bacteroids.A study was performed to investigate the most commonly associated nodule bacteria and the rhizospheric micro organisms associated with the fenugreek plant .In this present study the effective and in effective root nodules ,the root nodulating tropical legumes,Trigonella foenum-graecum both effective and ineffective nodules, was selected. The seeds of trigonella foenum-graecum and Bradyrhizobium strains S24(effective)and S24A06(ineffective)were obtained from Tamil Nadu Agricultural university (TNAU),Coimbatore.*

Keywords: *Root nodules (effective and ineffective), Rhizobium, Bradyrhizobium, Fenugreek, Tropical legumes*

I. Introduction

Our biosphere consists of 19,000species of legumes of which a only one six of have been studied for nodulation (Allen &Allen 1981).Nodules are in fact 'SHELTERED &CONTROLLED ENVIORNMENT".Root nodule bacteria provide a source of nitrogenous compounds .Which are avoidly pickled up by the host plant &translocated rapidly to growing plant. One of the earliest botanical descriptions of root nodules on legumes is attributed to Dale champ. Research on the morphology of the nodules on essential understanding of limitations to symbiotic nitrogen fixation on an organism &crop community.

The Rhizobium a nitrogen fixing bacteria is the essential feature of leguminous plants. Increased cultivation of legumes is essential for the regeration of nutrient-deficient soils and providing needs nutrients to human beings and animals.

Soil contain many types of micro organisms such as bacteria ,fungi&alage which are important because they effect the all properties of soil (Physical, chemical &biological) .Among the soil bacteria a unique group called Rhizobia has a beneficial effect on the growth of plants. It can live either in the soil or within the root nodules of host legumes.

Some alkaline soils have fertility problems due to poor physical properties .Which adversely affect the growth and the yield of crops and inoculation with the Rhizobium is more effective for promoting growth of feenu Greek in the alkaline soil.Rhizobia characterized into two groups on the basis of growth rate. First group is fast growth Rhizobia.Second group is slow grower Rhizobia. The slow growing bacteria have mean generation time greater than 6h and fast growing bacteria have less than 6h in selective broth medium (Elkan1992)

II. Biological Nitrogen Fixation

Biological nitrogen fixation by leguminous green manure crops in symbiotic association with *Rhizobium* is a low cost input for rice crop. The cultivation of nitrogen fixing legume green manure crop would be an efficient way of improving soil fertility. The formation of nodules in the roots is an external manifestation of the symbiotic association of a bacterium, *Rhizobium*, with the roots of the leguminous plants. As rightly pointed out by Fred *et al.* (1932), the credit for the first report of the root nodules goes to Fuchs, (1542) who first described the root nodules from *Aphaca*, *Vicia faba* and *Trigonella foenumgraecum* in the first edition of the book entitled *Historia Stirpium Commertarii Insignes* published in 1542. The actual insight into the origin and the function of the root nodules in relation to the utilization of atmospheric nitrogen by leguminous plants was given and explained by Hellriegel in 1886.

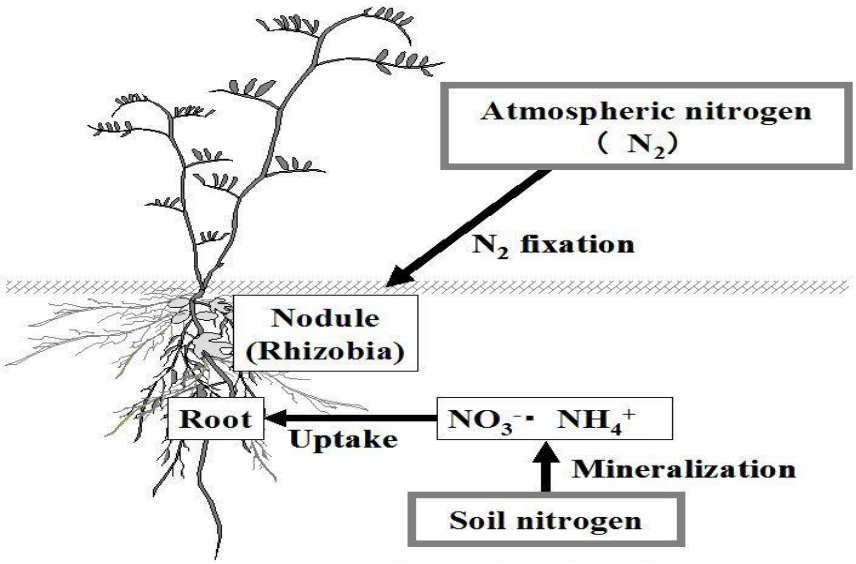

Fig. Biological nitrogen fixation with root nodules

Nevertheless, the role of bacterium in the nitrogen utilization was not established until 1888. It was only in 1888, Beijerink, first isolated the bacterium (*Rhizobium*) from the root nodules and cultured on the media in the laboratory. Since then, the attention of the microbiologists is centred around understanding the morphology of the bacterium, its distribution, ecology and its physiological and genetical relationship with the roots and nature, morphology and distribution of nodules in the roots of the host plants in relation to different habitats and environmental conditions; and the research works on these aspects have been reported and reviewed from time to time (Allen and Allen, 1936, 1947; Martin, 1948; Bowen, 1956; Gordon *et al.*, 1999; Djordjevic *et al.*, 2003; Udvardi., 2001; Rasanen, 2003; Serraj, 2003; Vij, 2003; Tajima *et al.*, 2004; Liu Miller *et al.*, 2006, Larrainzar *et al.*, 2007).

III. Rhizobium - Legume Symbiosis

Symbiosis is a biological phenomenon involving dynamic changes in the genome, metabolism and signaling network. A multidirectional comprehension of these interactions is required when studying symbiotic organisms. In plant–microbe interactions, two symbiotic systems have been actively studied for many years (Kistner and Parniske, 2002; Bonfante and Genre, 2010).

Rhizobia have two different life-styles, either as free-living soil bacteria or as nitrogen-fixing endosymbionts within root nodules of legume host plants. In a well-balanced physiological interaction, the microsymbiont fixes atmospheric nitrogen and provides ammonia as a nitrogen source to the plant in exchange for a carbon and energy source generated by photosynthesis (Udvardi and Day, 1997; Djordjevic *et al.*, 2000; Long, 2001; Prell and Poole, 2006; Pessi *et al*., 2007).

Legume crops are important for the development of sustainable agriculture and legume nodules provide an excellent model for studying fundamental aspects of plant–microbe signaling and cell morphogenesis **(**Limpens *et al.,* 2003). The three subfamilies of Leguminous (Caesalpinioideae, Mimosoideae and Papilionoideae) contain species that form root nodules as described by Sprent (2001) Nodulation in Legumes**.** Green gram (*Vigna radiata*) is an important short duration pulse crop. Due to its high nutritive value, it is grown throughout the tropical countries of South and Southeast Asia, particularly in India (Akhtar *et al.,* 2005).

In *Rhizobium*-legume symbiosis, host-specificity is decided in both ways, by plant flavonoids as well as by the rhizobia. The nodulation genes (nod, nol and noe) encode proteins involved in the synthesis and secretion of lipochito oligosaccharides, called Nod factors, play a pivotal role in the induction of all early responses (Spaink, 1995; Bladergroen and Spaink 1998; Schultze and Kondorosi 1998; Zuanazzi *et al.,* 1998; Hirsch *et al*., 2001; Geurts and Bisseling, 2002; Jones *et al.,* 2007; Kouchi *et al.,* 2004). However, other rhizobial systems such as ethylene biosynthesis regulation (Okazaki *et al.,* 2004; Gresshoff *et al.,* 2009), protein secretion systems (Deakin and Broughton, 2009) and BacA (LeVier *et al.,* 2000) are often required for the establishment of symbiosis with legumes, probably because they are involved in bacterial release into the host cytoplasm and bacteroid development.

Two routes of *Rhizobium* infection have been described for root-nodule formation in legume roots: entry via root hairs and via cracks. Root-hair entry occurs in most legumes, e.g. soybean and common bean (*Phaseolus vulgaris*). Crack entry occurs in a few legumes: peanut and *Sesbania.* In peanut, root nodules

develop only at the sites of lateral-root emergence (Uheda *et al*., 2001), where the epidermis and cortex of the parent root are broken by emergence of the lateral root (Boogerd and Van Rossum, 1997).

For root nodule symbiosis, an exchange of signal molecules between the host and rhizobia is required. After root colonization, rhizobia enter roots of legumes via root hairs and they induce morphological changes in the epidermis and migrate to the root cortex via infection threads and induce the formation of nodules typically in the susceptible region of the root. The formation of a nodule requires the reprogramming of differentiated root cells to form a primordium, from which a nodule can develop. Furthermore, the bacteria must infect the root before the nitrogen-fixing root nodule can be formed. It is generally thought that a compatible symbiotic interaction involves an inhibition of host defense mechanisms to permit the establishment of N -fixing soil bacteria within the host cells (Sprent and Faria, 1988; Sprent, 2001; Hadri *et al.,* 1998; Geurts and Bisseling, 2002; Tesfaye *et al.,* 2006).

IV. Root Nodules-Nodule Status

Microscopic studies indicate that the lateral roots of all *Acacia* and *Prosopis* species examined have root hairs. Only very young seedlings have hairs on the tap roots. Nodules grew singly or in groups on the lateral roots and occasionally on the top roots or on the short and thin rootlets that arise from the tap root. Young nodules are spherical but later become elongated and being able to grow from several points (Rasanen *et al.,* 2001). Legume nodules are of two types – determinate and indeterminate – viewed in terms of the growing periods of the individual nodules. The determinate nodule is oval, whilst the indeterminate nodule has an axis and is elongated by the meristem at the apical part of the nodule (Puppo *et al.,* 2005).

Nodules are classified as effective and ineffective. Even with very young nodules, which are small and indistinguishable externally, the presence of leghemoglobin (nodule internal tissue pigmented pink) will help to identify effective nodules. Ineffective strains of rhizobia form ineffective nodules which are generally small and contain poorly developed bacteroid tissue showing accumulation of starch in host cells which do not contain *Rhizobium* and of dextran in host cells infected by *Rhizobium.* The bacteroids of ineffective nodule contain glycogen. On the other hand, effective nodules formed by effective stains are well developed and pink in colour due to leghemoglobin and the bacteroid tissue is well organized with plenty of bacteroids (Vincent, 1982; Venkataraman and Kannaiyan, 1993).

Leguminous plants strictly control nodule numbers, because nodulation and nitrogen fixation are an energy drain on the host. To maintain the symbiotic balance with rhizobia, plants have evolved negative feedback systems known asautoregulation of nodulation (AON). AON involves long-distance signaling via shoot–root communication and is mediated by CLAVATA1-like receptor kinases such as *L. japonicus* HAR1, *Glycine max* NARK and *M. truncatula* SUNN (Oka-Kira and Kawaguchi, 2006; Ferguson *et al.,* 2010).

Nodulation tests with bacterial strains isolated from *Acacia* nodules in the field have shown that although nodules are formed on the roots, not all are capable of fixing N . In Australia, only 36 per cent of the strains isolated from *Acacia* spp. significantly increased plant growth. Also a later study with rhizobia isolated from Australian acacias showed a very wide variation in effectiveness, some host rhizobia combinations being close to parasite. Vegetative rhizobia, being capable of saprophytic growth in soil and nodule debris, have been found in both effective and ineffective nodules (Serraj, 2003).

Both plant and bacterial genomes interact to produce ineffective nodulation. The same *Rhizobium* strain can form effective nodules on one species and ineffective nodules on another closely related species from the same cross-inoculation group. The symbiosis may "fail" at virtually any stage in its development for any particular association. Generally a plant forms more ineffective nodules than effective and these are usually smaller and spread over the whole root system. In ineffective nodules rhizobia or infection threads are rarely found, the whole nodules being formed of roughly isodiametric "parenchyma" cells Development of vascular traces is restricted (Hardy and Silver, 1974; Rasanen, 2003). It is characteristic of *Acacia* and *Prosopis* spp. that they are easily nodulated by incompatible rhizobia inducing ineffective nodules. Five African strains, isolated from *A. senegal, P. chilensis* and *A. mollisima* nodules in *Sudan* and *Senegal* and representing four *Sinorhizobium* species, formed effective nodules on African acacias and Latin American *Prosopis* spp. But induced in-effectice nodules on African *P. africana* and nodule-like structures on Australian *A. holocericeae*. *Sinorhizobium* and *Mesorhizobium* strains isolated from *A. tortilis* subsp. *radiana* nodules from various sites in the North and South Sahara formed effective nodules on its host but, induced usually ineffective ones on *A. senegal, P. juliflora, Leucacna leucocephala, F. albida* and *A. mangium*. Hence, the two latter tree species usually form N *Bradyrhizobium* spp. (Rasanen, 2003) fixing nodules with Sprent (2001) suggested that at the community level both plants and bacteria may gain advantage from the formation of ineffective nodules. Ineffective as well as effective *Acacia* nodules may be a way of maintaining rhizobial populations under stress conditions (Rasanen, 2003).

V. Materials & Methods

Composition of Yeast Extract Mannitol Broth (Vincent, 1970)

Mannitol	- 10.0 g
Potassium hydrogen phosphate	- 0.5 g
Magnesium sulphate	- 0.2 g
Sodium chloride	- 0.1 g
Calcium carbonate	- 1.0 g
Yeast extract	- 1.0 g
Distilled water	- 1000 ml

For solid medium 16 g agar was added to the above broth.

1. Pot Culture Experiment

1.1. Inoculum Preparation

About 100 ml of broth was taken in a 250 ml Erlenmayer flask and 1 ml of pure suspension culture containing 6×10^{-7} cells was inoculated. It was kept on a rotary shaker to produce heavily turbid suspension and incubated at 28 ± 2°C for 4 to 6 days. The population of the test isolate was determined by dilution plate method (Hoben and Somasegaran, 1982). After the quantitative determination of population in the inoculum suspension, the broth cultures (containing 6×10^{-7} cells ml^{-1}) (both effective and ineffective) were mixed with sterilized lignite carrier for seed inoculation.

1.2. Seed Inoculation

Prior to sowing, the seeds of *Trigonella foenum*-graecum were mixed with rhizobial cluture-carrier material and made air dry. These inoculated seeds were sown in pots which had already been prepared.

1.3. Preparation of Earthen pots

Soil from fallow plots was mixed well, sieved and filled in earthenware pots at the rate of 10 kg per pot. The pots were watered to the level of 50 per cent moisture holding capacity of the soil and sterilized in a large horizontal autoclave at 20 lbs pressure for 2 h. They were then allowed to incubate in a pot culture house for 4 days and the soil in each pot was loosened and mixed well with the help of a stout glass rod. The physico-chemical properties such as EC, pH, bulk density (g/cc) and organic carbon (%) nitrogen, phosphorous, potassium and micronutrients such as zinc, copper, manganese and iron of sterilized soil samples were analysed (Table 1) following the methods of Barnes (1959) and Muthuvel and Udhayasoorian (1999). The following experimental conditions were employed.

- Seeds without test isolate – Control (C)
- Seeds treated with test isolate (T)

The *Bradyrhizobium* inoculated seeds were sown in the pot and watered well. The plants were uprooted at different stages (vegetative, flowering and pod filling) of growth for morphometric analysis.

2. Host Response Studies

The isolate was tested for its host response effectiveness by conducting morphometric analysis on the host plant and histological and histochemical studies on the effective and ineffective root nodules of host plant.

2.1. Morphometric Analyses

The morphometric analyses such as root length, shoot length, nodule status and nodule dry weight were done during vegetative, flowering, and pod filling stages, both in control and *Bradyrhizobium* inoculated plants.

VI. Histological Studies of Root Nodules

Collection and Preparation of Samples for Sectioning

The required samples of effective and ineffective root nodules were cut and removed from the plant and fixed in FAA (Formalin -5ml+Acetic acid5ml+70 Ethyl alcohol-90ml). After 24 h of fixing, the specimens were dehydrated with graded series of tertiary-butyl alcohol (TBA) as per the schedule given by Sass (1940). Infiltration of the specimens was carried out by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super-saturation. The specimens were cast into the paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10-12 µm. Dewaxing of the sections was carried by following customary procedure (Johansen, 1940). The sections were stained with toluidine blue as per the methods published by O'Brien *et al*. (1964). Since toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytological

reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells and dark green to suberin.

Histochemical Studies of Root Nodules

The sections were also stained with safranin, fast-green and potassium iodide for histochemical studies. Microscopic description of tissues were supplemented with photomicrographs wherever necessary. Photographs of different magnifications were taken with Nikon Labphot 2 Microscopic unit. For normal observations, bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was used. Magnification of the figures was indicated by the scale- bars. Descriptive terms of the anatomical features were as given in the standard Anatomy book (Easu, 1965).

Estimation of Leghemoglobin Content of Root Nodules (Sadasivam and Manickam, 2007)

The washed and weighed (200 mg nodules) (both effective and ineffective) were crushed in phosphate buffer (pH 7.4), macerated in a mixer separately and then centrifuged at 10,000 xg for 10-30 min. The supernatant was made up to 4.0 ml with phosphate buffer and 2.0 to 5.0 ml of pyridine reagent was added. Then, it was divided equally between two tubes. To one portion was added a few crystals of sodium dithionite and the optical density was measured at 556 nm after 2-5 min in spectronic 20 using the blank without extract. To the other portion, a few crystals of potassium hexacyanoferrate was added to oxidize the hemochrome and the optical density was measured at 539 nm. The quantity of leghemoglobin was calculated by comparing with the standard graph prepared using pyridine and expressed in mg/g.

Nitrogenase Activity (Hardy *et al.,* 1968)

The nitrogenase activity of the root nodules was determined by acetylene reduction method. Nodulated root system was gently freed from soil particles, washed briefly in running tap water and dried by blotting with filter paper. The nodules, both effective and ineffective, were then transferred separately to serum bottles of 15-20 ml capacity. The container was closed air tight with rubber stopper through which gas injection and gas sampling were done using micro syringe. The air in the container was evacuated and then equal quantity of acetylene was injected into it. The reaction was allowed to proceed for 1 h at 28 ± 2°C after which, 0.5 ml gas sample was withdrawn and fed to the gas chromatography (Perkin Elmer F33 model) having Poropak column 80-100 mesh, column temperature 150°C and oven temperature 95°C.

The acetylene reduction activity (ARA) was graphically represented on the gas chromatograph chart. It was quantified by measuring the height of the peak in mm. The obtained results were compared with that of the standard graph and the nitrogenase activity in terms of ethylene produced was determined.

VII. Discussion

The world population is expected to cross the ten billion mark by 2050. This is likely to create unprecedented pressures on the limited natural resource base of the planet, earth, making it pretty difficult to produce additional requirement of food, fibre and raw materials for the huge population. Organic farming has emerged as an important priority area globally in view of the growing demand for safe and healthy food and long term sustainability and concerns on environmental pollution associated with indiscriminate use of agrochemicals (Suman *et al.,* 2015). The chemicals used in agriculture are supposed to be taking a very heavy toll of this source of energy. The agricultural importance of nitrogen fixation is not only to provide ammonium to the crops but also to minimize pollution.. Chemical fertilizers and their exploitation cause air and ground water pollution by eutrophication of water bodies (Youssef *et al.,* 2014).

Though biofertilizers are ecofriendly and cost effective, its production, use and quality are to be strengthened for better exploitation under sustainable agriculture systems

VIII. Conclusion

Scientists are looking for more viable and sustainable alternatives. They have identified microorganisms that convert atmospheric nitrogen and fix it into soils as a source. It has been observed that the total world biological nitrogen fixation by the microorganisms is about three times that of the industrially produced nitrogen. This proves that if the natural biological nitrogen cycles are harnessed through enhanced microbial population and activity, the need for nitrogen can be met more sustainably and economically (Sahoo and Bhardwaj Tuteja, 2013). Organic farming has emerged as an important priority area globally in view of the growing demand for safe and healthy food and long term sustainability and concerns on environmental pollution associated with indiscriminate use of agrochemicals (Suman *et al.,* 2015).In this study, the main aim is effective and in-effective root nodules with deal strain of Brady hizobiumSpp,and also observe the morphological characters and histo-chemical characters.

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