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# Exploration of the Indigenous Rhizobial Strains from the Soils of Tripura for Increased Nodulation Efficiency in Pigeon Pea (*Cajanus cajan*)

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**ABSTRACT**

The huge fertilizer usage in agricultural fields has an irreparable impact on the environment. One of the ways to reduce fertilizer dependency is the use of the biofertilizers. The leguminous crop plants have root nodules which are involved in atmospheric nitrogen fixation. The type of bacterial strain residing in nodules which are involved in nitrogen fixation depends on both plant species as well as edaphic factors. The soils of Tripura with acidic pH have indigenous strains associated with different leguminous crops as well as other leguminous plant species in the wild need to be explored. One of the important leguminous crops in Tripura is pigeonpea, the pods of which are consumed as a vegetable. The present study involved exploration of the Rhizobial strains associated with pigeonpea cultivated in Tripura as well as Sesbania rostrata (a legume plant used in green manuring). 16 strains were isolated on YEMA medium and were characterized. The nodule forming efficiency of these strains was studied by plastic cup experiments in the laboratory. The yield parameters were also observed in the plants inoculated with these strains. The promising Pigeonpea variety from the trials of AICRP-Pp centre at Tripura was used in the present study. The few strains showed better nodulation and yield parameters. The future work includes studying these selected strains by pot culture experiments and later into the field conditions. The novelty of these strains would be ascertained by their molecular characterization.

**Keywords :** Rhizobial strains, Nodulation efficiency, Pigeonpea.

## Introduction :

The chemical fertilizers provide specific formulations of NPK to treat nutrient-deficient soils and plants. A long-term and large-scale use of chemical fertilizers gives rise to environmental issues such as change in the soil pH, upset beneficial microbial ecosystems, toxic accumulation of harmful chemicals, run off to water sources leading to eutrophication and

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ground water pollution. Biofertilizers propose a sustainable solution to the reduction in the use of chemical fertilizers while meeting the demands of the growing population. The use of biofertilizers in place of chemicals is likely to reduce the impact on soil, air and water and also has the potential to improve human health. They are not only high in nutritional value but also add effective microbes to the soil.

All organisms use the ammonia ($NH_3$) form of nitrogen to manufacture amino acids, proteins, nucleic acids, and other nitrogen-containing components necessary for life. Biological nitrogen fixation is the process that changes inert $N_2$ into biologically useful $NH_3$. This process is mediated in nature only by N-fixing rhizobia bacteria (Rhizobiaceae, á-Proteobacteria) (Sørensen and Sessitsch, 2007). Other plants benefit from N-fixing bacteria when the bacteria die and release nitrogen to the environment, or when the bacteria live in close association with the plant. In legumes and a few other plants, the bacteria live in small growths on the roots called nodules. Within these nodules, nitrogen fixation is done by the bacteria, and the $NH_3$ they produce is absorbed by the plant.

The *Rhizobium* application increased crop growth by improving plant height, seed germination, leaf chlorophyll, and N content [Sara *et al.*, 2013]. Datta *et al.* (2015), isolated *Rhizobium* strains and concluded that growth and yield parameters were significantly enhanced by *Rhizobium* application in comparison to the control. *Rhizobium* is commonly used in agronomic practices to ensure adequate nitrogen (approximately 80% of biologically fixed N comes from symbiosis) and have the potential to replace chemical N fertilizers [Rubio-Canalejas *et al.*, 2016]. *Rhizobium* maintains the soil fertility along with higher crop yields [Arora *et al.*, 2017]. *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium,* and *Mesorhizobium* are collectively called rhizobia. They can act directly by fixing nitrogen or influencing plant hormones or indirectly by decreasing the inhibitory effects of pathogens [Mabrouk *et al.*, 2018]. Seed inoculation of rice with different strains of *Rhizobium* at graded levels of N increased straw yield by 4% to 19% and rice grain yield by 8% to 22% [Sammauria *et al.*, 2020].

To meet the increasing food demand of the ever-increasing world's population, crops with minimum inputs and maximum output are the best suited. The leguminous crops are one of those which has a lesser nitrogen input requirement due to symbiotic association with $N_2$-fixers and they have high nutritional status. In all regions of the world where food consumption exceeds production or where nitrogenous fertilizer must be imported, leguminous crops have a special relevance. Self-sufficiency for nitrogen supply and the high protein and calorific values of food, forage and feed legumes make them increasingly attractive. Greater use of legumes can have a significant beneficial impact in tropical countries where population increase and food production are most out of balance, and where the purchasing power for imported fertilizers is less adequate.

Pigeon pea is an important *kharif* pulse crop cultivated in Tripura with an area, production and productivity of 4832 ha, 3873.03 MT and 802 kg/ha respectively (https:// agri. tripura. gov.in /basic statistics). The acidic soils of Tripura harbour important indigenous Rhizobial strains which need to be explored and utilised as a biofertilizer for cultivation of pulses including pigeonpea. The present study was aimed to isolate such strains from root nodules of crops viz. pigeonpea and wild plants viz. *Sesbania rostrata* and bring them under nutrient management regime of pigeonpea cultivation in the state.

**Materials and methods :**

***Isolation of Rhizobial strains :*** The isolation of *Rhizobium* was done from the rhizosphere soil of Pigeonpea field, root nodules of field grown Pigeonpea and root nodules of *Sesbania rostrata*. The main species nodulating Pigeon pea in India is *Bradyrhizobium yuanmingense* (Jorrin *et al.*, 2021). *Sesbania rostrata* is one of the few legumes that forms nodules on both stems and roots (Dreyfus *et al.*, 1981). *Azorhizobium caulinodans* forms stem and root nodules in *Sesbania rostrata* (Ndoye *et al.*, 1994).

***Isolation of rhizobia from rhizosphere soil :*** The rhizosphere soil of a healthy pigeonpea plant (45 days old) was collected aseptically from the field and brought to the laboratory. 10.0g rhizosphere soil was weighed and suspended in 90ml sterile water blank in a conical flask. 1ml aliquot was taken from the soil suspension and serially diluted in 9 ml water blanks in test tubes. 1ml aliquot from every dilution was spread on Yeast Extract Mannitol Agar (YEMA) plates with 1% Congo Red. The plates were incubated at 28°C under dark for 3-4 days. The plates were observed for the appearance of colonies typical of rhizobia which show little or no Congo Red absorption (Vincent, 1970; Somesegaran and Hoben, 1994).

***Isolation from root nodules :*** A healthy pigeonpea plant (45 days old) with root nodules was collected from the field and brought to the laboratory. The plant root was washed under running tap water and blot dry under laminar air flow. The root nodules were excised and immersed in 0.1% $HgCl_2$ for 5mins. The nodules were washed repeatedly with sterile water for 3-4 times and then placed in 70% ethanol for 2-3 minutes. The nodules were then washed with sterile water 3-4 times and dried using sterile blotting paper. The nodules were then crushed aseptically in 1ml sterile water in a test tube using a sterile glass rod. The crushed root nodule extract was serially diluted in 9ml water blanks in test tubes. 1ml aliquot from every dilution was spread on Yeast Extract Mannitol Agar (YEMA) plates with 1% Congo Red. The plates were incubated at 28°C under dark for 3-4 days. The plates were observed for the appearance of colonies typical of rhizobia which show little or no Congo Red absorption (Vincent, 1970; Somesegaran and Hoben, 1994). The isolation from the root nodules of *Sesbania rostrata* was done by following the above-mentioned procedure.

***Morphological study of Rhizobial isolates :*** The isolated rhizobial strains were analysed for their morphology by Gram staining technique.

**Plant infection experiments :**

***Soil Preparation :*** Soil from pigeonpea fields under AICRP-Pp at No. 9 Tilla of College of Agriculture was collected and brought to the laboratory. It was dried, sieved, and sterilized in hot air oven at 120°C for 3 hours. The recommended fertilizer dose of urea, MOP and SSP was added to the sterilized soil. 200g of the above soil were filled with transparent plastic cups of 250ml volume and plugged with non-absorbent cotton and sealed with aluminium foil. The above plastic cups were autoclaved for 30 mins at 15psi.

***Seed material and inoculation :*** Seeds of UPAS-120 variety of Pigeon pea were soaked with sterile distilled water overnight before the experimental setup. Overnight culture (16-18 hours old) of the sixteen rhizobial isolates was prepared in culture tubes. OD values of the rhizobial cultures were measured at 610 nm to determine the optimum concentration for inoculation. The soaked seeds were surface sterilized with 0.1% $HgCl_2$ and washed 3-4 times with distilled water (Vincent, 1970). 25-30 seeds were aseptically added to every 16 different rhizobial broth culture tubes and kept for one hour incubation at 28°C. There were 8 replicates for every 16 different rhizobial isolates and 3 seeds were sown in every replicate. 25 ml sterilized distilled water added with fungicide @2.0g/L was put in every cup after sowing. The set up was placed in the south-eastern corner of the laboratory with direct sunrays falling. The germination and seedling emergence was observed on the next day. Sterilized distilled water was used for irrigation as and when required. Data harvesting was done after 20 days and 45 days. The data were analysed for ANOVA followed by Duncan's Multiple Range Test.

**Results and discussion :**

A total of 16 representative rhizobial strains with 8 isolates from Rhizosphere soil of Pigeonpea field, 5 isolates from root nodules of field grown Pigeonpea and 3 isolates from root nodules of *Sesbania rostrata* were isolated (Table 1). It was found that all the 16 rhizobial isolates were either rod-shaped or coccobacillus and Gram-negative. All the inoculated Rhizobial isolates were able to form root nodules at 20 DAS (Table 2). No nodulation was observed in the control plants as the soil used in the study was thoroughly sterilized before the experimental set-up. The statistical analysis revealed that rhizobial isolates from the root nodules of Pigeon pea, namely $T_9$, $T_{10}$ and $T_{12}$, were more efficient in root nodule formation at 20 DAS in the Pigeon pea variety under study. These strains were found to be slow growing on culture medium YEMA. However, it was also found that one of the rhizobial strain, $T_8$, which was isolated from the rhizosphere soil and found to be a fast grower was equally efficient in nodulating the plants. The differences for other growth parameters were not statistically significant from the control plants. All the treatment inoculated with Rhizobial isolates showed root nodulation at 45 DAS (Table 3). No nodulation was observed in the control plants as soil was sterilized to remove any inherent rhizobial strain in it. The statistical analysis revealed that rhizobial isolates from the root nodules of Pigeon pea were more efficient in root nodule formation at 45 DAS viz. $T_{10}$ and $T_{12}$ (Figure 1). It was found that one of the rhizobial strain, $T_4$, isolated from the rhizosphere soil was equally efficient in nodulating the plants under study. It was found to be a fast grower on culture medium. The differences for other growth parameters were not statistically significant from the control plants.

There have been many studies performed to determine the composition and characteristics of indigenous strains isolated from cultivated legumes (Carelli *et al.*, 2000). The main aim of the present study was to isolate and characterize the indigenous rhizobial strains from the soils of Tripura which may be utilized for obtaining efficient root nodulation in Pigeon pea. The Pigeon pea cultivated in Northern India showed poor nodulation as reported by Raghuwanshi *et al.* (1994), with 80 % of Pigeon pea plants were either not nodulated or very poorly nodulated. Gaur and Sen, 1979 studied that *Sesbania* has strong affinity for the "cowpea group" (one of the cross inoculation groups under classification of *Rhizobium*) to which Pigeon pea belongs. Subba Rao, 1980 reported that *Rhizobium* isolated from the nodules of *Sesbania* and *Arachis* were able to nodulate pigeon pea. Nautiyal *et al.* (1988), studied the nodulation efficiency in members of "cowpea group". Anand and Dogra, 1991 reported that the pigeon pea plants can be nodulated both by slow and fast growing rhizobial strains. However, the fast-growing rhizobia are more important in $N_2$ fixation in sub-tropical and tropical soils (Boakye *et al.*, 2016). The present study involved both types of rhizobial strains and it was found that the slow growing rhizobial strains isolated from root nodules of pigeon pea were more efficient in terms of nodulation. The fast grower strains either from rhizosphere soil or root nodules of Sesbania were able to form nodules but not as efficiently as slow growers. The rhizobial strain isolation studies in Indian soils suggested that pigeon pea can be nodulated by *Rhizobium spp.* (Rajendra *et al.*, 2008; Singh *et al.*, 2018; Singha *et al.*, 2018), *Bradyrhizobium spp.* (Nautiyal *et al.*, 1988), *Sinorhizobium/ Ensifer* (Dubey *et al.*, 2010; Gosai *et al.*, 2020; Jain *et al.*, 2020), *Mesorhizobium* or even *Burkholderia* (Singha *et al.*, 2018). However, inoculation of Pigeon pea with efficient rhizobial strains does not necessarily result in nodulation or yield increases (George *et al.,* 2007), there may be some plant growth promoting traits in the rhizobial strain instead of efficient nodulation, enhanced nitrogen uptake and increased yield.

**Conclusion :**

The present study helped in identifying the nodulation efficiency of few indigenous Rhizobial isolates obtained from rhizosphere soil of pigeonpea, root nodules of Pigeonpea and Sesbania spp. The nodulation efficiency of a Rhizobial isolate depends on many factors viz. crop variety, soil pH, temperature, etc. hence a better knowledge of ecological aspects would be necessary. The indigenous Rhizobial isolates would be better in nodulation efficiency and plant growth promotion as they are more suited to the given soil factors. The plastic cup experiments helped us in identifying the nodulation efficiency of few indigenous Rhizobial isolates, but an elaborate pot culture experiments and subsequent field-based studies would help in getting a better picture of the nodulation efficiency as well as plant growth promotion.

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**Table1 : The Rhizobial isolates in the present study with their important features**

| Sl. No. | Rhizobial isolate | Source of isolation | Gram staining | Morphology | Growth habit on YEMA |
|---|---|---|---|---|---|
| 1 | $T_1$ | Rhizosphere soil of Pigeon pea | Gram negative | Coccobacillus | Fast grower |
| 2 | $T_2$ | ” | ” | ” | ” |
| 3 | $T_3$ | ” | ” | Rod shaped | ” |
| 4 | $T_4$ | ” | ” | Coccobacillus | ” |
| 5 | $T_5$ | ” | ” | ” | ” |
| 6 | $T_6$ | ” | ” | ” | ” |
| 7 | $T_7$ | ” | ” | ” | ” |
| 8 | $T_8$ | ” | ” | ” | ” |
| 9 | $T_9$ | Root nodules of Pigeon Pea | ” | Rod shaped | Slow grower |
| 10 | $T_{10}$ | ” | ” | Rod shaped | ” |
| 11 | $T_{11}$ | ” | ” | Rod shaped | ” |
| 12 | $T_{12}$ | ” | ” | Coccobacillus | ” |
| 13 | $T_{13}$ | ” | ” | Rod shaped | ” |
| 14 | $T_{14}$ | Root nodules of Sesbania rostrata | ” | Rod shaped | Fast grower |
| 15 | $T_{15}$ | ” | ” | Rod shaped | ” |
| 16 | $T_{16}$ | ” | ” | Rod shaped | ” |

**Table2 : The observations at 20 DAS**

| Treatment | No. of leaves | No. of nodules | Root length(cm) | Shoot length(cm) | Root dry weight(g) | Shoot dry weight(g) |
|---|---|---|---|---|---|---|
| $T_1$ | $11.2^{abc}$ | $8.4^{bc}$ | $14.6^{bcde}$ | $33.06^{abc}$ | $0.086^{cdefg}$ | $0.48^{abcd}$ |
| $T_2$ | $9.8^{bc}$ | $1.4^{c}$ | $14.42^{bcde}$ | $32.22^{abc}$ | $0.099^{abcdefg}$ | $0.483^{abcd}$ |
| $T_3$ | $10.8^{abc}$ | $2.8^{c}$ | $15.56^{bcde}$ | $30.82^{abc}$ | $0.13^{abcd}$ | $0.484^{abcd}$ |
| $T_4$ | $12^{ab}$ | $0.4^{c}$ | $13.98^{cde}$ | $34.94^{a}$ | $0.12^{abcde}$ | $0.535^{ab}$ |
| $T_5$ | 7 | $3^{c}$ | $11.68^{e}$ | $22.34^{d}$ | $0.057^{g}$ | $0.213^{f}$ |
| $T_6$ | $10.4^{bc}$ | $1.4^{c}$ | $13.28^{cde}$ | $31.38^{abc}$ | $0.105^{abcdefg}$ | $0.536^{ab}$ |
| $T_7$ | $9.6^{c}$ | $4.6^{c}$ | $18.34^{ab}$ | $25.78^{cd}$ | $0.082^{defg}$ | $0.324^{cdef}$ |
| $T_8$ | $11^{abc}$ | $14.2^{ab}$ | $14.16^{bcde}$ | $30.68^{abc}$ | $0.116^{abcdef}$ | $0.407^{abcdef}$ |
| $T_9$ | $9.8^{bc}$ | $17^{a}$ | $15.24^{bcde}$ | $30.14^{abc}$ | $0.092^{bcdefg}$ | $0.38^{bcdef}$ |
| $T_{10}$ | $11.2^{abc}$ | $17.8^{a}$ | $15.1^{bcde}$ | $32.02^{abc}$ | $0.101^{abcdefg}$ | $0.458^{abcd}$ |
| $T_{11}$ | $12.8^{a}$ | $5.6^{c}$ | $20.22^{a}$ | $34.24^{ab}$ | $0.142^{a}$ | $0.501^{abc}$ |
| $T_{12}$ | $12^{ab}$ | $16.6^{ab}$ | $14.56^{bcde}$ | $32.16^{abc}$ | $0.139^{ab}$ | $0.462^{abcd}$ |
| $T_{13}$ | $10.8^{abc}$ | $2.6^{c}$ | $16.16^{abcd}$ | $28.82^{abcd}$ | $0.078^{efg}$ | $0.291^{def}$ |
| $T_{14}$ | $9.8^{bc}$ | $0^{c}$ | $12.56^{de}$ | $25.98^{cd}$ | $0.069^{fg}$ | $0.244^{ef}$ |
| $T_{15}$ | $11.4^{abc}$ | $2.8^{c}$ | $14.66^{bcde}$ | $29.5^{abcd}$ | $0.132^{abc}$ | $0.413^{abcde}$ |
| $T_{16}$ | $9.4^{c}$ | $0^{c}$ | $14.26^{bcde}$ | $27.12^{bcd}$ | $0.085^{cdefg}$ | $0.385^{bcdef}$ |
| Control | $11.4^{abc}$ | $0^{c}$ | $16.76^{abc}$ | $34.06^{ab}$ | $0.125^{abcde}$ | $0.598^{a}$ |
| C.D. (0.05) | 2.3531 | 8.5250 | 4.1902 | 7.2862 | 0.0498 | 0.1966 |

**Table 3 : The observations at 45 DAS**

| Treatment | No. of leaves | No. of nodules | Root length (cm) | Shoot length (cm) | Root dry weight(g) | Shoot dry weight (g) |
|---|---|---|---|---|---|---|
| $T_1$ | $11.2^{abcde}$ | $0.8^{e}$ | $10.46^{d}$ | $28.42^{cdef}$ | $0.075^{ef}$ | $0.276^{efg}$ |
| $T_2$ | $13.2^{a}$ | $3^{de}$ | $14^{abcd}$ | $39.06^{a}$ | $0.173^{a}$ | 0.762 |
| $T_3$ | $12.2^{abc}$ | $3.6^{de}$ | $11.68^{abcd}$ | $36.98^{ab}$ | $0.134^{abc}$ | $0.539^{a}$ |
| $T_4$ | $11.4^{abcd}$ | $15.6^{ab}$ | $12.4^{bcd}$ | $31.96^{abcde}$ | $0.111^{def}$ | $0.503^{ab}$ |
| $T_5$ | $11.2^{abcde}$ | $10.6^{bc}$ | $10.46^{cd}$ | $34.5^{abc}$ | $0.113^{def}$ | $0.459^{abcd}$ |
| $T_6$ | $12.6^{ab}$ | $3^{de}$ | $15.66^{abc}$ | $32.46^{abcde}$ | $0.112^{def}$ | $0.444^{abcde}$ |
| $T_7$ | $10.8^{bcdef}$ | $7.6^{cd}$ | $16.66^{ab}$ | $31.12^{bcde}$ | $0.13^{abcd}$ | $0.329^{bcdefg}$ |
| $T_8$ | $9.8^{def}$ | $10.6^{bc}$ | $12.3^{bcd}$ | $25.7^{efg}$ | $0.098^{def}$ | $0.27^{efg}$ |
| $T_9$ | $10.4^{cdef}$ | $10.4^{bc}$ | $15.34^{abcd}$ | $30.04^{bcdef}$ | $0.1^{def}$ | $0.325^{cdefg}$ |
| $T_{10}$ | $10^{def}$ | $12.4^{abc}$ | $12.68^{abcd}$ | $19.5^{g}$ | $0.091^{def}$ | $0.299^{cdefg}$ |
| $T_{11}$ | $10.6^{bcdef}$ | $1.6^{de}$ | $16.52^{ab}$ | $28.1^{cdef}$ | $0.108^{def}$ | $0.321^{cdefg}$ |
| $T_{12}$ | $9.8^{adef}$ | $18.2^{a}$ | $12.82^{abcd}$ | $27.04^{def}$ | $0.067^{f}$ | $0.243^{fg}$ |
| $T_{13}$ | $10.4^{cdef}$ | $1.8^{de}$ | $15.96^{ab}$ | $33.22^{abcd}$ | $0.117^{bcde}$ | $0.29^{defg}$ |
| $T_{14}$ | $9^{f}$ | $3.4^{de}$ | $14.34^{abcd}$ | $20.62^{cdef}$ | $0.083^{def}$ | $0.2^{g}$ |
| $T_{15}$ | $10^{def}$ | $0.4^{e}$ | $15.06^{abcd}$ | $20.38^{cdef}$ | $0.113d^{ef}$ | $0.391^{abcdef}$ |
| $T_{16}$ | $9.2^{ef}$ | $0^{e}$ | $14^{abcd}$ | $23.56^{fg}$ | $0.069^{ef}$ | $0.252^{fg}$ |
| Control | $12.6^{abf}$ | $0^{e}$ | $17.5^{a}$ | $37.16^{ab}$ | $0.162^{ab}$ | $0.474^{abc}$ |
| C.D. (0.05) | 2.1074 | 6.2137 | 5.0433 | 7.7447 | 0.0487 | 0.1777 |