Characterization of nodulation and nitrogen fixation potential of selected wild legumes of semiarid region, Tirupati, India

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Abstract
Bioavailability of nitrogen is one of the major nutrients limiting crop plant growth and yield in agroecosystems worldwide. Symbiotic / Biological Nitrogen Fixation (BNF) by legumes and associative, endosymbiotic and endophytic nitrogen fixation in non-legumes play major role in reducing the use of synthetic nitrogen fertilizer in agriculture, increased plant nutrient content and soil health reclamation and potential economic gains to farmers in terms of input cost. Fifteen native wild legumes and cultivated Arachis hypogaea were selected from the semi-arid region, Tirupati to study nodule morphology and nitrogen fixation potential. The similarity matrix was used for cluster analysis to construct a phenogram using the unweighted pair group method with averages (UPGMA). The wild species included Indigofera mysorensis, a new addition to the global list and a report of nodulation in Albizia lebbeck, Mimosa pudica and Tephrosia tinctoria from India.

Morphological features of the nodule are characteristic of the species. There is a positive correlation between intensity of nodule interior colour and nitrogenase activity. All the species are highly effective nitrogen fixers and three-fold variation was observed among the species. Nodule morphological features and nitrogenase activity grouped 16 legumes into 5 distinct clusters at 65% level indicating the diversity.

Keywords: Semiarid region, Wild legumes, Nodule morphology, Nitrogen fixation, Cluster analysis.

Introduction

Environmental friendly, sustainable and low-cost agricultural practices to enhance food production are highly essential to meet the needs of the ever-increasing global population. In this context, legumes which provide dietary proteins and fats for human consumption, feed and fodders to the animals and above all as a partner in Biological Nitrogen Fixation (BNF) are vital for global agriculture sustainability. The family Leguminosae, with an estimated number of species of 20000 in 727 genera, is one of the most diverse and widely distributed flowering plant families[28]. The members of Leguminosae form symbioses with rhizobia which are very diverse ranging from field grain annual legumes, perennial trees, lines, shrubs and herbs with a natural distribution on all continents except Antarctica[21].

Biological nitrogen fixation involving Legumes-Rhizobia symbiosis is of major ecological importance that occurs on all continents and accounts for a fourth of the nitrogen fixed annually on Earth[6]. BNF is the major way for nitrogen input into ecosystems and is an efficient source of fixed nitrogen which plays an important role in land remediation[39]. The fixation of symbiotic nitrogen plays an important role in increasing plant biomass[7]. By enhancing and extending the use of symbiotic nitrogen fixation, we can improve the quality and quantity of agricultural products, reduce the cost of chemical fertilizer inputs and maintain soil health and fertility.

Rapid global climatic change, adverse soil conditions and production-based intensive agriculture emphasize the need for the exploration of non-agricultural areas, indigenous soils and nodulating wild plants to identify the most effective nitrogen-fixing strains to perform under limiting conditions. Nodulated wild legumes with their biodiversity and adaptation to arid/semi-arid climatic conditions are vital to the soil fertility and nitrogen cycle and are an important source for elite rhizobial strains. The nodulated wild (herb and tree) legumes have the potential for nitrogen fixation, reforestation and to control soil erosion[2]. With climate change and an increasing world population, there is an urgent need to develop a diverse range of nodulated legumes native to dry environments[46].

A characteristic of the legume-rhizobia interaction is the formation of a special organ, the nodule, where the reduction of N_2 to ammonia (NH_4^+) by the enzyme nitrogenase takes place[54]. Sprent[49] reported that nodulation is a robust taxonomic character, both at the presence/absence level and the structural and physiological level. Because of this ability, legumes can grow in unfertile, arid soils with low N content, a property that makes them pioneer plants suitable for revegetation programs and enhancement of soil fertility. The effectivity of the nitrogen-fixing wild herb legumes and their significance to soil fertility in arid regions were reported[22,61]. Many of the tree legumes have proven to have effective nodules[23,31,56] and fix nitrogen more than grain legumes[12].

Based on nitrogen fixation and chemical composition of wild annual legumes in Egypt, Sherif et al[41], concluded that wild herb legumes were characterized by a higher rate of nodulation and higher nitrogen-fixing ability than their cultivated relatives. Zahran[61] reported nodulation (nodule number, type and structure) and symbiotic nitrogen fixation of five herb legumes *(Medicago intertexta, Melilotus indicus, Trifolium resupinatum, Trigonella hamosa* and *Alhagi murarum) and* reported that these wild legumes showed a great variation in nodulation and nitrogen fixation and suggested that wild legumes could be significant nitrogen-fixers, especially when grown under drought conditions.

Variation in nodulation, symbiotic polymorphism of forage legume populations (*Hedysarum* ssp., *Medicago* ssp., *Trifolium* ssp., *Scorpirus* ssp.) and diversity of rhizobia from arid regions of North Africa and the Mediterranean Area were reported[34]. Sprent et al[44] evaluated the biogeography of nodulated legumes and their associated nitrogen-fixing microsymbionts in terms of both longitudinal and latitudinal trends.

Characterization of nodulation pattern and nitrogen fixation potential of native wild legumes is essential for their utilization in the management of soil fertility and to extend agriculture to new areas. Zahran[62,63] reviewed rhizobium legume symbiosis and its superiority and safety over chemical nitrogen fertilizers and emphasized its significance for agriculture in arid regions and their rehabilitation.

Several workers studied the nodulation pattern and few analyzed the nitrogen fixation potential of wild legumes from arid/semi-arid regions of the world including India. The significance of nodulated wild legumes from arid and semi-arid regions for dry environments was reviewed by Sprent and Gehlot[46]. Nodulated legumes from the Thar Desert of India were investigated by Gehlot et al[19]. Nodule characteristics of five pastoral legumes (*Loteae*, *Psoraleae* and *Trifolieae* tribes), including the first report of two taxa (*Lotus pusillus* and *L. arabicus*) from Tunisia were investigated by Rejili et al[39]. Nodulation pattern from two native wild legumes of the Aravalli ranges of Rajasthan, India was reported by Rathore et al[38]. Panwar et al[35] reviewed the native nodulating legumes and associated rhizobia of western Rajasthan. Nodulation and nitrogen fixation in 67 *Mimosa* spp. from two biomes in Brazil were investigated[16].

Legumes are not commonly seen in high quantities in Asian tropical woods[51]. They do exist as pioneer plants on deteriorated ground, particularly wasteland[59]. Native legumes, particularly those with abundant nodulating bushes, can help to restore barren terrain such as mine sites and man-made slopes. Legume species with high nodule formation and nitrogen fixation ability should be prioritized in reforestation[4]. This study's nodulation data aids in the selection of species for this purpose. Many natural leguminous trees may be used as priority pioneer species for the rehabilitation of degraded and overexploited rainforests due to their nitrogen-fixing association with rhizobia[15].

Nodulation of native woody legumes in Hong Kong, China was reported[4] in which nodulation patterns, nodule morphology and nitrogen-fixing ability were studied along with seasonal patterns of nodulation and morphology of nodules. Twenty-eight native woody legume species were examined for their nodulation status in which five new nodulating species were added to the world's list of nodulation inventory.

The need for a nodulation survey is not exclusively due to our lack of knowledge of legumes. Only around 20% of all known Leguminosae species have been studied for nodulation[48]. The fact that nodulation is diverse and influenced by a variety of edaphic and climatic conditions emphasizes the need for local nodulation investigations. Water availability[53], soil pH[8] and soil nutrient availability, particularly phosphorus and other minerals[50], are environmental factors known to affect nodulation and nitrogen fixation in legumes. Therefore, nodulation examinations are important for the exploitation and sustainable management of nitrogen-fixing legumes.

A survey of nodulation was carried out in seven regions of Brazil and the nodulation status of 131 legume species was reported[14]. Nodulation is reported in 46 species and six genera, representing 35% of the examined species. A large and systematic survey of the occurrence of nodulation was carried out in six different natural forest areas in the south of Guinea[15] to determine nodulation in 156 leguminous species. It was concluded that of the 97 plant species and 14 genera that had never been examined before this study, 31 species and four genera were reported to be nodulated.

Tirupati, situated near the foothills of Tirumala, a part of Rayalaseema is a tropical region with a semi-arid climate, experiencing high temperatures (Max. 42ºC and Min. 20ºC). During most of the year, soils are dry (annual average rainfall, 950 mm**)** and red loamy soil with poor organic content. Even under these harsh conditions, the biodiversity of legumes is high and rhizobia associated with these legumes might exhibit tolerance to adverse edaphic and climatic conditions. The present investigation was aimed at studying nodulation patterns, nodule characterization and estimation of the nitrogen fixation potential of selected wild legumes.

Material and Methods

Study site: The study site, Sri Venkateswara University campus is in Tirupati (13º62' N and 79º40' E) at the foothills of the Seshachalam (Tirumala) hill range covering nearly 1000 acres (Fig. 1). The climate is semi-arid temperate, experiencing three seasons including extended hot summer (April to June) and winter (November to February). The rainy season (July to October) separates summer and winter. The mean average annual rainfall is 950 mm and the mean annual maximum temperature is 40 ± 2ºC with a minimum of 20 ± 2 ºC. The soil is red loamy with a low water holding capacity and poor in organic content. The site of the *Arachis hypogaea* collection has a similar soil profile but under cultivation for many years.

Soil analysis: Soil samples at 5-10 cm depth were collected at six different places with a high number of legumes and were analyzed for nutrient, physical and chemical properties by ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy) analysis in Agricultural Development Laboratory, Zuari Agro Chemicals Ltd., Tirupati. The soil sampling sites had no record of exogenous fertilizer input. The physical, chemical and nutrient status of soil are presented in table 1.

Plant collection and analysis of nodule morphology: Wild legume plants including herbs, shrubs, climbers and trees were uprooted for the observation and collection of nodules. About ten plants for each species were analyzed. Root nodules were differentiated from other nodule-like structures using a standard procedure[57]. The size, shape and type of nodules were assessed by the standard methods[3,9,45-47]. The specimens of nodulated plants were deposited in the herbarium of the Botany Department, Sri Venkateswara University, Tirupati.

Estimation of nitrogen fixation potential: At the plant collection site, some nodules were cut open to examine the interior color. Pink coloration indicates the presence of the nitrogen-fixing enzyme nitrogenase activity/leghemoglobin, whereas green or white indicates ineffective nitrogen fixation[50]. The amount of nitrogen fixation was analyzed through an Acetylene Reduction Assay (ARA). For nitrogenase assay estimation, soil was removed gently with careful shaking from the roots containing nodules. Nitrogenase activity was estimated by following the standard method[26] with minor modifications. The excised nodulated roots without soil particles were sealed in a serum bottle with a rubber cap and were incubated for 30 min with C_2H_2 (Acetylene) gas which was obtained from CaC_2 (Calcium carbide) crystals.

From this, 5 ml of gas was taken and injected into another sealed serum bottle with 1.5 ml oxidant solution (Composition: 80 mL of 0.05M $NaIO_4$, 10 mL of 0.005M $KMnO_4$, pH adjusted to 7.5 with KOH and diluted to 100 mL). These bottles were agitated on a rotatory shaker at 300 rpm for 90 min at 28 ºC. The reaction was terminated by injecting 0.5 ml of 2N NaOH[5]. This mixture was incubated with Nash reagent (Composition: 150 g of ammonium acetate, 3 mL of acetic acid, 2 mL of acetylacetone diluted to 1 liter) for 60 min and then O.D. was measured at 412 nm. The standard graph was prepared using known amounts of ethylene (obtained from Chemtron Science Laboratories Pvt. Ltd., Navi Mumbai, India) for calibration.

Cluster analysis: The results of the nodule morphology and nitrogenase activity of legume plants were converted into a binary dataset (1 for positive and 0 for negative) which was used to estimate the simple matching similarity coefficient (Sms) of each legume to generate a similarity matrix[43] computed with Jaccard coefficient. The similarity matrix was used for cluster analysis to construct a phenogram using the unweighted pair group method with averages (UPGMA)[43].

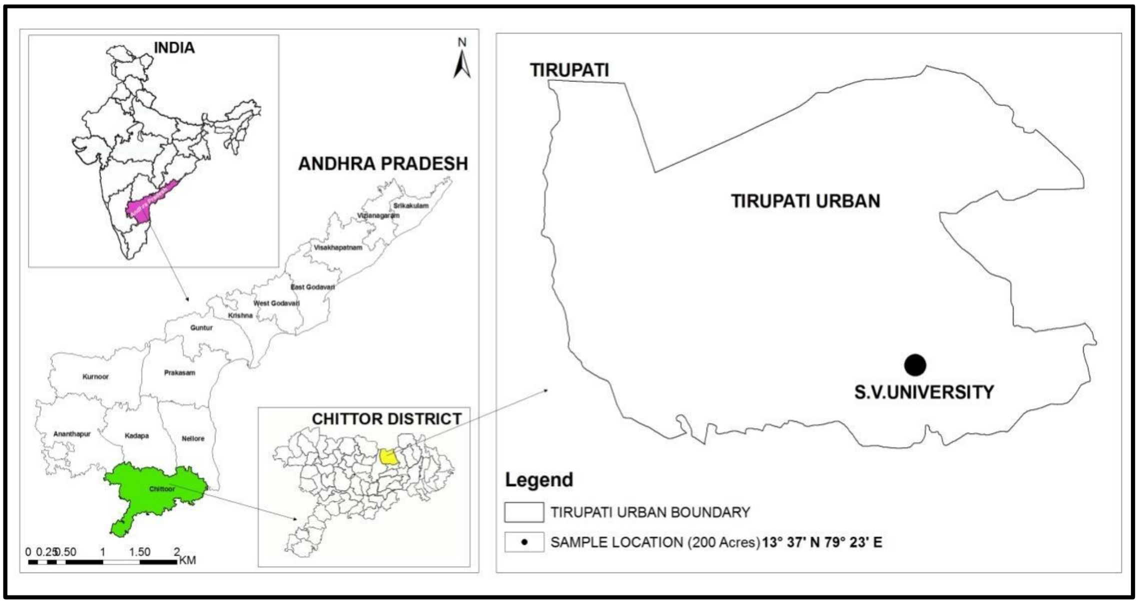

Fig. 1: Study site

Table 1
Study site soil characteristics

Soil type	pH	E.C.	Organic content	Total nitrogen	P_2O_5	K_2O	Zn	Fe	Cu	Mn
Red loamy	6.3 ± 0.08	0.03 ± 0.001	0.69 ± 0.02	0.07 ± 0.006	15 ± 0.81	60 ± 1.94	1.05 ± 0.03	21.7 ± 0.31	0.6 ± 0.03	6.88 ± 0.02

Note: Means are values of six replications

Results and Discussion

Plant samples: Fifteen nodulating native wild legumes and cultivated *Arachis hypogaea* were selected to study nodule morphology and nitrogen fixation potential. The 15 wild legumes belonging to 12 genera comprised of 11 species, belong to Papilionoideae, two each to Mimosoideae and Caesalpinioideae. The habits of the plant species include nine herbs, two each of shrubs, climbing shrubs and trees (Table 2).

Nodulation status: According to the GRIN database (http://www.ars-grin.gov/~sbmljw/cgi-bin/taxnodul.pl), here we report nodulation in *Indigofera mysorensis* which is a new addition to the global list and in *Albizia lebbeck, Mimosa pudica* and *Tephrosia tinctoria* from the Indian subcontinent. Regarding the nodulation status of *Cassia tora* according to the GRIN database contrasting reports were there but here we report nodulation (http://www.ars-grin.gov/~sbmljw/cgi-bin/taxnodul.pl).

Nodulation in *Cassia tora* may be due to the presence of compatible rhizobia or *Cassia tora* genotype or prevailing environmental conditions forcing the symbiosis through coevolution. Nodulation was also reported in the remaining species by others.

Nodule morphology: Nodule characters are highly conserved and are being used in higher-level taxonomy of legumes.

Table 2
Nodule morphology of wild legumes

S.N.	Plant name	Isolate	Family	Habit	Nodule characteristics			
					Distribution	Shape	Size*	Number /plant
1	*Albizia lebbeck*	Ale	Mimosoideae	Tree	Fine roots	Elongate	Small	3
2	*Alysicarpus monilifer*	Amo	Papilionoideae	Herb	Fine roots	Globose	Medium	8
3	*Arachis hypogaea*	Ahy	Papilionoideae	Herb	Main and fine roots and at their junctions	Aeschynomenoid	Medium	50
4	*Chamaecrista absus*	Cab	Caesalpinioideae	Shrub	Fine roots	Elongate	Medium	7
5	*Cassia tora*	Cto	Caesalpinioideae	Shrub	Fine roots	Palmate	Big	3
6	*Clitoria ternatea*	Cte	Papilionoideae	Climbing shrub	Main and fine roots	Globose	Big	10
7	*Crotalaria hebecarpa*	Che	Papilionoideae	Herb	Fine roots	Elongate	Small	9
8	*Desmodium trifolium*	Dtr	Papilionoideae	Herb	Main and fine roots and at their junctions	Aeschynomenoid	Small	40
9	*Indigofera linnaei*	Ili	Papilionoideae	Herb	Fine roots	Elongate	Small	8
10	*Indigofera mysorensis*	Imy	Papilionoideae	Herb	Fine roots	Elongate	Small	3
11	*Indigofera trifoliata*	Itr	Papilionoideae	Herb	Fine roots	Elongate	Small	9
12	*Millettia pinnata*	Mpi	Papilionoideae	Tree	Main and fine roots	Globose	Big	18
13	*Mimosa pudica*	Mpu	Mimosoideae	Herb	Main and fine roots	Bifurcate	Medium	20
14	*Rhynchosia minima*	Rmi	Papilionoideae	Climbing shrub	Fine roots	Globose	Small	10
15	*Tephrosia purpurea*	Tpu	Papilionoideae	Herb	Fine roots	Elongate	Small	9
16	*Tephrosia tinctoria*	Tti	Papilionoideae	Herb	Fine roots	Elongate	Small	8

* Big – 16 mm above; Medium – 7-15 mm; Small – Below 6 mm

The location of root nodules, their number, shape and size are characteristic features of the legume species and are not related to the species of bacterium[42]. The number, shape and size of nodules of the 15 species were recorded during the peak flowering time of the respective species. About 10 plants from each species were analyzed. The nodules were found on the roots located within the top 5 – 15 cm soil. Six morphological features of nodules were considered for analysis (Table 2).

The shape of the nodule showed variation from palmately lobed in *Cassia tora,* spherical in *Desmodium trifolium,* globose in *Alysicarpus monilifer, Clitoria ternatea, Millettia pinnata, Rhynchosia minima* and cylindrical in the remaining species. The nodule type is astragaloid in *Cassia tora* and crotalaroid in the remaining species.

In the majority of species, nodules were present only on the secondary roots and three species had nodules on both primary and secondary roots. However, in *Desmodium trifolium,* the nodules were present on primary and secondary roots and at their junctions. The position of nodules on the root system is not related to plant habit. Even among the tree species and woody climbers, variation was observed in the location of the nodules. In *Albizia lebbeck* nodules were located on secondary roots whereas, in *Millettia pinnata* nodules were present on both primary and secondary roots. Authenticity of nodule forming ability of Albizia lebbeck was reported earlier[29]. The occurrence of nodules in tree species on fine roots was reported by Qadri and Mahmood[37] and Kaur et al[24].

In shrubs and herbs, nodules were confined to secondary roots. However, in the case of *Desmodium trifolium* which is a herb, nodules were located on both types of root systems and at their junctions similar to that of *Arachis hypogaea*. Angie et al[4] reported the occurrence of nodules on primary as well as secondary roots in shrubs and woody climbers.

Generally, the external color of the nodule depends on the root and soil color. In all the species, the color of the nodule is brown like that of the external color of the root. The same was also reported by Mahmood and Iqbal[30]. However, Rejili et al[40] reported that the color of the nodule was not related to root but depends on soil quality, color and the presence of Leghemoglobin. In the native woody legumes of Hong Kong, nodule colour ranged from pale yellow to brown like the root color[4].

In the region of present plant collection, quality and colour (brown) of the soil are the same and incidentally, the colour of the root is also brown, hence the influence of root color/soil quality on nodule color could not be ascertained.

Generally, the nodules are spherical to globose in the case of determinate nodules and elongated to coralloid in indeterminate nodules[3,45,47,52]. In the present investigation, both determinate (globose and aeschynomenoid) and indeterminate (elongate, bifurcate and palmate) types of nodules were observed (Fig. 2). Elongate and bifurcate nodules were observed in *Albizia lebbeck* and *Mimosa pudica* of Mimosoideae respectively. The bifurcate nodules of *Mimosa pudica* (Fig. 2F) agree with the nodule type reported in tribe Mimoseae[45].

Mimosoid legume nodules are all indeterminate and often branched[44]. The two species *Cassia tora* and *Chamaecrista absus* belonging to the tribe Cassieae under Caesalpinioideae have palmate (Fig. 2E) and elongate nodules respectively. Nodules of varied morphologies were observed in the species belonging to Papilionoideae.

Elongate nodules observed in *Tephrosia* (Fig. 2G) species are in agreement with the literature[45]. The two species representing the Desmodieae i.e. *Alysicarpus monilifer* and *Desmodium trifolium* have globose (Fig. 2C) and aeschynomenoid (Fig. 2B) type nodules respectively. Globose nodules were observed in *Clitoria ternatea, Millettia pinnata* (Fig. 2D) and *Rhynchosia minima.* Elongate nodules observed in *Crotalaria hebecarpa* in the present investigation are different from the lupinoid / palmate type reported earlier in the tribe Crotalarieae[60]. The three *Indigofera* species have elongated nodules (Fig. 2H). This agrees with the literature, that the *Indigofera* nodule is unique which is indeterminate type with lenticels[44]. In *Arachis hypogaea*, characteristic dalbergioid nodules i.e. aeschynomenoid (Fig. 2A) type were observed.

In the present study, the majority of the herbs possessed elongated nodules of indeterminate type. Both the woody climbers and shrubs had globose nodules as reported by Angie et al[4]. No relation was found between the habit of the plant and shape of the nodule, as the shape is related to plant taxonomy[42]. Rejili et al[39], Nutman[32] and Dart[13] also proposed that nodule shape is characteristic of legume but independent of Rhizobium strain.

The size of the nodule varied from 6 – 16 mm. The nodules were big (>16 mm) in *Cassia tora, Clitoria ternatea and Millettia pinnata,* medium (7-15 mm) in *Chamaecrista absus* and *Mimosa pudica and* small (< 6 mm) in the remaining species. The nodules' size varied from millimetre to centimetre observed from wild legumes.

Number of nodules per plant varied from species to species. In majority of species, number of nodules varied from 6 – 10 per plant (Table 2). A maximum of 40 were observed in *Desmodium trifolium* followed by 20 in *Mimosa pudica* and a minimum of 2 – 5 were observed in *Cassia tora, Albizia lebbeck and Indigofera mysorensis.* The number of nodules per plant is controlled by the host plant through an autoregulatory mechanism[18] and depends on environmental factors[40]. From the nodulation study of native legumes in Tunisia, the highest of 31 nodules and lowest of 11 nodules per plant were reported[39].

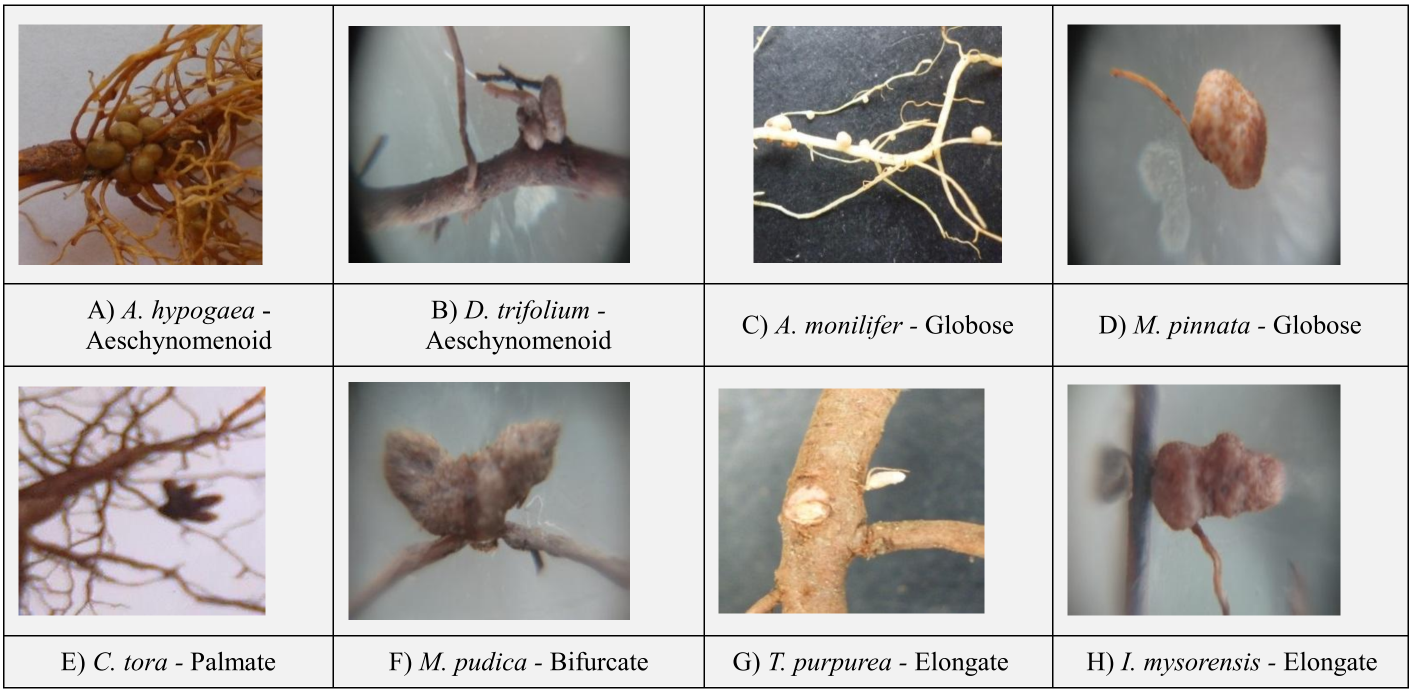

Fig. 2: Nodule morphology

In the present study, nodulated plants were collected from the same region and hence the reported variation in nodule number per plant is solely dependent on the host plant. No relation was found between the size and number of nodules per plant.

Kulkarni et al[25] suggested that the total nodule biomass is plant-dependent, irrespective of the number and size of nodules. Generally, fewer nodules are usually large but the total mass of the nodules per plant would stay constant. The results of the present study were also in agreement with the earlier studies. The observed polymorphism in the nodule characters in the 16 host species is under the control of their genetic determinants and not by the rhizobia. This was also confirmed by the isolation of spontaneously nodulating *Lotus japonicum* mutants by Tirichine et al[55], even in the absence of rhizobium.

Seasonal effect in nodulation: Nodule pattern was studied in three seasons of the year and seasonal change did not affect the morphology of nodules and the proportion of nodulated plants of a species. However, more nodules were observed to senesce in the summer season than in the monsoon and winter seasons. Low availability of water in dry season might lead to the senescence of nodules, due to the fact the deficiency of water affects the nodule development/maintenance and nitrogen fixation. This is in accordance with the previous findings, in which seasonal difference in the number of senescent nodules was noted in most of the woody legumes in Hong Kong, China[4].

The overall nodulation pattern was consistent. Based on study on native Australian legumes, Lawrie[27] reported that nodular activity reaches maximum in spring, declined to a minimum in late summer and continued at a lower lever during winter. Based on these findings, it was suggested that variations in rainfall and temperature were the major factors influencing variations in nodular activity.

Nitrogen fixation potential: The application of wild legumes in the management of soil fertility depends on their nitrogen fixation potential. This important character can be evaluated based on nodule interior color and acetylene reduction assay (ARA) for nitrogenase activity. The interior color of the nodules varied from pink-red to pale pink. In most of the species, the color is pale pink, three species each had pink and pink-red nodules. In *Desmodium trifolium*, pink-red nodules were observed, in *Indigofera mysorensis, Mimosa pudica and Rhynchosia minima*, pink nodules were observed and in *Albizia lebbeck, Alysicarpus monilifer, Chamaecrista absus, Cassia tora, Crotalaria hebecarpa, Indigofera linnaei, Indigofera trifoliata, Tephrosia purpurea* and *Tephrosia tinctoria*, pale pink nodules were observed. This variation is due to the amount of leghemoglobin present in the nodular cells.

The pink-red nodules contain highest leghaemoglobin which indicates an active N_2-fixation[39]. Leghemoglobin is synthesized by the plant in response to rhizobial infection[58] which accumulates in the cytoplasm of infected plant cells and is crucial for symbiotic nitrogen fixation as it transports the oxygen for the bacteroid respiration and protects sensitive nitrogenase enzyme from inhibition by regulating oxygen pool[33].

The most important parameter in nitrogen fixation potential is nitrogenase activity. The nitrogenase activity of the nodules of the species ranged from 1000 to 2967 nmoles of C_2H_4/plant/hour (Table 3 and fig. 3). In the acetylene reduction test, maximum nitrogenase activity i.e. 2967 nmoles C_2H_4/plant/hour was recorded in *Desmodium trifolium* closely followed by *Clitoria ternatea* and *Milletia pinnata and* a minimum of 1000 nmoles C_2H_4/plant/hour was observed in *Albizia lebbeck* and *Tephrosia purpurea*. *Arachis hypogaea* with a maximum number of pink-red nodules i.e. 50 per plant showed maximum nitrogenase activity i.e. 3275 nmoles C_2H_4/plant/hour when compared to the wild species.

The three species *Desmodium trifolium, Clitoria ternatea and Millettia pinnata,* had almost similar nodule morphology and nitrogenase activity. Even the minimum value i.e. 1000 nmoles observed in the present investigation is 100 times greater than the value of effective nitrogen fixers' i.e. 10 nmoles of C_2H_4/plant/hour[17].

The physiological relationship between leghaemoglobin content and nitrogen fixation effectiveness in soya bean and common bean was shown by Dakora et al[12] where nitrogen fixation is high when the leghaemoglobin content is high. The same relationship was also observed between the color intensity of the nodule and the extent of nitrogenase activity in the present investigation.

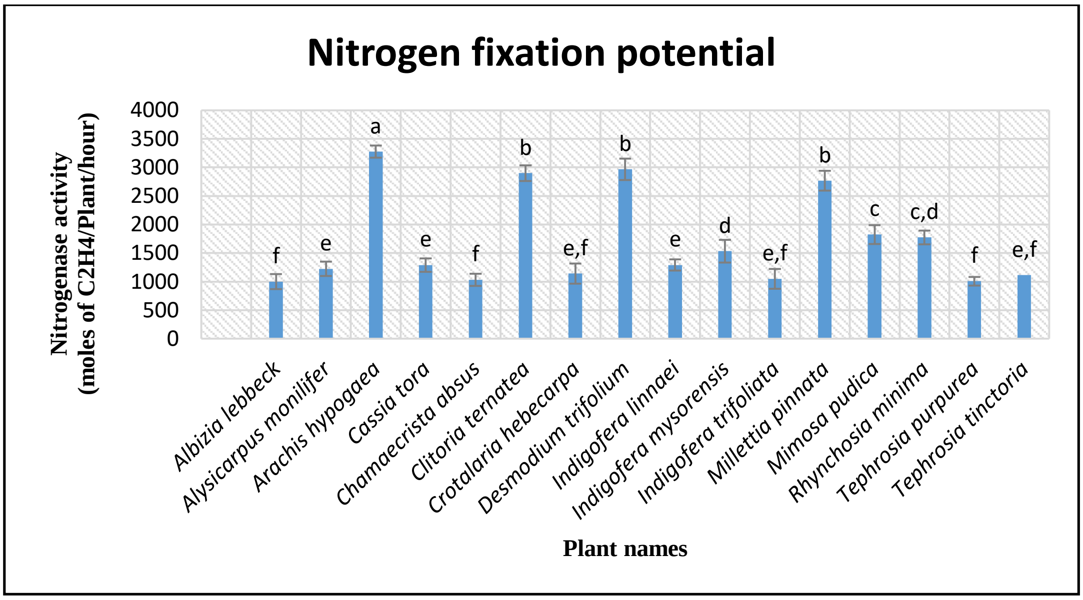

Fig. 3: Nitrogen fixation potential of wild legumes and cultivated plant

Table 3
Nitrogen fixation potential of wild legumes

S.N.	Plant name	Nitrogen fixation potential	
		Internal colour	Nitrogenase activity (moles of C_2H_4/Plant/hour*)
1	*Albizia lebbeck*	Pale pink	1000^{f} ± 119.02
2	*Alysicarpus monilifer*	Pale pink	1225^{e} ± 131.49
3	*Arachis hypogaea*	Pink-red	3275^{a} ± 125.01
4	*Chamaecrista absus*	Pale pink	1033^{f} ± 117.85
5	*Cassia tora*	Pale pink	1292^{e} ± 105.73
6	*Clitoria ternatea*	Pink-red	2900^{b} ± 108.01
7	*Crotalaria hebecarpa*	Pale pink	$1142^{e,f}$ ± 136.68
8	*Desmodium trifolium*	Pink-red	2967^{b} ± 177.17
9	*Indigofera linnaei*	Pale pink	1292^{e} ± 185.78
10	*Indigofera mysorensis*	Pink	1534^{d} ± 098.61
11	*Indigofera trifoliata*	Pale pink	$1050^{e,f}$ ± 197.91
12	*Millettia pinnata*	Pink-red	2767^{b} ± 172.41
13	*Mimosa pudica*	Pink	1825^{c} ± 175.12
14	*Rhynchosia minima*	Pink	$1775^{c,d}$ ± 165.21
15	*Tephrosia purpurea*	Pale pink	1008^{f} ± 120.47
16	*Tephrosia tinctoria*	Pale pink	$1116^{e,f}$ ± 074.54

* Means are values of six replications; Means followed by the same letter in a column are not significantly different but by different letters are significantly different (p = 0.05)

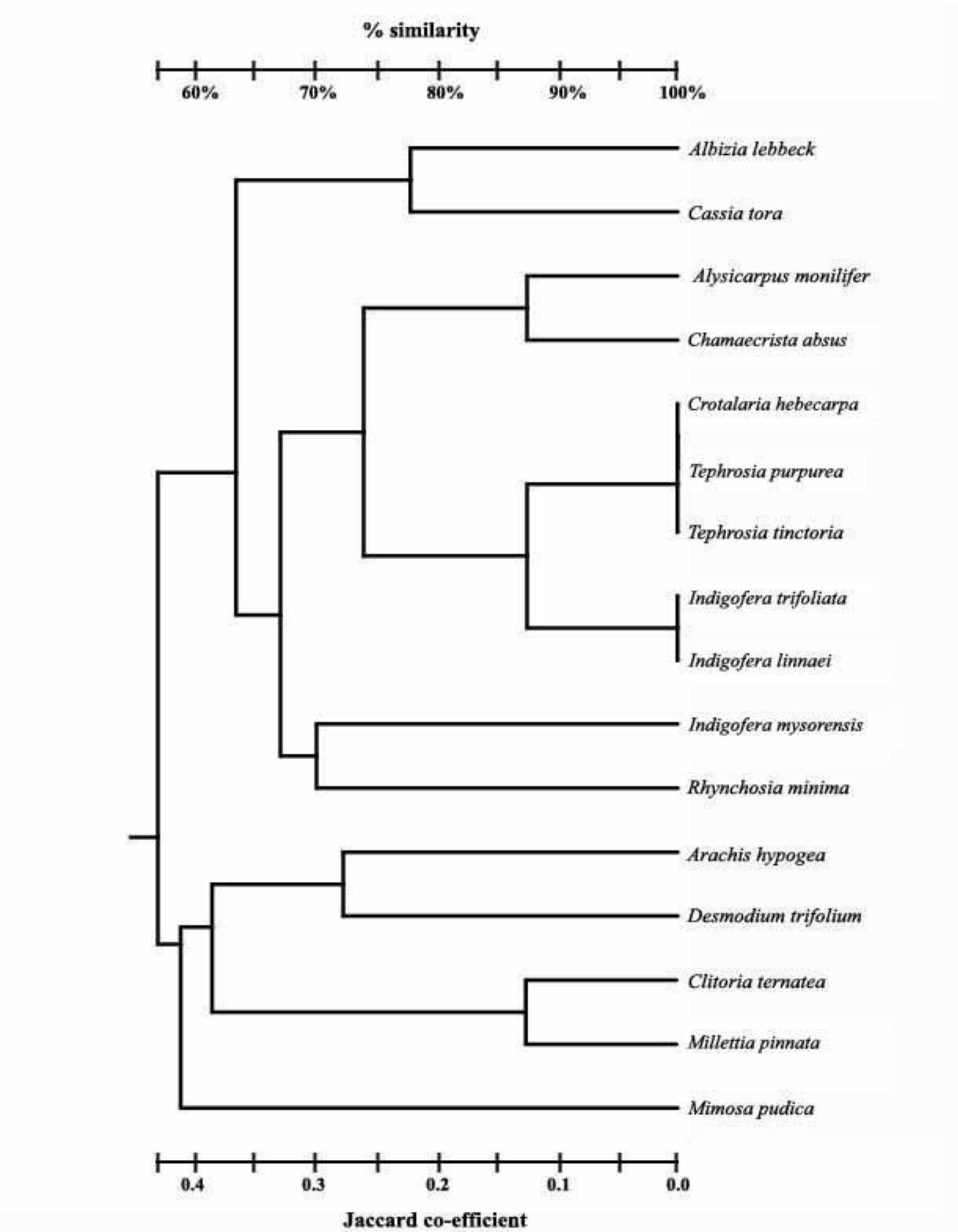

Fig. 4: UPGMA dendrogram showing the clustering of wild legumes based on nodulation and nitrogen fixation potential

Nitrogenase activity is maximum in pink-red nodules followed by pink nodules and minimum activity was observed with pale pink nodules. Pinkish nodules are more effective nodules with high nitrogen-fixing activity and a high amount of leghemoglobin as reported by Angie et al[4]. The high amount of leghemoglobin may be protecting the nitrogenase activity.

Simms et al[42] reported that the smaller size nodules are generally occupied by the poor/mediocre strains with low nitrogen fixation. However, in the present investigation, no such correlation was observed between nodule size and nitrogenase activity. Even the smaller pinkish nodules showed high nitrogenase activity. Nodule number and nitrogen fixation are maximum in *Arachis hypogaea* due to its effective compatibility with rhizobia resulting from the co-evolution of partners and a favourable cultivated soil environment.

Cluster Analysis: Nodule morphological features and nitrogenase activity grouped 16 legumes into 5 distinct clusters at 65% level indicating the diversity and Mpu as a loner at a distance of 0.33 (Fig. 4). Clusters 1, 3, 4 and 5 consisted of two species each and the second cluster consisted of seven species. *Albizia lebbeck* and *Cassia tora* formed cluster 1 with 83% similarity at a distance of 0.23. The seven species in cluster 2 were grouped into two sub-clusters at a distance of 0.136, one with two species and the other with five species. *Alysicarpus monilifer* and *Chamaecrista absus* joined as one subcluster and further remaining five species sub-clustered into two subgroups. Species in each subgroup have 100% similarity and two subgroups joined at a distance of 0.125.

Indigofera trifoliata and *Indigofera linnaei* showed 100% similarity formed by one subgroup. *Crotalaria hebecarpa* showed 100% similarity with the two *Tephrosia* species i.e. *Tephrosia purpurea* and *Tephrosia tinctoria* forming the second subgroup (Fig 4). However, *Indigofera mysorensis* separated from the other two *Indigofera* species and clustered with *Rhynchosia minima* due to the variation in nodule internal color and nitrogenase activity and formed cluster 3. This may be due to variation at the species level.

Cultivated *Arachis hypogaea* joined *Desmodium trifolium,* at a distance of 0.278, as cluster 4. *Clitoria ternatea* clustered with *Millettia pinnata,* at a distance of 0.40 and formed cluster 5. The diversity observed is due to the high amount of variation in nodule characteristics and nitrogen fixation observed among the studied legume species.

The observed diversity among the wild legumes is in partial agreement with Sprent et al[44]. Legumes display a range of nodulation phenotypes (infection, morphology and structure) that are characteristic of different tribes and may be related to the evolution of nodulation in legumes[44].

The ability of legumes to nodulate may confer other benefits, enabling them to maximize the use of available resources and colonize new areas. The nodules' presence can modify root plasticity independent of any effects of nitrogen fixation[20]. Arid conditions appear to be particularly beneficial to nodulated legumes[36].

Adams et al[1] showed that nodulation can be correlated with greater water use efficiency in certain areas, an important property in the context of climate change. Crisp et al[10] have also reported on the expansion of Australian legumes into more arid areas.

Conclusion

Analysis of nadulation status revealed 15 wild legumes belonging to 12 genera comprising of 11 species belonging to Papilionoideae, two each to Mimosoideae and Caesalpinioideae, nodulated in the SVU campus. High amount of variation in nodulation pattern and nitrogen fixation potential was observed among the studied wild legume species. This study adds *Indigofera mysorensis* a new nodulating legume to the global list and provides detailed description of nodule morphology and nitrogen fixation potential in thirteen species from semiarid region of Andhra Pradesh.

The reported fifteen wild legumes are effective nitrogen fixers with three-fold variation among species and *Desmodium trifolium, Clitoria ternatea* and *Millettia pinnata* being more efficient in that order. This may be due to better compatibility between nodule bacteria and legume species specificity. These species can be utilized for the improvement / restoration of soil fertility.

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