

Nodule formation potential in forage and grain legumes from rhizobia indigenous to Sancti Spiritus, Cuba

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The potential of nodule formation from rhizobia isolated from *Centrosema spp.* and *Stylosanthes viscosa* was evaluated for its future application in agricultural practice. A nodule collection was made from which rhizobia were isolated, identified and assessed morphoculturally. The number of nodules/plant was measured. The number of effective isolates was determined on inoculating forage and grain legumes. The percentage of effective strains was calculated regarding the total of strains for each macrosymbiont and geographical zone. All isolates formed nodules in *Macroptilium atropurpureum*. However, the isolates obtained from the legumes of the central zone in *Lupinus angustifolius* only formed nodules between 14 to 29 % while this value was between 20 to 67 % in those from the southern zone in this species. There was no nodulation in *Glycine max.* or in *Medicago sativa*, except with the reference strains used. It is concluded that the effective potential of the inoculated strains in different legumes could have been related to typical biochemical mechanisms of the process of radicular infection, rather than with the plant taxonomy. These results could be used in agricultural practice with the inoculation of forage legumes useful for cattle feeding and of legumes from temperate zones that can be inoculated with isolated rhizobia in tropical zones. Other trials, under semi-controlled conditions, are recommended for selecting the most efficient strains.

Key words: *Bradyrhizobium*, legumes, radicular infection, nodulation

Leguminosae family approximately includes more than 750 genera and more than 18,000 species from which only 8 % are cultivated plants. There is poor information on wild legumes and rhizospheric bacteria associated with them (Giongo *et al.* 2011). These bacteria (rhizobia) have the capacity of fixing atmospheric nitrogen through the intracellular symbiosis that they establish with the legumes. In Cuba, the wide biodiversity of native forage legumes, together with the stressing edaphoclimatic characteristics of the cattle raising ecosystems and to the urgent need of using our bioresources lead to the study and practical application of the legume-rhizobium symbiosis.

López and Sistachs (1986) and Tang (1996) among other authors have centered their investigations on natural nodulation of forage legumes and on the symbiotic efficiency of native rhizobia, inoculated in promissory legumes for cattle feeding. The majority of the tropical legumes evaluated in this study, as well as the natural macrosymbionts of the isolates, form part of the options of natural protein source in Cuban livestock production and are widely disseminated in the Cuban archipelago (Olivera *et al.* 2008). In the specific case of *Centrosema* and *Macroptilium*, they are also used as live cover in citrus plantations in Cuba (Fontes *et al.* 2009).

The forage legume of temperate climate, *Medicago sativa*, is also one of the most important nutrient sources for the cattle (Lowe *et al.* 2010). *Lupinus angustifolius* and other grain legumes can even substitute soybean (Howieson *et al.* 2008).

Sancti Spiritus province is divided in four edaphoclimatic zones (Hernández 1989). In each zone, there are ecosystems which are under environmental conditions exerting notable influence on the biodiversity of the plant species and microorganisms. Thus, the study of rhizobia adapted to these ecosystems brings about not only to the contribution of new knowledge on its genetic diversity, but also to strain selection with capacity for fixing atmospheric nitrogen, in symbiosis with legumes of interest for animal feeding. This latter was the main objective of this study.

Materials and Methods

Soil characteristics. The chemical properties of the soil were evaluated in the Laboratory of Chemical Analysis of the Experimental Station of Soils and Fertilizers “Barajagua”, Cienfuegos (table 1). Five samples were taken from each collecting place: banks of the river Jatibonico del Sur (central zone), Tuinucú river (Central zone), forest zone (central zone) and coastal grasslands (Southern zone). pH, cationic exchange capacity (Cec), P₂O₅, K₂O and organic matter (OM) were established.

Soils of the central zone corresponded to the alluvial category (Hernández *et al.* 1994). It was verified that the concentration of P₂O₅ and K₂O was higher in the soil of Jatibonico. pH varied from neutral (Tuinucú) to moderately alkaline (Jatibonico) (table 1). In this zone, soil analysis exhibited different values in the chemical composition, demonstrating the variability that can

Table 1. Chemical characteristics of the sampled soils

Zone	Place	Soil	pH (potentiometry)	Ccc, meq/100g (Mehlich)	M. Org., % (Walkley- Back)	P ₂ O ₅ mg/100g (Oniani)	K ₂ O mg/100g (Oniani)
Central	Jatibonico	Alluvial	8.01	14.80	2.94	6.74	23.60
	Tuinucú	Alluvial	6.80	23.20	4.01	3.63	15.10
South	San Pedro	Ferrallitic Quartzitic yellow lixiviated	4.40	9.65	3.63	0.99	12.24

present alluvial soils (Hernández *et al.* 1994). Southern soils are type yellow ferrallitic quartzitic lixiviated (Hernández *et al.* 1994) with acid pH. The OM, P₂O₅ and K₂O values determined were characteristic of this type of soil. They stand out by showing extremely low phosphorus values (table 1). It was also found NaCl concentration of 0.18 %. The capacity of cationic exchange in this type of soil was lower regarding the rest, which together with the acidity (pH 4.4) and to the NaCl concentration, could have negatively influenced on plant development.

Climatological data throughout the screening period. They were taken from the climatological database of Sancti Spiritus (Provincial Meteorological Center, CITMA). The most relevant data showed that in the central zone there were no rainfall and the temperature was of 23.90 °C, with 77 % of relative humidity. In the southern zone fell 92.50 mm. Mean temperature was high (28.70 °C), as well as the relative humidity (80 %). This confirms the stressing character of some environments where the nodules were collected.

Nodule collection. A screening was carried out, according to the methodology of Hernández *et al.* (1999). The areas belonging to two edaphoclimatic zones were evaluated: central and southern zones. Vigorous plants were selected at the beginning of the flowering stage without symptoms of pest attack presence of diseases. Five plants per sampled species were taken. The areas selected had no history of inoculation with rhizobia and each one showed different topographic characteristics. For the assessment of the type of radical nodulation, size, color and nodule form, the methodology of Bergerson (1982) was used. Nodule extraction from the roots and their later preservation was carried out according to Somasegaran and Hoben (1994). For that, a soil section was cut with a shovel, at not least of 15 cm around the plant and 20 cm depth. Nodules were preserved in containers with CaCl₂.

Rhizobia isolation. Nodules were sterilized in ethanol at 95 % (30 s) and sodium hypochlorite 4-6 % (2-3 min). They were rinsed in sterile distilled water. Later, the isolates were obtained from a compression made with a sterile glass macerator (Somasegaran and Hoben 1994). Sowing was made in yeast-mannitol solid medium (Vincent 1970).

Identification of rhizobia. The identity of the

rhizobia was confirmed by their capacity of provoking nodulation in *Macroptilium atropurpureum* (Vincent 1970) in 150 x 25 cm test tubes containing agar based nutritive solution of Norris and Date (Date 1982). Fresh inocula (108 CFU/mL) were used of each one of the isolates from which 1 mL was added to each tube. Seeds were sterilized with sodium hypochlorite (2.5 %) and scarified in hot water at 80-90 °C (2-3 min), before the sowing (Somasegaran and Hoben 1994).

Seedling grew in a chamber under controlled conditions SIOH (Convicon Co., Canada), with luminous periodicity of 16 h-light (300 microeinsteins/m²/s) at 26 °C during the day and 22 °C in the night. Relative humidity was of 75 to 85 %. Nodulation was evaluated at four weeks.

Gram tinting and growth in solid peptone-glucose medium were used for finding contaminants (Somasegaran and Hoben 1994). Smears were observed with the aid of an optical stereoscopic microscope.

Crossed inoculation tests in different legume species. The nodulation capacity of the isolates was evaluated in *Macroptilium atropurpureum*, *Glycine max.*, *Lupinus angustifolius*, *Medicago sativa*, *Neonotonia wightii* and *Centrosema plumieri*. Seeds of *M. atropurpureum*, *G. max.*, *L. angustifolius* and *M. sativa* were disinfected with ethanol 95 % application (30 s), sodium hypochlorite 4-6 % (2-3 min) and some successive rinses in sterile distilled water. They were scarified in hot water (80-90 °C) for 2-3 min. *N. wightii* and *C. plumieri* seeds were disinfected and scarified in sulphuric acid (20 min). They were rinsed in sterile distilled water. For their germination, they were placed in containers with sterile humid vermiculite in the darkness at 25 °C for 72 h. Later they were transferred to 25 x 200 mm tubes (*N. wightii* and *M. sativa*) and to 500 mL Erlenmeyers (*G. max.*, *L. angustifolius* and *C. plumieri*) with sterile vermiculite soaked up in nitrogen-free Hoagland solution (Prévost *et al.* 1987).

Pre-germinated seeds were inoculated with 1 mL of rhizobia suspension (10⁸ CFU/mL). For their growth they were submitted to a regime of luminous periodicity of 16 h-light (300 μE/m²/s) at 26 °C during the day and 22 °C during the night. Relative humidity was adjusted from 75-85 %. At the end of four weeks, the assessment

started (presence or absence of radical nodules).

Experimental design and statistical analysis. For evaluating the nodulation a completely randomized design with six treatments was used for each experiment (central zone, first experiment), (southern zone, second experiment) and ten replications. Treatments consisted of the different host legume genera: *Neonotonia wightii*, *Medicago sativa*, *Glycine max.*, *Lupinus angustifolius* and *Centrosema plumieri*. The number of effective isolates/plant variable was evaluated, since it is a variable with digit counting with zeros, was transformed by $\sqrt{x} + 0.5$ (Ruesga *et al.* 2005). To the transformed data that fulfill the statistical suppositions, an analysis of variance was applied. Mean comparison was calculated by Duncan (1955). The percentage of host-trap legumes infected by the isolates was determined.

Results and Discussion

In the central zone radical nodules of *Centrosema virginianum*, *C. plumieri* and *Neonotonia wightii* were collected while in the Southern the nodules belonged to *Centrosema sp.*, *C. virginianum*, *C. molle* and *Stylosanthes viscosa* (table 2).

According to Cuadrado *et al.* (2009) diverse factors can affect the symbiosis and prevent the appearance of nodules and consequently, the legume can die, excepting if the soil is very rich in nitrogen derivatives. The nodulation of legumes collected in the southern zone was scarce and in some instances, of small size. These indicators could have been influenced by the acid pH of the soil. Martínez-Viera (1986) maintains that soil acidity exerts decreasing effect on the symbiotic system, among other causes by the low molybdenum assimilation. Vance and Graham (1995) stated that soil acidity affects not only the rhizobia, but also the macrosymbiont by the same concentration of the hydrogen ion as for the deficiency induced of macro and microelements. If taken as basis these statements it could be inferred

that phosphorus deficiency, together with soil acidity of the Southern zone were the main barriers for the formation of radical nodules of greater size in the natural macrosymbionts.

In the central zone, the number of nodules was medium (10-50), of intermediate size (4.0 mm) and predominant internal color varying from pink to brown (figure 1). The greatest localization was found in the secondary roots. The accompanying vegetation consisted of shrubs and trees (table 2).

Nodulation in the roots of the legumes in the southern zone was characterized for being scarce (2-8 nodules), the size of the nodules medium or small (2.5-4.0 mm), with internal pink color. They were chiefly localized at the secondary roots (figure 1). The accompanying vegetation was characterized by poor or bare grasslands (table 2). In general, nodules collected were considered desmoids with defined oblong growing habit and spherical meristem (Bergerson 1982).

To all isolates, their identity as rhizobia was verified, on producing nodules in *Macroptilium atropurpureum* seedlings by their Gram (-) characteristic and poor or null growth in PGA (Vincent 1970). All showed negative response to Gram tinting and non-sporulated bacilli were observed as well as bacteroids in the smear, typical properties of rhizobia (Garrity and Holt 2001). In studies of polyphasic taxonomy it was determined that these isolates belong to *Bradyrhizobium sp.* (Bécquer 2002).

Lupinus angustifolius and *Neonotonia wightii* showed different level of radicular infection with the two groups of isolates (figures 2 and 3). From the 63 isolates evaluated, all were capable of forming nodules in *Macroptilium atropurpureum*. Nonetheless, *Lupinus* (10 nodules/plant) resulted statistically poorer ($P < 0.001$) than *Neonotonia* (16 nodules/plant), on being inoculated with isolates of the central zone, while in turn *Neonotonia* (1 nodule/plant) was lower ($P < 0.001$) than *Lupinus* (12 nodules/plant) on being inoculated

Table 2. Characteristics of the nodulation in the natural macrosymbionts

Natural macrosymbionts	Nodulation	Distribution, %		Size	Color	Shape	Accompanying vegetation
		Secondary root	Main root				
Zona Centro							
<i>N. wightii</i>	Medium	90	10	Medium	Rosado	Esférica	Shrubs trees
<i>C. plumieri</i>	Medium	85	15	Medium	Rojo	Esférica	Shrubs trees
<i>C. virginianum</i>	Scarce		10	Medium	Rosado	Esférica	Shrubs trees
<i>C. molle</i>	Scarce	90	15	Medium	Rojo	Esférica	Grassland
Zona Sur							
<i>Centrosema sp.</i>	Scarce	95	5	Medium	Rosado	Esférica	Grassland
<i>C. virginianum</i>	Scarce	90	10	Medium	Rosado	Esférica	Grassland
<i>C. molle</i>	Scarce	80	20	Medium	Rosado	Esférica	Grassland
<i>S. viscosa</i>	Scarce	95	15	Small	Rosado	Esférica	Grassland

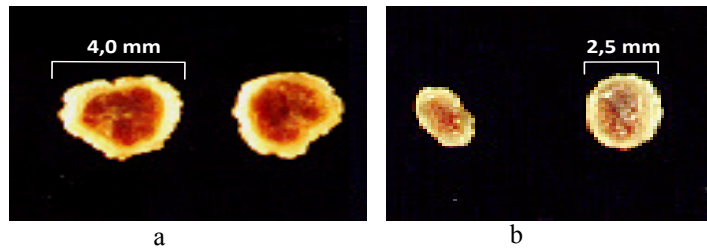


Figure 1. Morphology of representative nodules of the *Macrosymbiont plumieri* (Central zone) (a) y *C. molle* (southern zone) (b)

with isolates of the southern zone. *Centrosema* (38 nodules/plant) shared common indices with *Macroptilium* (40 nodules/plant) regarding effective isolates of the central zone in these genera. However, *Centrosema* was statistically poorer (21 nodules/plant) ($P < 0.001$) than *Macroptilium* (23 nodules/plant), when inoculated with isolates of the southern zone.

Olivera *et al.* (2008) found *Centrosema* widely

disseminated in three Cuban provinces. Although Gómez *et al.* (2010) in legume screenings carried out in Cauto Valley, Cuba, found that *C. plumieri* exhibited high natural nodulation indices. Pérez *et al.* (2008) established high infective promiscuity in *Centrosema*. Results found in this study demonstrated that the capacity of nodule formation of the isolates from *Centrosema* and *Neonotonia*, on inoculating in *Macroptilium*

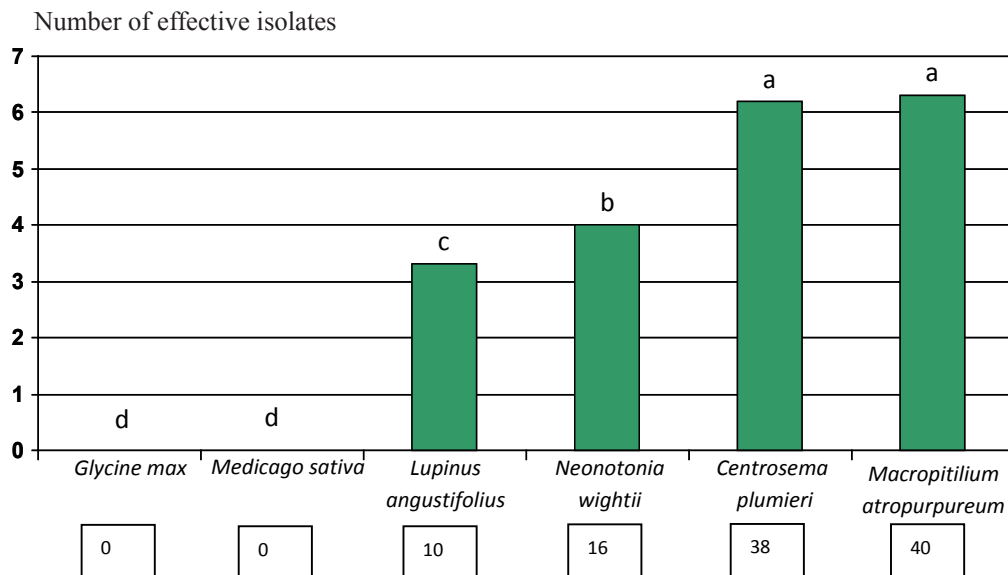


Figure 2. Number of the central zone that formed nodules in the host –trap legumes. Transformed data (\sqrt{x}). S. E.: 0.66. $P < 0.001$.

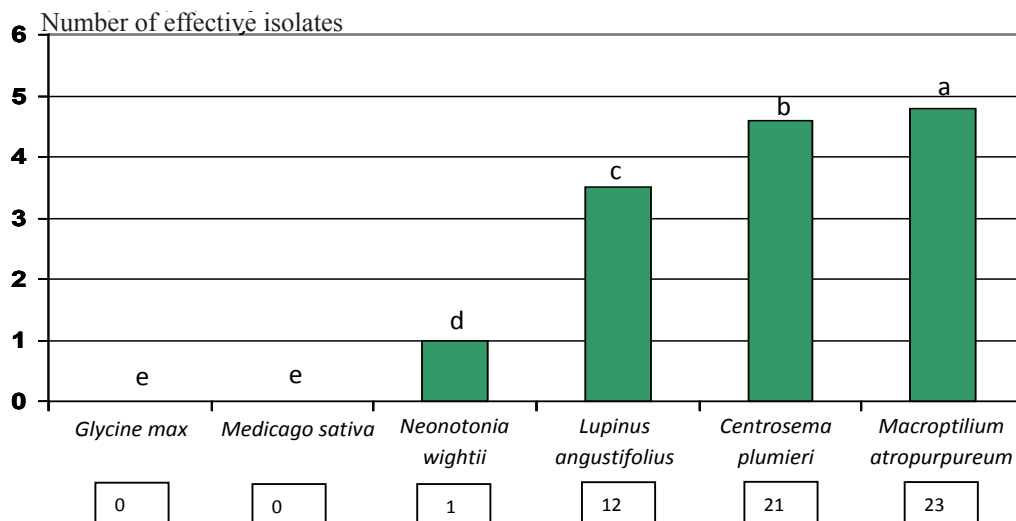


Figure 3. Number of the southern zone that formed nodules in the host –trap legumes. Transformed data (\sqrt{x}). S. E.: 0.56. $P < 0.001$

atropurpureum, *C. plumieri* and *Lupinus angustifolius*, did not have close relationship with geographical zone from which they came from. Date (2010) reported native forage legume accessions with already established strains of Bradyrhizobium in the soil. In table 3 are shown the percentage of isolates that infected the host legumes used in the evaluation. The isolates from the central zone which were microsymbionts of *N. wightii*, *C. virginianum* and *C. plumieri* formed nodules in *Lupinus angustifolius* and infected this legume (29, 26 and 14 %, respectively). Those of the southern zone (*C. virginianum*, *Centrosema sp.* and *C. molle*) infected *L. angustifolius* (20 to 67 %). The isolates of *S. viscosa* did not form nodules in *Lupinus*, *N. wightii* and *C. plumieri*.

C. plumieri was efficiently infected by the greatest

Frank, 1987). Nonetheless, in Neonotonia, besides its own microsymbionts, only one *Centrosema molle* isolate formed effective nodules and in *G. max.* no nodulation was found. Plants belonging to *Lupinus* were infected by isolates from the legumes native to Sancti Spiritus province, except for *Stylosanthes viscosa*. This is interesting from the taxonomic point of view, since *Lupinus* belongs to the Genistae tribu (ILDIS 2010) indigenous to temperate zones. Phaseolae tribu (*Centrosema spp.*, Neonotonia and others) belongs to so-called "old tropical world" (Young and Johnston 1989). However, Lezama (2010) stated that *L. carazensis*, *L. lindleyanus* and *L. aridulus* are nodulated by Mesorhizobium. The rest of the *Lupinus* species are nodulated by

Table 3. Percentage of isolates forming nodules in the host-trap legumes

Natural macrosymbionts from where the isolates came	Host trap legumes infected by the isolates, %			
	<i>M. atropurpureum</i>	<i>C. plumieri</i>	<i>N. wightii</i>	<i>L. angustifolius</i>
Central zone				
<i>N. wightii</i>	100	93	100	29
<i>C. virginianum</i>	100	95	5	26
<i>C. plumieri</i>	100	100	14	14
Southern zone				
<i>Centrosema sp.</i>	100	80	-	60
<i>C. virginianum</i>	100	100	-	20
<i>C. molle</i>	100	100	8	67
<i>S. viscosa</i>	100	-	-	-

part of the isolates of the central zone, in a range varying between 93 and 100 %, while those of the southern zone was infected between 80 and 100. From these, those of maximum infective power came from macrosymbionts belonging to the *Centrosema* genus. Bécquer *et al.* (2013) found a high nodulation index in *C. molle* induced by the rhizobia strains from other *Centrosema* species, although this fact did not necessarily implicated high symbiotic efficiency.

Ample range results were verified in the legume *N. wightii* in which nodules from 5 to 100 % were formed from the isolates coming from *C. virginianum*, *C. plumieri* and *N. wightii* (central zone) and only 8 % of the isolates of the southern zone (coming from *C. molle*). The macrosymbiont with an absolute percentage of effective radicular infection was *M. atropurpureum* with 100 %, *G. max.* and *M. sativa* did not show nodules (this datum was not included in table 3).

It was inferred that the phylogenetic position of the plants, according to their systematics, did not influence either in a notable way on the mechanism of radicular infection, since *G. max.*, *N. wightii* and *C. molle* belong to the tribu Phaseoleae (Zindler-

Bradyrhizobium.

The effective radicular infection of *Lupinus* with symbionts isolated in *Centrosema spp.* and Neonotonia, could have been related to the production of common isoflavonoids between both legume tribus. Ingham (1981) assured that in spite that Phaseoleae is characterized by the production of pterocarpan in some representatives of both tribus luteon and wighteon isoflavons were found. According to Olivares (2008) the isoflavonoids are one of the most important factors in the mechanism of radicular infection in the legume-rhizobium symbiosis.

There was no nodulation in *Glycine max.* or in *Medicago sativa*, except with the reference strains used, agreeing with what was reported by Vincent (1970) on their close symbiotic affinity.

It is concluded that the infective affinity of the inoculated strains in different legumes phylogenetically far of the original host legumes of these strains could have had connection with the biochemical mechanisms typical of the radicular infection process rather than with the plant taxonomy. These results could be useful in agricultural practice, specifically in the inoculation of forage legumes valuable for bovine cattle feeding, as

well as in the inoculation of plants of temperate zones with rhizobia strains isolated in tropical zones. However further studies under semi-controlled conditions are required for selecting the most efficient strains.

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