Symbiotic efficiency of native rhizobia from Sancti Spíritus, Cuba, inoculated in Centrosema molle

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ABSTRACT

An essay was conducted in greenhouse, in order to determine the symbiotic efficiency of 39 native strains of bradyrhizobia from Sancti Spíritus. The isolations were made from root nodules of *Centrosema molle, Centrosema virginianum* and *Centrosema plumieri*, which are macrosymbionts from the central and southern areas of Sancti Spíritus. The obtained strains were inoculated in *C. molle*, promising forage legume for livestock feeding in the territory. The inoculation methods were standard, according to the recommendations in the international literature. A randomized block experimental design was used, with 41 treatments and three replications. Variance analysis (ANOVA) was made and the dry weight of the aerial part of the plant, root dry weight, nodulation index and nitrogen yield were evaluated. It is concluded that there was high symbiotic efficiency of the native strains from the central area, by being inoculated in a legume from the same zone; while the ones from the southern area were inefficient in such legume. On the other hand, the strains JK3 (*C. virginianum*), HG1 (*C. plumieri*) –both from the central area– and SP20 (*C. molle*, southern zone) and JJ2 (*C. virginianum*, central zone), showed high efficiency levels in the evaluated legume, which makes them the most promising ones. The high nodulation indexes of JH4, JJ2, JJ7, JK6, JK1 and JK5 (*C. virginianum*) did not imply high symbiotic efficiency. A selection phase in the field is recommended, with different macrosymbionts and under stressful soil and climate conditions.

Key words: *Bradyrhizobium* sp., *Centrosema* sp., inoculation

INTRODUCTION

Centrosema belongs to the *Faboideae* subfamily, *Phaseoleae* tribe, and it is a neo-tropical genus which contains very important species from the pasture and forage point of view. It is original from southern tropical America and is abundant in the tropics, where it constitutes an important forage legume as protein and mineral source for ruminants (Lukiwati, 2007). It is said that it can fix up to 280 kg of N/ha/year associated with grasses (Sylvester-Bradley *et al*., 1983).

In Queensland, Australia, *Centrosema molle* is considered one of the most used forage legumes by livestock, to produce high live weight gains (English *et al.,* 2009). It is moderately specific in nodulation, for which its inoculation is recommended. In Sancti Spíritus, Hernández *et al*. (1999) found that *Centrosema* was one of the genera of higher appearance frequency and more adapted to the soil and climate conditions of the region. Olivera, Machado and Fung (2008) also found this genus widely disseminated in three provinces from the eastern part of Cuba.

The symbiotic fixation of atmospheric nitrogen in *C. molle* was previously studied in Cuba by Tang and Menéndez (1988) and Tang, Menéndez, Cantillo and Gazó (1988), who obtained significant increases in dry matter with the strain CIAT-1670 of *Rhizobium* spp. Also Tang, Rodríguez and Ávila (1994) determined that *C. molle* (IH-129, CIAT-482 and CIAT-5151) had a positive response to the action of the soil natural rhizobia. This kind of study requires to be continued based on the isolation of new strains of rhizobia in different ecosystems. That is why the objective of this work was to evaluate, under greenhouse conditions, the symbiotic efficiency of *Bradyrhizobium* sp., from livestock ecosystems of Sancti Spíritus, inoculated in *C. molle* to choose the most outstanding ones for their future evaluation in the field.

MATERIALS AND METHODS

The isolations were made from root nodules of *Centrosema plumieri, Centrosema virginianum* and *C. molle*, all naturalized legumes from livestock ecosystems of Sancti Spíritus. The obtained strains, after being taxonomically located as belonging to *Bradyrhizobium* sp. (Bécquer, Prévost, Cloutier and Laguerre, 2002), were denominated according to the geographic origin of their macrosymbionts (table 1).

The strains were preserved in wedges of solid yeast-mannitol medium at 4 °C (Vincent, 1970). The inoculants were prepared in Erlenmeyer flasks of 250 mL, that contained100 mL of solid yeast-mannitol medium and they were inoculated at 29-30 °C, from five to eight days, in a rotary shaker (160 rpm) until reaching a titer of 10^6-10^8 CFU/mL.

The seeds (*C. molle*) came from the Experimental Station of Pastures and Forages of Sancti Spíritus, Cuba. They were sterilized and scarified with H_2SO_4 (95 %) during 20 minutes and were successively rinsed in sterile distilled water. Afterwards, from four to five seeds were planted per pot. After germination, they were thinned to leave two plants per pot (each pot was considered a replication). Sterile vermiculite was used as substratum, imbibed in nitrogen-free Hoagland solution (Prévost *et al*., 1987).

The plants were inoculated in the phase of cotyledon breaking with 10 mL of bacterial suspension, with a titer of 10^6-10^8 CFU/mL. For the growth in greenhouse they were subject to a regime of light periodicity of 16 light hours (300 μE/m^2/s), at 26 °C during the day and 22 °C at night. The relative humidity was adjusted at 75-85 %. Three weeks after sowing, 30 mg/L de KNO_3 were added to the substratum as source of N, to facilitate the plant establishment (Prévost *et al*., 1987).

A randomized block experimental design was used, with 41 treatments: 39 native strains of *Centrosema* spp., a control (reference strain: 25B6) and an absolute control (non-inoculated treatment), with three replications. A fertilized control was not used, because the experiment was conducted under strictly controlled conditions; the only amount of nitrogen received by the plants came from the symbiotic fixation made by the inoculated strains. A variance analysis (ANOVA) for inoculation experiments was applied (Somasegaran and Hoben, 1994) and the differences between means were determined by Duncan´s test (1955).The dry weight of the aerial part of the plant (DWAPP) (g/pot) and the dry weight of the root (DWR) (g/pot) were evaluated, in the second cutting (120 days); because in this stage forage legumes express better their agro-physiological parameters (Álvarez *et al*., 1997). The plants were dried at 80 °C during 24 hours in an air circulating oven.

The nodulation index (NI) was calculated according to the indicators shown in table 2 and it was considered high when it was equal to or

Table 1. Denomination of the isolated strains, identification and geographic zone of the natural macrosymbionts

Natural macrosymbiont	Denomination of the strains	Geographic zone
C. plumieri	HA1, HA2, HA3, HG1, HG2, HG3, HG4	Central area, Sancti Spíritus
C. virginianum	JH1, JH2, JH3, JH4, JI1, JI2, JI3, JJ2, JJ3, JJ4, JJ5, JJ6, JJ7, JK1, JK2, JK3, JK4, JK5, JK6	Central area, Sancti Spíritus
C. virginianum	SP7, SP8, SP9, SP10, SP11	Southern area, Sancti Spíritus
C. molle	SP12, SP13, SP14,SP19, SP20, SP21, SP22, SP23	Southern area, Sancti Spíritus
C. molle	25B6	Australia

Table 2. Indicators for the determination of the NI.

Characteristics of the nodule	Value
Diameter (A)	
Large	3
Medium-sized	2
Small	1
Color (B)	
Red	3
Pink	2
White	1
Quantity (C)	
Much	3
Little	2
None	0

NI= A x B x C

higher than 15 (Bordeleau, Antoun and Lachance, 1977).

Although Date (2010) and Date and Eagles (2010) considered the dry weight of the whole plant as reference for the measurement of the legume-rhizobium symbiosis, to calculate the symbiotic efficiency (SE) of the strains the methodology proposed by Bordeleau *et al.* (1977) was used. The evaluation criterion to determine the most outstanding ones was based on the treatments that showed high symbiotic efficiency:

1. When the DWAPP is lower than the result of the subtraction of the standard error from the mean of all treatments, the strain was considered inefficient (*i*) DWAPP< x – SE.
2. When the DWAPP was within the range of the treatment mean and the standard error, the strain was considered moderately efficient (*m*) DWAPP = x ± SE.
3. When the DWAPP was higher than the addition of the mean of all treatments and the standard error, the strain was considered highly efficient (*h*) DWAPP> x + SE.

RESULTS AND DISCUSSIONS

Symbiotic efficiency and dry weight of the aerial part of the plant

During the evaluation conducted in the second cutting, variable symbiotic efficiency of the strains was observed with respect to the inoculated macrosymbionts (table 3). Except JJ5 (*C. virginianum*, central zone), SP7, SP8, SP9, SP11 (*C. virginianum*, southern zone), SP21 (*C. molle*) and the non-inoculated treatment –which turned out to be inefficient–, the rest of the strains (including the reference strain 25B6) showed moderate efficiency, or high in the case of JK3 (*C. virginianum*), HG1 (*C. plumieri*) –both from the central zone–, SP20 (*C. molle*, southern zone) and JJ2 (*C. virginianum*, central zone).Only JJ2 showed high efficiency, with a significant difference (p<0,05) in the DWAPP. The reference strain 25B6 and five strains from the southern zone (SP23, SP19, SP13, SP12 and SP10), in addition to the ones from the central zone (JH1, JH2, JH3, JI1, JI2, JI3, JJ4, JJ6, JJ7, JK5, JK6, HA1, HG3), in spite of sharing common superscripts in the DWAPP with the highly efficient strains, did not show high symbiotic efficiency. In general, the strains from the central zone, which were microsymbionts in *C.virginianum* and *C. plumieri*, were observed to show moderate efficiency in *C. molle*; while the ones from the southern zone, isolated from *C. virginianum* and *C. molle*, were mostly inefficient.

In this sense, Sprent (2001) stated that the microsymbionts with the same geographic origin as their macrosymbionts can show higher symbiotic efficiency, probably due to a legume-rhizobium coevolution. Also, Thrall, Burdon and Woods (2000) found that most of the macrosymbionts inoculated with rhizobia of the same species showed better productive results, although with considerable variation. These concepts are applicable in this study, because Hernández *et al.* (1999) demonstrated that *Centrosema* has the highest appearance frequency in the central zone of the Sancti Spíritus province.

Likewise, it is interesting that most of the *C. virginianum* (central zone) and *C. plumieri* (central zone) strains were more efficient in *C. molle* than their own microsymbionts, for which the higher influence of the geographic influence is evident. Although the symbiotic efficiency was the basis for the evaluation of the inoculated strains, it cannot be ignored that some strains in both legumes showed values that shared common superscripts with the highly efficient treatments; however, they were not considered outstanding due to the lower value obtained in the applied formula. This fact suggests that the criterion of the researcher must prevail over any pre-established formulation.

Although 25B6 (reference strain) showed moderate efficiency, the native strains showed a higher symbiotic potential, in spite of having proved the inefficiency of some of them. Howieson *et al.* (2008) claimed that even in the relatively non-disturbed ecosystems the rhizobia with little symbiotic capacity prevail, but at the same time others can be highly competitive.

Root dry weight

In the variable RDW the treatments with values that did not differ statistically among themselves predominated, although the JK3 (*C. virginianum*, central zone) and SP20 (*C. molle*, southern zone) strains were statistically higher (p < 0,001) than JH3, JH4, JJ3, JJ5, JK1, JK2, JK4, HA2, HA3, HG2, HG4, SP7, SP8, SP9, SP11, SP14, SP21, SP22, 25B6 and the non-inoculated control (table 3). The rest of them shared common superscripts with JK3 and SP20. It is important to emphasize that the strains that showed statistical superiority in this variable were considered highly efficient according to the

Table 3: Symbiotic efficiency of the strains from the central and southern zone in *C. molle.*

Treatment-strain	DWAPP, 2nd cutting (g/pot)	RDW, 2nd cuttting (g/pot)	NI, 2nd cuttting
JH1	m 5,66 abcde	1,38 abcdefghi	12,00 abcd
JH2	m 6,34 abcde	1,55 abcdefgh	8,00 bcdef
JH3	m 6,32 abcde	1,13 bcdefghi	14,00 abc
JH4	m 7,61 abcd	1,60 bcdef	18,00 a
JI1	m 6,43 abcde	1,94 abcd	9,00 bcdef
JI2	m 5,30 abcde	1,62 abcde	8,00 bcdef
JI3	m 5,77 abcde	1,56 abcdefgh	12,00 abcd
JJ2	h 9,96 a	2,17 abc	18,00 a
JJ3	m 3,47 def	0,78 defghi	14,00 abc
JJ4	m 6,82 abcde	1,85 abcd	14,00abc
JJ5	i 2,26 ef	0,97 cdefghi	8,00 bcdef
JJ6	m 4,88 abcdef	1,44 abcdefgh	12,00 abcd
JJ7	m 7,74 abcd	1,94 abcd	19,00 a
JK1	m 7,42 abcd	1,20 bcdefghi	15,00 ab
JK2	m 4,90 abcdef	1,17 bcdefghi	14,00 abc
JK3	h 8,82 abc	2,50 a	13,00 abcd
JK4	m 4,06 cdef	1,12 bcdefghi	13,00 abcd
JK5	m 6,29 abcde	1,26 abcdefghi	15,00 ab
JK6	m 6,48 abcde	1,56 abcdefgh	18,00 a
HA1	m 7,27 abcde	1,47 abcdefgh	8,00 bcdef
HA2	m 5,90 abcde	1,14 bcdefghi	9,30 bcdef
HA3	m 6,54 abcde	1,02 bcdefghi	10,00 bcd
HG1	h 9,68 ab	2,20 abc	10,00 bcd
HG2	m6,67 abcde	1,19 bcdefghi	10,00 bcd
HG3	m 5,48 abcde	1,24 abcdefghi	10,00 bcd
HG4	m 5,38 abcde	0,92 efghi	6,70 cdefg
SP7	i 0,40 f	0,30 ghi	2,00 fg
SP8	i 0,28 f	0,29 ghi	2,30 efg
SP9	i 0,36 f	0,29 ghi	2,30 efg
SP10	m 7,70 abcd	1,38 abcdefghi	10,00 bcd
SP11	i 0,44 f	0,47 efghi	2,00 fg
SP12	m 5,51 abcde	1,68 abcde	8,00 bcdef
SP13	m 6,60 abcde	2,07 abcd	9,00 bcdef
SP14	m 4,20 cdef	1,16 bcdefghi	6,30 defg
SP19	m 7,27 abcde	1,55 abcdefgh	6,00 defg
SP20	h 8,02 abcd	2,30 a	7,00 cdefg
SP21	i 0,40 f	0,32 fghi	2,00 fg
SP22	m 3,57 def	1,01 bcdefghi	9,70 bcde
SP23	m 5,36 abcde	1,68 abcde	6,00 defg
NI	i 0,13 f	0,26 hi	0,00 g
25B6	m 6,66 abcde	0,15 i	2,00 fg

Table 3 (Continuación)

Treatment-strain	DWAPP, 2nd cutting (g/pot)	RDW, 2nd cuttting (g/pot)	NI, 2nd cuttting
ES±	2,48*	0,63***	3,66***

h: highly efficient, *m*: moderately efficient, *i*: inefficient, NI: non-inoculated treatment.
Values with non-common superscripts differ at p < 0,05 (Duncan, 1955).
*p < 0,05 ***p < 0,001

DWAPP. This result is not surprising, because it is known that rhizobia can positively influence the plant development, not only through nitrogen fixation, but also by means of the production of plant growth stimulating substances, which can act on the different plant parts (Avis, Gravel, Antoun and Tweddell, 2008). Independently from the effect of nitrogen fixation on the aerial part of the plant, it is not discarded that in these treatments a higher absorption of easily assimilable nutrients occurred through the root system.

Nodulation index

The NI of JH4, JJ2, JJ7, JK1, JK5 and JK6 (*C. virginianum*, central zone) was high (table 3). In the case of JH4, JJ2, JJ7 and JK6, such level was statistically higher (p<0,001) than most of the strains, especially the ones from the southern zone. In general, the strains that showed lower values for this variable were those that resulted inefficient in the DWAPP. The strain JJ2 showed a high efficiency in this variable, as well as a high nodulation index. The reference strain 25B6 had moderate efficiency in DWAPP and its NI was low.

The high NI of the above-mentioned strains from the central zone does not imply, necessarily, higher symbiotic efficiency or highly significant values in the DWAPP (for example: JH4, JJ7, JK1, JK5 and JK6). This could be related to an imbalance of the nutrient exchange among the symbionts. It could be also considered that the capacity of root infection of the rhizobia –expressed by the common *nod* genes (which regulate nodulation)– not always implies high symbiotic capacity, which in turn constitutes the expression of other genes such as the *nif* genes (that codify for nitrogenase) and the *fix* genes (which regulate nitrogen fixation) (Martínez-Romero and Palacios, 1990).

On the other hand, among the signals used by rhizobia in their "molecular dialogue"with the plant, which play an important role in the formation of N-fixing nodules, are the exopolysaccharides (EPS), the LPS (lipopolysaccharides), as well as the signs of quorum sensing (Fraysse, Courdec and Poinsot, 2003). Therefore, if there are mutants of inefficient rhizobia in the synthesis of these compounds, the result would also be deficient infection of the plant or low N fixation. The results suggest that the differences in the biochemical interaction of every strain with the macrosymbiont determined the kind of response in each treatment.

The fact that there were strains from *C. plumieri* and *C. virginianum* (central zone) that showed a statistically higher effect on the studied variables, places them in a much more strict selection range. It also demonstrates the existence of a high potential of nitrogen fixation, together with low specificity towards the macrosymbiont.

The symbiotic efficiency of the strains from the central zone of Sancti Spíritus was concluded to be high or moderate when they were inoculated in a macrosymbiont from the same zone; while the ones from the southern zone were inefficient. On the other hand the strains JK3 (*C. virginianum*), HG1 (*C. plumieri*); both from the central zone; SP20 (*C. molle*, southern zone) and JJ2 (*C. virginianum*, central zone), showed high levels of efficiency in the evaluated legume, which makes them the most outstanding ones.It is remarkable that the high nodulation indexes of JH4, JJ2, JJ7, JK6, JK1 and JK5 (*C. virginianum*), in all cases were not in correspondence with high symbiotic efficiency. A subsequent phase of selection under field conditions is recommended, with different macrosymbionts and under stressed soil and climate conditions, typical of Cuban livestock ecosystems.

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