Selection of rhizobia isolates from nodules of the forage legume *Pueraria phaseoloides* (tropical kudzu)

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Ten rhizobia isolates were used for selecting those showing potentialities for the preparation of a biofertilizer allowing a better establishment of tropical kudzu in livestock production soils. Their possible taxonomic distribution and their capacity of growing in six carbon sources and produce polyhydroxybutyrates, were determined. Also, *in vitro* nodulation bioessays were conducted in the host plant. A completely randomized design with ten observations per treatment was used. All isolates resulted possible members of the Bradyrhizobium genus. Six of them consumed all the sugars as only carbon source, galactose was the least consumed. Polyhydroxybutyrate concentrations produced by these isolates were between 0.001 and 0.009 $g.L^{-1}$, what was significantly higher than the rest of the isolates. Plants inoculated with two of the isolates showed greater aerial dry mass which tallies with those having greater effective nodulation. From the ten isolates, four showed greater potentialities for the preparation of an effective inoculant for tropical kudzu culture.

Key words: *identification, biofertilizer, sugars, PHB, nodulation*

In Cuba, the strategy of cattle production is based on the use of pasture and forages as main feeding source (Hernández *et al*. 2006). The utilization of the forage legume *Pueraria phaseoloides* (tropical kudzu) is known which, associated with grasses, improves dry matter production, increases the protein level of the cattle diet and increases the quality and forage digestibility (Rodríguez 2006). By adding this legume to the daily diet milk yield and calf weight increase (Chacón *et al.* 2000) have been observed. Tropical kudzu has also been used as plant fertilizer for improving soil fertility and, consequently, pastures productivity in tropical areas (Unkovich *et al*. 2008). However, the majority of grasslands present poor quality regarding their feeding benefits, since grasses predominate regarding legume cultures. This is mainly due to the poor legume establishment in cattle production areas with soils presenting, in general, poor fertility and which are affected by biological, physical and chemical factors (Senra 2002).

An alternative ecologically sustainable, that could improve tropical kudzu establishment in cattle production soils, is the inoculation of this legume with a biofertilizer based on rhizobia that establish an effective symbiosis with the kudzu. In Cuba there is not this type of bioproduct which besides diminishing the application of nitrogen fertilizers augments the yield of the cattle mass. For the selection of rhizobia isolates must be considered their survival capacity in the soil and their competitive abilities with native rhizobia populations besides their symbiotic efficiency. These are highly desirable characteristics in rhizobia strains recommended for legume inoculation.

The objective of this study was to select rhizobia isolates with potentialities for the preparation of a biofertilizer allowing the improvement tropical kudzu establishment in cattle production soils.

Materials and Methods

Microbial material. The study was carried out at the National Institute of Agricultural Sciences (INCA) in the period 2010-2011. Ten rhizobia isolates (1-1, 1-2, 2-4-, 2-5, 11-5, 12-4, 12-5, K1, K2 and K14) were used from the nodules of the forage legume *Pueraria phaseoloides*, cultured in the region of Cascajal, Villa Clara province, Cuba. Presently, these bacterial isolates belong to the rhizobium strain collection of the Microbiology Laboratory of the Department of Physiology and Plant Biochemistry of the National Institute of Agricultural Sciences of Cuba.

Morpho-cultural and physiological characteristics. The micro-morphological characteristics studied were form and size of the bacterial cell, response to Gram tinting and spore presence. These traits, as well as the purity of each one of the bacterial cultures, were determined by Gram tinting (Norris and Dates 1976).

For studying the cultural characteristics, the isolates under study were cultivated in yeast-mannitol-agar exhaustion mean (YEM) (Vincent 1970), with Congo red and pH 6.8. They were incubated for 10 d at 28º C. Later, the form, color, texture, mucus production and colony diameter (mm) were determined. Also, the growth rate of each isolate was specified and the colony appearance or not in the plates were controlled every 24 h

during the ten incubation days. The isolates with visible colonies in the medium at two or three days of incubation were considered of fast growth. However, those with colonies appearing at five or seven days were considered of slow growth (Wang and Martínez-Romero 2001).

Acid and base excretion. In order to know if the different isolates excrete acid or base to the culture medium the YEM medium was used at pH 6.8 with bromothymol blue (0.5 % in NaOH 0.016 N) indicator. Once the growth time of each isolate elapsed, a color change in the culture medium was observed. The change of color from green (original color of the medium after being formulated) to yellow, agreed with the acid excretion to the medium. The change from blue green was equivalent to the base excretion to the culture medium by the microorganism (Wang and Martínez-Romero 2001).

Sugar assimilation. For this, the solid medium Bergensen (Bergensen 1961) with pH 6.8 and Congo red addition was utilized. This medium contained individually as carbon sources sucrose, glucose, rhamnose, lactose, galactose and mannitol at a concentration of 10 g L^{-1}. The inoculated isolates were incubated at 28ºC for 10 d.

Polyhydroxybutyrate (PHB) production. PHB determination was carried out according to the methodology described by Finkelstein (Finkelstein *et al*. 1997). PHB ($g.L^{-1}$) concentration was calculated according to the pure PHB pattern curve by means of the expression $y = 0.02x$, $r^2 = 0.89$.

In vitro nodulation essays. They were realized from *Pueraria phaseoloides* (tropical kudzu) plants, host legume of these bacterial isolates. The seeds were donated by the EEPF of Cascajal, Villa Clara. For the scarification seeds were maintained in ethanol at 70 % for 5 min. Later, they were washed with distilled water and submitted to a treatment with concentrated sulfuric acid for 10 min. Immediately afterwards, they were submerged in sodium hypochlorite at 25 % (v/v) for 15 min and washed several times with sterile distilled water. Next, seeds were placed in plates with Agar-Water (0.75 %) (m/v) and incubated at 28º C in the darkness for 24 h. Seeds with emergent roots (of approximately 1-2 cm) were placed in 200 mL flasks (5.5 cm of diameter and 12.0 cm of height) containing 50 mL of Norris' and Date's medium (Norris and Dates 1976) at a rate of one seed per flask. Three days later, plant roots were inoculated with 1 mL of inoculum of the different isolates cultivated in liquid YEM medium, pH 6.8 and A = 0.1 (measurement at $\lambda = 600$ nm) which corresponded to a cell concentration of $10^7 – 10^8$ CFU mL^{-1}. A non-inoculated control treatment was used.

Plants grew under controlled conditions, with photoperiod of 16 daylight hours/8 h of darkness, at a day/night temperature of 26/22º C and relative humidity of 70 %. Four weeks later, the number of nodules in the main root, total nodulation, nodule effectiveness, dry mass of the total nodules (g) and aerial dry mass of the plants (g) were determined. Nodule effectiveness was visually established. Through this, the presence or absence of reddish coloration inside each nodule, characteristic of the leghemoglobin protein (Rodríguez and López 2009) was observed. Dissection of each one of the nodules was made with the utilization of stainless steel scalpel blades.

Design and statistical analysis. In the PHB production essay three replications for each one of the rhizobia isolates were used. Data obtained were submitted to the normality test (Bartlett's 1937 test) and variance homogeneity (Kormogorov's-Smirnov's test) (Chakravarti *et al*. 1967). A simple classification analysis of variance was also applied. The *in vitro* nodulation essays were made twice. A completely randomized design with ten observations per treatment was utilized. Data from the variables number of total nodules per plant, total effective nodules, nodules in the main root per plant and effective nodules per plant were transformed according to the expression root of X, where X is the value of each one of these variables.

For both essays a simple classification analysis was applied. The mean comparison test of Tukey for $\alpha < 0.05$, for discriminating differences between means (Sigarroa 1985) was used. Data from the variables total nodule dry matter and plant aerial dry mass were represented graphically by the program SigmaPlot (2001).

Results and Discussion

All isolates grew slowly in YEM medium (from five to seven days). Colonies were characterized by being small; lower than 1.0 mm for the isolates 1-2, 2-4, 11-5, 12-4, 12-5, K1, K2 and K14; between 1.0 and 2.0 mm for the isolate 2-5 and of 2.0 mm of diameter for the 1-1. Also they were characterized by their translucent color, occasionally white toward the center and circular with irregular borders, with the exception of the K2 isolate which presented irregular borders. In addition, there was scarce or none mucus production. In all cases it was evidenced the base excretion to the culture medium. There was also a coloration change from green to blue. All the characteristics observed in these bacterial isolates coincide with what described by Wang and Martínez-Romero (2001) for the Bradyrhizobium genus. Therefore, they could be considered as possible members of this genus.

In the literature the promiscuity of tropical kudzu has been stated on establishing the symbiosis with native and efficient rhizobia strains of Rhizobium and Bradyrhizobium genera. However, it is common its establishment with species of this latter bacterial genus (Silva 2007). On the other hand, Cascajal soils are known by their acidity, with pH values between 4.7 and 4.9 (González *et al*. 2009). Greater tolerance to the acidity has been observed in strains of slow growth and

base producers belonging to the Bradyrhizobium genus, regarding other of rapid growth (Bordeleau and Prévost 1994). This explains that, generally, this rhizobium genus is associated to soils with this restriction, where it can be found to prevail over other groups (Norris 1967).

Also it is recognized that rhizobia populations in the nodules are determined, to a great extent, by the edaphoclimatic conditions of the ecosystems where they are developed. In the literature there are reports on stressing conditions, as soil acidity. These influence on rhizobia populations and favor the type of the most adequate species for the symbiosis, according to their tolerance to this factor (Morón *et al*. 2005).

The carbon is found among the most important macronutrients for the microorganisms, since large amounts are required for forming part of the cell structures and of the molecules making possible the cell metabolism. For chemoheterotroph microorganisms as rhizobia, the sugars are one of the carbon sources by which this element is incorporated in large amount inside the cell. From the monomers of this family of compounds, many bacteria, among them rhizobia, obtain energy for the metabolism, besides synthesizing their own cell structures (Madigan *et al*. 2011). For that, in the media proposed for the culture of these microorganisms, one of the most important components is the carbon source. This is the case of the mannitol in the YEM medium (Vincent 1979) and molasses in the Bradyfat (Nápoles 2003).

In table 1 are shown the results from the utilization of different carbon sources by the rhizobia isolates. The isolates 2-4, 2-5, 11-5, K1, K2 and K14 had the capacity of consuming all the carbon sources evaluated. Counting on bacterial isolates fit for consuming a large variety of carbon sources is a very important advantage for developing inoculants that can vary their carbon source or having in their composition extracts of natural origin substituting totally or partially the synthetic media. In this way biofertilizer production for these legumes is more profitable.

Galactose was the least consumed sugar by rhizobia isolates. However, in the literature there are studies in which 100 % of rhizobia isolates from *Pueraria mirifica* consumed galactose as only carbon source (Pongsilp *et al*. 2010).

The specificity of the enzymes involved in the oxidative metabolism of sugars, above all in the isolates 1-1. 1-2, 12-4 and 12-5, is important to highlight, since these microorganisms used glucose as carbon source and not galactose, in spite of the structural similarity between both monosaccharides.

The polyhydroxyalkanoates (PHAs) are biodegradable polymers, synthesized by numerous bacterial genera in response to nutritional unbalances and to environmental stress (Khanna and Srivastava 2005). The polyhidroxybutyrate (PHB) is the member which best characterizes this compound family and, in many cases, is used as energy source by the microorganisms producing it as in the case of Rhizobium and Bradyrhizobium (Nair *et al*. 1993).

In figure 1 is presented the PHB concentration generated by rhizobia isolates. The 1-2 was that producing the greatest concentration of this compound (0.009 $g.L^{-1}$), while isolate 1-1 had barely 0.001 $g.L^{-1}$. Rhizobia with capacity for producing and accumulating greater concentrations of this biopolymer, and above all to degrade them rapidly, have greater adaptation advantages regarding other microorganisms. This explains why they count on a carbon and energy reserve that promotes the processes of cell division and colonization of the rhizosphere. This reserve will be used for surviving under stressing environmental conditions, as high temperatures, ultraviolet radiation, osmotic shock and the presence of oxidant agents (Kadouri *et al*. 2003).

In previous studies the influence of different abiotic factors on PHB production such as temperature, pH

Table 1. Utilization of different carbon sources by rhizobia isolates

Isolates	Carbon sources					
	Sacarose	Glucose	Rhamnosa	Lactose	Galactose	Mannose
1-1	+	+	+	+	-	+
1-2	+	+	+	+	-	+
2-4	+	+	+	+	+	+
2-5	+	+	+	+	+	+
11-5	+	+	+	+	+	+
12-4	+	+	+	+	-	+
12-5	+	+	+	+	-	+
K1	+	+	+	+	+	+
K2	+	+	+	+	+	+
K14	+	+	+	+	+	+

+ Growth
± Poor growth
- Nil growth

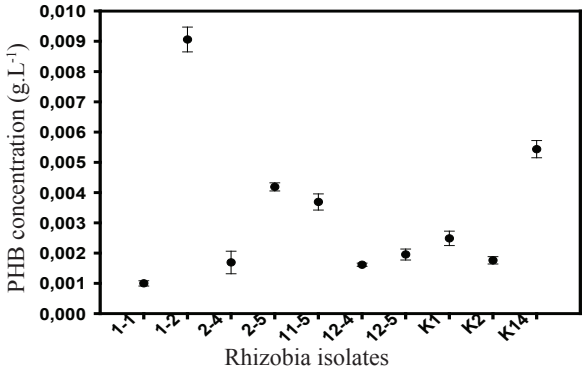

Figure 1. PHB production ($g.L^{-1}$) by rhizobia isolates

and sugars (glucose, sucrose and arabinose) (Sita Lakshmi *et al*. 2012) has been determined. All rhizobia isolates analyzed in this experiment had the capacity for consuming glucose as carbon source (table 1). Therefore, they could be also used for the synthesis and accumulation of this biopolymer in the cytoplasm by these microorganisms.

For increasing PHB production by certain Rhizobium strains multiple studies have been conducted for utilizing fermentable compounds, as in the case of molasses. *Rhizobium meliloti* has been used for the fermentation of sugar beet molasses, with 56 % yield of PHB accumulation (Naranjo 2010). Considering that molasses a by-product from sugar production in Cuba contains, approximately, 36 % sucrose and 10 % glucose (Agüero and Alfonso 1990), which are sugars that these rhizobia isolates consume (table 1); further optimization studies on culture media, similar to those cited in the literature (Gómez *et al*. 2008) could be carried out. However, in this case rhizobia used in this study will be used with the purpose of increasing the potentialities of these microorganisms in PHB production.

In tables 2 and 3 are shown the effect of rhizobia isolates inoculation on the number of total nodules and their effectiveness in tropical kudzu plants. In the treatments where 1-1, 2-4, 2-5 and K2 isolates were employed there was a greater number of total nodules and total effective, regarding the treatments in which 12-4, 12-5 and K14 were used.

In tables 4 and 5 is shown the effect of rhizobia isolates inoculation on the number of nodules in the main root and its effectiveness.

In spite that there were no significant differences between the majority of the treatments, results were similar when the number of total nodules per plant was analyzed, since again the 12-4 and 12-5 isolates created lower amount of nodules. Even so, the same than in the remaining treatments, for these rhizobia isolates the efficiency of the nodulation process on the main root, resulting from the difference in absolute value of created and effective nodules, was 100 %. These results suggest that the isolation process of these microorganisms was successful, since isolates with ineffective capacities, competent for producing effective nodules in biological nitrogen fixation were obtained.

These rhizobia isolates could be used for the preparation of biofertilizers for promoting the growth of this legume, savings in the utilization of chemical

Table 2. Effect of rhizobia isolates inoculation on the number of total nodules of tropical kudzu plants

Isolates	Mean true value	Mean transformed value	Standard error ±	Statistical significance
Non-inoculated control	0.00	0.00	0.00	d
1-1	3.02	1.57	0.29	a
1-2	1.70	1.14	0.21	ab
2-4	3.00	1.60	0.22	a
2-5	3.47	1.54	0.36	a
11-5	2.70	1.43	0.27	ab
12-4	0.09	0.34	0.23	cd
12-5	0.11	0.38	0.20	cd
K1	2.09	1.32	0.34	ab
K2	4.05	1.58	0.28	a
K14	1.01	0.77	0.18	bc

Equal letters do not differ significantly (Tukey $\alpha = 0.05$ and $n = 10$)

Table 3. Effect of inoculation of rhizobia isolates on the number of total effective nodules of tropical kudzu plants

Isolates	Mean true value	Mean transformed value	Standard error ±	Statistical significance
Non-inoculated control	0.00	0.00	0.00	e
1-1	2.36	1.66	0.34	a
1-2	1.37	0.92	0.22	bcd
2-4	2.70	1.43	0.27	ab
2-5	3.19	1.47	0.34	ab
11-5	1.90	1.12	0.27	abc
12-4	0.09	0.34	0.23	de
12-5	0.50	0.38	0.20	de
K1	1.98	1.13	0.27	abc
K2	3.27	1.44	0.26	ab
K14	0.70	0.57	0.00	cde

Equal letters do not differ significantly (Tukey $\alpha = 0.05$ and $n = 10$)

Table 4. Effect of inoculation of rhizobia isolates on the number of main root nodules of tropical kudzu plants

Isolates	Mean true value	Mean transformed value	Standard error ±	Statistical significance
Non-inoculated control	0.00	0.00	0.00	c
1-1	0.90	0.77	0.18	a
1-2	0.60	0.60	0.16	ab
2-4	1.20	0.88	0.22	a
2-5	0.71	0.59	0.20	ab
11-5	1.00	0.88	0.16	a
12-4	0.30	0.24	0.16	bc
12-5	0.20	0.20	0.13	bc
K1	1.10	0.86	0.20	a
K2	1.20	0.90	0.21	a
K14	0.90	0.77	0.18	a

Equal letters do not differ significantly (Tukey $\alpha = 0.05$ and $n = 10$)

Table 5 Effect of inoculation of rhizobia isolates on the number of effective nodules in the main root

Isolates	Mean true value	Mean transformed value	Standard error ±	Statistical significance
Non-inoculated control	0.00	0.00	0.00	c
1-1	0.80	0.81	0.26	a
1-2	0.60	0.60	0.16	ab
2-4	0.81	0.84	0.21	a
2-5	0.61	0.64	0.23	ab
11-5	0.90	0.78	0.18	a
12-4	0.30	0.24	0.16	bc
12-5	0.20	0.20	0.13	bc
K1	1.10	0.86	0.20	a
K2	1.10	0.87	0.20	a
K14	0.70	0.97	0.20	a

Equal letters do not differ significantly (Tukey $\alpha = 0.05$ and $n = 10$)

fertilizers and increasing yields (Olivera *et al* 2010).

In spite that the K2 isolate was one of those producing the highest number of total nodules, its dry matter was lower than in treatments utilizing 2-4, 2-5 and K1 isolates with capacity for creating nodules in the culture similarly (figures 2 and 3). This result is due, seemingly, to the presence of nodules in secondary roots from inoculated plants with the K2 isolate, although abundant and effective for biological nitrogen fixation, they were of small size. This isolate did not show performance significantly higher than 2-4, 2-5 and K1 isolates in the nodulation at the main root or in its effectiveness (tables 4 and 5).

The contribution of inoculation to the nitrogen contents of plants was evidenced when compared to the non-inoculated control (figure 3), there was positive effect on the aerial dry mass of these plants, by inoculating with rhizobia isolates. Similar results were found by Ray and Valsalakumar (2009), on inoculating Rhizobium isolates from tropical kudzu nodules in the legume Mung bean (Green Gram (*Vigna radiata*). Also they were similar to those reported by Abbsi *et al*. (2010) in soybean studies (*Glycine max*). Inoculated plants with 2-4, 2-5 and K1 isolates were the ones with greater aerial dry mass, while those inoculated with 12-4 and 12-5 had lower aerial dry weight.

Plants inoculated with the isolates 2-4 and 2-5 had greater aerial dry mass (figure 3), fitting with those showing greater number of total nodules, total of effective nodules and dry mass of total nodules (tables 2 and 3) (figure 2). Similarly, this correspondence was found for the treatments using 12-4 and 12-5, but in this case with the minimum values of these variables. These results are based on the fact the plants require relatively high nitrogen levels for their growth and development. Precisely, the main source of this element in legumes is the biological nitrogen fixation (BNF) which, in this case, takes place by rhizobia present inside the radical nodules (Vance 1998). Consequently, kudzu plants with greater amount of effective nodules tend to develop greater plant biomass.

Tropical kudzu is a legume with capacity for establishing symbiosis with rhizobia from other legumes (Norris 1967). However, an increase in nitrogen

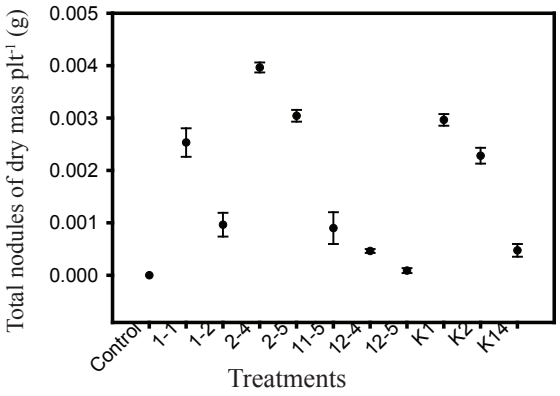

Figure 2. Effect of rhizobia isolates inoculation on the dry mass of total nodules of the tropical kudzu plants. Bars indicate mean confidence intervals for $\alpha = 0.05$ and $n = 10$

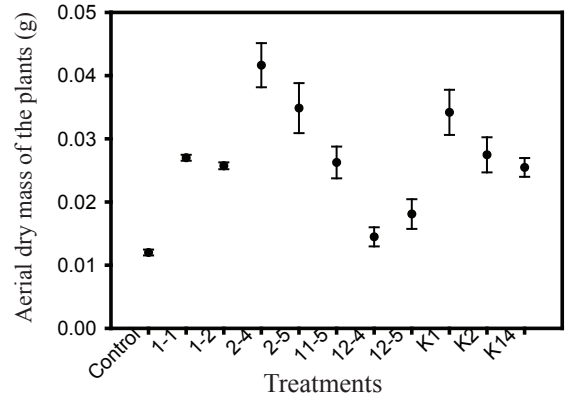

Figure 3. Effect of rhizobia isolates inoculation on the aerial dry mass of tropical kudzu plants. Bars indicate mean confidence intervals for $\alpha = 0.05$ and $n = 10$

concentration is reported when the culture is inoculated with competitive and specific strains for itself (Silva 2007). In this sense, the use of biopreparations from rhizobia from tropical kudzu nodules could increase plant biomass of this culture under field conditions. This will positively influence its use as animal feed and thus, on meat and milk yields of cattle. This increase in the foliar area of the culture as a result of the inoculation of these rhizobia isolates, could promote the kudzu capacity as cover culture in permanent plantations, as well as its abilities for soil protection and improvement, due to its high capacity for fixing atmospheric nitrogen and weed control (Rodríguez 2006).

It is concluded that the ten rhizobia isolates studied could be identified as belonging to the Bradyrhizobium genus. They are of slow growth and base secretors to the culture medium. Four of these bacterial isolates (2-4. 2-5, K1, K2) are those with greater probability for being utilized for the preparation of effective inoculants allowing the improvement of tropical kudzu establishment. These rely on physiological characteristics allowing them not only to tolerate stressing environmental conditions, but also to establish an effective symbiosis with this culture.

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Received: febrary 22, 2013