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RESEARCH ARTICLE

USE OF RHIZOBIUMBACTERIA TO INCREASE THE PRODUCTION OF FIVE TROPICAL LEGUMES WITH FORAGE POTENTIAL

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Abstract

The objective of the work was to evaluate the effect of the application of *Rhizobium* bacteria in the biomass production of forage legumes. *Rhizobium*strains were isolated and selected from the rhizosphere of five forage species. Characterization and subsequent cultivation were carried out to inoculate plants in a greenhouse. Subsequently, the biofertilizer was prepared to apply it to five forage legumes: *C. ternatea, L. leucocephala, C. macrocarpum, M. pruriens and C. cajan*. Plants were germinated in petri dishes and on substrate, after 10 days of germination, they were transplanted into Leonard's Jugs and watered with distilled water. The plants were placed in a completely randomized design with three replicates. The evaluations were carried out every week measuring plant heightand at the end of the experiment root weigh, dry matter of aerial part and radicular volume. The best values obtained were in M. pruriens which showed from 49 to 50 cm of plant height. For cross inoculation in weight variable was observed that the strains from *C. ternatea* and *L. leucocephala* showed the best results with 0.22 and 0.25 g/plant respectively. Although the best data of dry matter of aerial part was observed in *L. leucocephala*with 0.40 g, better response of Radicular volume and plant height was observed in strains that came from C. ternatea with 2 mL and 7 cm respectively. In this study it can be conclude that the use of biofertilizers can be an alternative for low-cost forage production, as long as it contains *Rhizobium* strains capable of associating with legumes and fixing atmospheric nitrogen.

Introduction:-

One of the problems that limits growing of livestock is the lack of quality and good production forage species (Ford et al., 2019). furthermore, in order to improve the dry matter production of forage species is necessary to use high quantity of inorganic nutrients (Martin, 2018). Particularly in tropical regions, the nitrogen in soils is not available for plants (Figueroa et al., 2020) and the costs to obtain this nutrient is higher(Khadda, 2021; Obando et al., 2010). One of the solutions to this problem is the use of legumes forage species which are nutritive source for ruminants(Castro-Montoya and Dickhoefer, 2020; Gaviria-Uribe et al., 2020), moreover, it has the potential to

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reduce the CO_2 emitted during the manufacturing of chemical nitrogenous fertilizers through their biological nitrogen fixation (BNF) capacity (Romanyà and Casals, 2020; Kumar et al., 2018).

An alternative to reduce the use of inorganic fertilizers in forage production is the application of biofertilizers (Itelima, 2018). The use of this products offers an economic and ecologic possibility to farmers(Bhowmik and Das, 2018). The benefic microorganisms usually interact with plant in the rhizosphere (Mishra et al., 2017). In this area occurs a wide biologic and chemical processes that provide maintenance, operation and stability of agricultural production systems (Aguirre, 2006). furthermore improve the uptake of nutrientsand water for the plant as well as nitrogen fixation (Hu et al., 2021), transport and solubilization of phosphorus (Stamenković et al., 2018), production of growing regulatory substances in roots, control of pathogenic microorganisms etc. (Aguirre, 2006).

Biofertilizers in forage species could represent an alternativefor improving the productivity or maintaining similar production when inorganic fertilizers are used (Feyissa et al., 2018). The use of biofertilizers is a technology of great importance in sustainable systems because of the creation of microhabitats in rhizosphere that is benefic to plants (Patel et al., 2021). On the other hand, the use of biofertilizers can contribute to reducethe inorganic fertilizers (Divan et al., 2008;Mayz-Figueroa, 2004;FAO y GTIS, 2015).

One of the biofertilizer that is used in nitrogen fixation is the *Rhizobium* bacteria which its use in legumes forage species could improve the productivity of the plant (Etesami, H. and Adl, 2020). These bacteria infect the Legume plants and create nodules when they fix the no atmospheric nitrogen.(Allitoet al., 2021; Mabrouk et al., 2018; Reed et al., 2011).In this case a symbiotic process occurs, the plant provides the nutrients necessary to the bacteria and bacteria provide the nitrogen necessary to the plant (Liu et al., 2018). It is worth mentioning that quantity of atmospheric nitrogen fixed depends on the bacteria and Leguminosae symbiosis species(Mabrouk j et al., 2018).Instead of the capacity of this symbiosis, some bacteria strains can behave as parasites or have an ineffective capacity to create fixation nitrogen nodules (Benezech et al., 2020). Essays preselection of 36 strains of *Rhizobium*were isolated from 15 legumes species of peanut (Arachis hypogea) in order to select the nitrogen fixation efficient. In this study only detected 13 strains that created nodules and 8 fixed atmospheric nitrogen which were similar to the control (Giardini and López 1978). On the other hand, Dobereiner (1971) observed that tropical forage legumes can create nodules with *Rhizobium* strains which were isolated from different species, however the capacity of nitrogen fixation was different. This different was because some bacteria acted like parasites and other as fixing bacteria. Moreover, this author observed that rhizobia strain that nodule and fixing certain quantity of nitrogen with a specific legume specie can be effective when is with association with other species.Due to the afore mentioned, the need arises to perform the isolation, selection and evaluation of *Rhizobium* strains and fungi of the genus *Glomus* in the laboratory and greenhouse in order to efficiently use the symbiosis between the legume plant and soil microorganisms.

Methodology:-

Study area, soil and climate conditions

The present study was carried out in latoratory of Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), campo experimental Chetumal and plot of co-worker farmer from Xul-Ha that is located in Othon P. Blanco, Quintana Roo at 3.5 km of Xul-Ha community with 21°30' N and 89°29' W coordinates at 10 masl. Climate conditions are 27.6° C and 62.3% of relative humidity on average, annual medium precipitation is 1300 mm and the period with the most precipitation was from June to November with 70% of precipitation.The soils of the study area are the so-called chromic luvisols, characterized by having a good content of organic matter.

Obtention, isolation and characterization of microorganisms

It was selected five better plants of the next species: *Clitoria ternatea, Mucura pruriens, Leucaena leucocephala, Sesbania sesbans, Pueraia phaseoloides, Centresema pubescens, Centrosema sp, Cajanus cajan y Macroptyllium atropurpureum.*Then, it was used the Hoffer barrier to obtain five samples per plant from rhizosphere. In total it was obtained 25 samples per species which were mixed to homogenize and then taken 500 g per species. The samples were collected in plastic bags to get in the laboratory.For isolation it was used the dilution technique. Ten grams of soil was diluted in 90 milliliters of distillate water sterilized to obtain the first dilution of 1×10^{-2}, then it was carried out more dilution to get on 1×10^{-6}.To culture the microorganism, it was taken 0.1 mL from 1×10^{-4}, $1x\ 10^{-5}$ y 1×10^{-6} and then was put and raked inside of petri dish with carbon medium culture. Microorganisms were cultured at room temperature for 72 hours. Then, they were subcultured to purified. Once the microorganisms were purified, it was carried out the characterization both macro and microscopically by Gram stain and biochemical tests.

Isolation and Culture ofRhizobiumgenerous bacteria

Plants from field with complete radicular system were extracted to obtain the radicular system with nodules. Then, plants were covered with paper moistened with distillate water and posteriorly carried to laboratory. In laboratory roots were washed with water from tube to eliminate the excess soil and expose the nodules. Then, number of nodules, coloration and location in primary or secondary roots were recorded. Finlay, from roots were selected big nodules with red coloration and easy detachment. Posteriorly, the nodules were disinfected with sodium hypochlorite at 4% and rinse three times with sterilized distillated water in laminar flow cabinet. Then, nodules were macerated in petri dishes with yeast-mannitol-agar and Congo red culture medium and cultured at 28°C for 9 days. Bacteria with Rhizobium characteristics were isolated and then characterized by Gram stain and biochemical tests. The purification of bacteria was carried out by colony replicate of Rhizobium (CIAT, 1988) in petri dishes. bacteria culture was incubated in essay tubes with Yeast Mannitol Agar(YMA)in inclined plane at 4°C.The five strains selected were mixed to be used in greenhouse tests.

Preparation of biofertilizer

Microorganisms cultured in specific mediums for growing were incorporated in an Erlenmeyer glass with liquid medium and mixed in a Eberbach reciprocal shaker for 7 days.Posteriorly, it was carried out a count of microorganisms in a milliliter of suspension by Visible-UV and using the McFarland Scale (Campbell et al., 1970).Then, it was taken 20 mL of suspension to inoculate in plastic bags with 30 g of sterile manure. These inoculated microorganisms were incubated in room temperature for 7 days. For control quality was used the dilution technique (CIAT, 1988) per each biofertilizer.This technique uses the Gram test in microorganisms which are cultured in LMA medium with Congo Red indicator (10mL/L) and Glucose-peptone-agar (GPA) with bromocresol purple (10mL/L). These mediums are cultured at 26°C for 14 days. To obtain the quality of this microorganism, the colonies count and nodules formation in plants are the variables that are evaluated.

Evaluation ofbiofertilizer in greenhouse conditions

Outstanding foragers from the adaptation and evaluation trials of forage species from the Chetumal Experimental Field were germinated in petri dishes and on substrate. After 10 days of germination, they were transplanted into Leonard's Jugs and watered with distilled water. The inoculation was carried out with the strains previously isolated and multiplied in the laboratory. Additionally, a cross inoculation test was carried out which consisted of preparing test tubes with yeast culture media, mannitol and agar, to cultivate the Macroptilium atropurpureum plant. The inoculated plants were grown in a greenhouse and fertilized with nitrogen-free nutrient solution media for irrigation.

Experimental design

The treatments used were: uninoculated soil, inoculation of isolated strains and inorganically fertilized soil. The plants were placed in a completely randomized design with three replicates. The evaluations were carried out every week measuring plant height, leaf-stem ratio, and at the end of the evaluation the performance of aerial dry matter and roots, as well as the nutritional content. An analysis of variance and Tuckey's test were performed for the difference of means.

Results and Discussion:-

Isolation and microorganism selection

The results in obtaining healthy plant nodules in the field for the isolation of nitrogen-fixing bacteria are shown in Table 1.

Table 1:- Number of nodules observed in the main and secondary roots, and the coloration shown by the nodules.

Especies	Number of total nodules	Total nodules in principal roots	Total nodules in secondary roots	Color
C. ternatea	8^{b}	4	9	Brown
L. leucocephala	12^{a}	7	5	Red
C. macrocarpum	16^{a}	9	7	Red
M. pruriens	07^{b}	2	5	Green
C. cajan	06^{b}	3	3	Green

Similar letters correspond to equal treatments accord Tukey test ($P \leq 0.05$).

The best number of nodules was observed in *L. leucocephala* and *C. macrocarpum*, this response could be to the capacity to generate nodules with many bacteria in soil (Velázquez et al., 2017).Cubillos-Hinojosa et al. (2021) mention that Leucaena that belongs to specific legumes requires certain strains to nodulate.On the other hand, the other species that had less nodulation could be due either to physical, chemical and biological effects that directly affect the Rhizobium population (Vidor, 1979; Valles et al 2003) or to the lack of symbiosis with acertain legume (Acosta-Jurado et al., 2021).With respect to L. leucocephala and C. macrocarpum, a greater number of nodules were observed in the main root of red color, which indicates a greater efficiency in nitrogen fixation due to the presence of the enzyme leghemoglobin responsible for fixation and that givesreddish coloration to the nodules.This agrees with that described by Velázquez et al., 2017. Furthermore, Cubillos-Hinojosaet al.(2019) mention that effective nodules are generally large and are grouped in the main root and in the upper secondary roots, in addition, they have a red color.

Resultsof experiment in greenhouse

For the root weight variable, it was found that the inorganically fertilized plants presented 0.21 g/plant and 0.14 g/plant for unfertilized treatment (P≤0.05), however, statiscticly it was equal to that obtained with the *C. ternatea*; similar result was observed in *C. macrocarpum*. The higher root weight value was obtained in L. leucocephala and C. cajana in respect with inorganically fertilized plants (P≤0.05). accord of our results M. pruriens (P≤0.05) showed the best root weight (1.20g/plant) as can be appreciate in Table 2.

Regarding the dry weight variable of the aerial part, the inorganically fertilized and unfertilized plants had values of 16.41 and 16.36 g respectively.The values of the *C. ternatea, L. leucocephala, C. macrocarpumand C. cajan*awere slightly lower (P≤0.05). on the other hand, the best dry weight was obtained with M. pruriens(P≤0.05 which showed 20.11 g of dry matter.This tendency was observed in *M. pruriens*plant height that showed the best value with 37.15 cm of plant height (Table 2).

In general, it is observed that biofertilizers had a positive effect in the plants inoculated.Plants evaluated showed better response that those with fertilized inorganically treatment. Despite the fact that some treatments with organic fertilizer were equal to those with biofertilizers, it is advisable to take strains with effective symbiosis since they were those that showed the best response in plant growth(Date, 1977)

Table 2:- Effect of biofertilizer application in legumes forage species development.

Species	Root weigh (g/plant)	Dry matter of aerial part (g)	Radicular volume (mL)	Plant height(cm)
C. ternatea	0.20^{c}	15.60^{c}	1.95^{c}	23.6^{b}
L. leucocephala	0.24^{b}	15.76^{c}	1.00^{c}	9.95^{e}
C. macrocarpum	0.19^{c}	15.78^{c}	0.50^{c}	11.0^{d}
M. pruriens	1.20^{a}	20.11^{a}	13.5^{a}	37.15^{a}
C. cajan	0.25^{b}	15.84^{c}	0.65^{c}	15.4^{c}
Fertilized	0.21^{c}	16.41^{b}	6.60^{b}	11.62^{d}
Unfertilized	0.14^{d}	16.36^{b}	5.40^{b}	11.27^{d}

Similar letters correspond to equal treatments accord Tukey test (P≤0.05).

Table 3 shows the results of the agronomic evaluation of species nodulation. For the total number of nodules, it was observed that *L. leucocephalaand M. pruriens*20 and 22 nodules in comparison with the other species. however, the coloration of these legumes was different (green and red respectively). Regarding *L. leucocephala*, it is the one that had the best results in nodulation, accordof Nicholas (1963), the presence of the host is important for bacterial multiplication. Furthermore, to be considered as forage species, the inoculation in Leucaena is necessary. On the other hand, López et al. (1983) showed thatnatural nodulation with different legumesnodulated successfully except in Leucaena, thus suggesting specificity regarding the Rhizobium strain. Although it is difficult to predict which strains will be effective, since the same plant can have effective and ineffective nodules (Mytton, 1984), it has been found that, in the selection of strains for tropical forage legume species, in almost all cases, at least one out of every 40 strains is effective (CIAT, 1986).

Table 3:- Nodules number in agronomic evaluation of strains inoculation.

Species	Number of total	N umber of toral	Number of total	Color

	nodules	nodules in principal roots	nodules in secondary roots	
C. ternatea	10[c]	04	6	Brown
L. leucocephala	28[a]	20	8	Green
C. macrocarpum	05[c]	03	2	Brown
M. pruriens	22[b]	15	7	Red
C. cajan	08[c]	05	3	Green

Similar letters correspond to equal treatments accord Tukey test (P≤0.05).

The results of cross inoculation in *Macroptilium atropurpureum* are presented in table 4 and 5. Data corroborate that the isolated and purified strains were *Rhizobium* due to the response obtained in experiment.

For the root weight variable (g/plant) it was observed that the strains from *C. ternatea*and L. leucocephalashowed0.22 and 0.25 g/plant respectively, these being much higher than treatments with inorganic fertilizer and strains that came from *M. pruriens*which showed values of0.13 and 0.16 g/plant respectively (P ≤0.05).

In the dry weight variable of the aerial part, the inorganically fertilized and unfertilized siratro plants had quite a distance in their values, since the inorganic fertilized (0.25 g) compared to the unfertilized (0.02g). The siratro plants inoculated with the *C. ternatea* and *C. cajan* strains had completely equal values between them, and also equal to those of the inorganically fertilized plants (Table 4). The highest values were observed in *L. leucocephala* and *M. pruriens*with 0.40 and 0.33g respectively, which indicates that they were the ones that showed the best performance in terms of the effectiveness in dry weight of the aerial part. The plants inoculated with strainsobtained from C. macrocarpumbehaved completely different that strains which came from L. leucocephala and M. pruriens, since they had values below the other treatments of selected strains, even below the values of the inorganically fertilized plants but slightly higher than unfertilized plants. This is important since we must not forget that to determine the effectiveness of the symbiosis it is necessary to know the nitrogen content in the plant tissue, that is why the dry matter yield was obtained, which can give an indication of the nitrogen content, although the relationship between the two parameters is not necessarily linear (Haydock et al., 1980).

The root volume variable showed that the inorganically fertilized and unfertilized siratro plants had equal values between them, and also slightly equal to the siratro plants inoculated with the *L. leucocephala, M. pruriensand C. cajans*trains. Those that had the lowest values compared to all the other treatments were those inoculated with strains from *C. macrcarpum* (P≤0.05).

Regarding height variable, the inorganically fertilized siratro plants had 6.0 cm differently that was observed in unfertilized with 2.5 cm of height (P≤0.05). the best height obtained was observed in plants which were inoculate with strains from C. ternatea with 7.5 cm of height.

Table 4:-.Effect of the application of biofertilizer (bacteria isolated from different legumes) on the development and effectiveness of siratro plants (Macroptyllium atropurpureum).

Species from which the strain was extracted	Root weigh (g/plant)	Dry matter of aerial part (g)	Radicular volume (mL)	Plant height(cm)
C. ternatea	0.22[a]	0.26[b]	2.0[a]	7.5[a]
L. leucocephala	0.25[a]	0.40[a]	1.33[b]	3.0[d]
C. macrocarpum	0.05[c]	0.04[c]	0.83[c]	3.8[d]
M. pruriens	0.16[b]	0.33[a]	1.63[b]	6.2[b]
C. cajan	0.02[c]	0.26[b]	1.63[b]	5.0[c]
Fertilized	0.13[b]	0.25[b]	1.33[b]	6.0[b]
Unfertilized	0.05[c]	0.02[c]	1.43[b]	2.5[d]

Similar letters correspond to equal treatments accord Tukey test (P≤0.05).

For the infectivity results, it was observed that the *L. leucocephala*and *M. pruriens*strainscaused a greater number of nodules in the siratro plants compared to the remaining strains. It can be seen that all the selected strains of the different plants under study managed to nodulate with the siratro plants. This confirms what is described by Dobereiner et al., (1967) and Halliday (1984) on the diversification of the symbiosis with the relation to the origin of

the strain and the plant under study. Also confirming what was described by Dobereiner (1971) who observed that tropical forage legumes have the ability to form nodules with *Rhizobium* strains isolated from other species. The only strains that managed to form nodules with red coloration were those of *C. ternatea* and *L. leucocephala*this could bebecause of these strains are more effective in siratro plants than the others, that although they manage to form nodules, since siratro is a promiscuous plant, they do not manage to form effective nodules in necessary quantities.

Table 5:- Infectivity in Siratro (Macroptyllium atropurpureum) by Rhizobium.

Species	Number of total nodules	Number of total nodules in principal roots	Number of total nodules in secondary roots	Color
C. ternatea	15[b]	10	5	Red
L. leucocephala	23[a]	19	4	Red
C. macrocarpum	10[b]	05	5	Brown
M. pruriens	27[a]	20	7	Brown
C. cajan	12[b]	03	9	Green

Similar letters correspond to equal treatments accord Tukey test ($P \leq 0.05$).

Conclusions:-

The use of biofertilizers can be an alternative for low-cost forage production, as long as it contains *Rhizobium* strains capable of associating with legumes and fixing atmospheric nitrogen. On the other hand,it was observed that forage legumes can present certain specificity towards certain strains of rhizobia to be able to nodulate efficiently for nitrogen fixation.

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