THE EFFECT OF INOCULATION OF AN INDIGENOUS BACTERIA ON THE EARLY GROWTH OF *Acacia farnesiana* IN A DEGRADED AREA

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ABSTRACT: Restoration of native vegetation and fuelwood production are important environmental pending goals for Mexico, where years of wrong management practices resulted in ecosystemic degradation and fuelwood scarcity. In degraded areas, native rhizobial strains are often undetectable, therefore, the restoration of natural vegetation associated with an effective nodulation of the leguminous trees is mostly appropriate. *Sinorhizobium americanum* is a native nitrogen-fixing bacteria isolated from nodules of the native *Acacia* species in the region. *Acacia farnesiana* is a multipurpose leguminous shrub from Mexican seasonally dry tropical forests (SDTF). In this study we analyzed the effect of inoculation with *S. americanum* on *A. farnesiana* growth in a greenhouse and in a very degraded area and compared with non-inoculated seedlings. In a greenhouse, we measured the biomass dry weight of different parts of the plant, using destructive sampling after 15, 20, 30, 45 and 120 days of growth. We also calculated the relative growth rate (RGR) and the resources allocation (root/shoot weight ratio and root length/root dry weight) of seedlings. In a degraded area we measured the seedling length and survival and calculated the RGR. In the greenhouse and in the degraded area, the inoculation positively affected the growth of seedlings. However in the greenhouse, the inoculation did not have effect on resource allocation patterns. Therefore, the inoculation with *Sinorhizobium americanum* could improve the *A. farnesiana* growth and the re-establishment of important plant-soil interactions in degraded areas, being a recommendable technique for land restoration and the improvement of fuelwood production.

Key words: Restoration, fuelwood, *Sinorhizobium americanum*, seasonally dry tropical forests, México.

O EFEITO DA INOCULAÇÃO DE UMA BACTÉRIA NATIVA NO CRESCIMENTO INICIAL DE *Acacia farnesiana* EM UMA ÁREA DEGRADADA

***RESUMO:** Restauração da vegetação nativa e produção de lenha são importantes metas ambientais pendentes para o México, onde anos de práticas de manejo equivocadas provocaram degradação ecossistêmica e escassez de lenha. Nessas áreas degradadas, estirpes de rizóbio nativas são muitas vezes indetectáveis e, portanto, a restauração da vegetação natural, associada com uma nodulação efetiva das árvores leguminosas é mais adequada. Sinorhizobium americanum é uma bactéria fixadora de nitrogênio isolada de nódulos de espécies nativas de Acácias na região do experimento de campo. Acacia farnesiana é um arbusto leguminoso com múltiplos usos, nativo das florestas tropicais estacionalmente secas do México. Neste estudo, analisamos o efeito da inoculação com S. americanum sobre o crescimento de A. farnesiana em estufa em uma área muito degradada e comparamos com mudas sem inoculação. Na estufa, medimos o peso da biomassa seca das diferentes partes da planta, utilizando amostragem destrutiva após 15, 20, 30, 45 e 120 dias de crescimento. Também calculamos a taxa de crescimento relativo (TCR) e a alocação de recursos (peso seco da raiz / peso seco da parte aérea e comprimento de raiz / peso seco de raiz). Em uma área degradada, medimos o comprimento e a sobrevivência das mudas e calculamos a TCR. Tanto na estufa quanto na área degradada a inoculação aumentou o crescimento das mudas. No entanto, na estufa, a inoculação não teve nenhum efeito sobre os padrões de alocação de recursos. Portanto, a inoculação com Sinorhizobium americanum poderia melhorar o crescimento de A. farnesiana e o re-estabelecimento de interações importantes solo-planta em áreas degradadas, sendo uma técnica recomendável para a restauração e para aumentar a produção de lenha.*

Palavras-chave: Restauração, lenha, Sinorhizobium americanum, florestas tropicais estacionalmente secas, México.

1 INTRODUCTION

Tropical regions worldwide suffer increasing pressure due to anthropogenic disturbances of their natural ecosystems. Mexico presented the third highest average annual deforestation by area of primary forests, 2000-2005 (395,000 ha), among tropical countries (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2005). In a more local scale, in the Tembembe river basin, in the Morelos state, the degradation of the seasonally dry tropical forest environment and mainly of its soils is evident, reaching 80% of erosion rates (GOMEZ-GARZÓN, 2002). In the area of study, Galindo-Escamilla (2006) also found a high

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soil bulk density, flooding events and a low microbiological activity. At this location, primary vegetation has been substituted or diminished because it is the only source of firewood, foliage and fruit for the local inhabitants (DORADO, 1983). Along with the forest degradation, damage to soil microbiota is widespread. Native rhizobium are often undetectable in eroded soils where native legumes have been cleared (THRALL et al., 2001) and so can become a handicap for plant establishment since microorganisms represent the major component of soil biomass and their activities have a key roll in nutrient cycling, affecting nutrients availability (COLEMAN et al., 1983). Therefore, the restoration of native vegetation and an effective nodulation of the leguminous trees could have a productive and ecological advantage.

In Morelos state, *Acacia* species provide shade, forage for animals, firewood, charcoal, and gums; and have great potential for increasing soil fertility and enhancing soil structure because of its symbiotic association with nitrogen-fixing bacteria (COMISION NACIONAL PARA EL CONOCIMIENTO Y USO DE LA BIODIVERSIDAD - CONABIO, 2009). Also, this genus represents 6-7 % of total known legume species (2,000 species) in the world. In Mesoamerica, there are 159 reported native species of which 46 out of 85 found in Mexico are endemic (RICO-ARCE, 2007). *Acacia farnesiana* (L.) Willd. (1806) was reported by Sprent (2001) as a neotropical nodulant species naturalized in the tropics region and the Mediterranean zone. Common in all warm zones of Mexico, including a great variety of climates and ecosystems (altitudinal range of 0-2600 m a.s.i., annual precipitations from 100 to 900 mm and temperatures from 5 to 30 °C). This species is a multipurpose shrub and the main source of fuelwood in the region of study where 88% of rural peasants use *A farnesiana* for fuelwood (VÁZQUEZ-PERALES, 2005) because its high caloric value (4.6 kcal.g^{-1}) (WEBB et al., 1980). This species is also able to adapt to a great variety of soils and is extremely tolerant to drought in regions with dry seasons of 4-6 months (HUANTE et al., 1992).

In the last decades, soil recovery projects have been initiated in Australia and Western Africa to analyze the symbiotic association of *Acacia* species and their specific rhizobial strains in order to develop inocula to use in forest plantations (DART et al., 2001; GALIANA et al., 1994). In Mexico and Latin America, studies with simbionts from *Acacia* species used in restoration projects are scarce (FOROUGHBAKHCH et al., 1987; FRIONI et al., 1998; ROSKOSKI et al., 1986).

On the other hand, among *Acacia* species there is a considerable specificity: rhizobial strains that were effective on one *Acacia* species, sometimes performed poorly on others (THRALL et al., 2001). Also, in some cases, introduced rhizobial strains did not survive because of competition with indigenous species, particularly under adverse soil conditions (ASAD et al., 1991; ELSAS; HEIJNEN, 1990). However, native populations are inefficient in nitrogen fixation and highly competitive in nodule formation, so it is necessary to perform the right selection of strains for inoculation, taking in account local environmental conditions, so that they do not take away surviving and competing opportunities against resident population (TURK et al., 1993). Therefore, to reach practical objectives there is the need to identify effective rhizobial strains that suite the host species to be used in restoration strategies. *Sinorhizobium americanum* has recently been isolated from nodules of native *Acacia* species in Sierra de Huautla which is located in southern Morelos state and where at least eight different *Acacia* species form part of the landscape (TOLEDO et al., 2003). Little is known about the development of *Acacia farnesiana* in a greenhouse and in degraded areas when inoculated with *Sinorhizobium americanum*.

The aim of this work was to evaluate *Acacia farnesiana*´s seedlings response (in terms of seedling growth and resources allocation) to direct inoculation of the nodulating rhizobia (*Sinorhizobium americanum*) under greenhouse conditions and the establishment and survival of these seedlings in experimental plots in a environmentally-degraded pasture. The knowledge obtained will contribute to the correct management of this species for future forest restoration and fuelwood production programs in STDF ecosystems.

2 MATERIAL AND METHODS

2.1 Study area

The field studies were conducted in Rio Tembembe Ecological Restoration Station (RTERS), in the Tembembe river basin cliff, in the northwestern region of Morelos state in Mexico (18° 54' 34" N and 99° 20' 23" W). This site has a monthly temperature average of 18-20°C (max. 30.1°C and min. 12.8°C), annual precipitation of 1000 and 1500 mm with a well-defined dry season from October to May. The predominant soil is feozem haplic with medium texture, slightly acid (pH 6.6) (GÓMEZ-GARZÓN, 2002). The predominant vegetation is the seasonally dry tropical forest (MIRANDA; HERNÁNDEZ, 1963).

This study zone has a previous record of deforestation to yield plots used for intensive cultivation. Currently, this area is occupied by very degraded induced grasslands. Because of an intensive grazing, periodical fires and absence of propagule sources; it is possible that the ecological succession process has been arrested (PUTZ; CALAHAN, 1992). According with previous studies made by Galindo-Escamilla (2006) in the study area, the average number of arbuscular mycorrhizal spores and the soil dehydrogenase activity (as a measure of microbial activity) along the year were low, even when compared with other areas of the RTERS (12.8 spores. g^{-1} and 171 µg INT g^{-1} h^{-1} respectively).

2.1.1 Seed collection and germination

Seed of *A. farnesiana* were collected in the protected natural forest of the Xochicalco Archaeological Zone (3 km from RTERS). In order to promote a synchronized germination the method of Toledo et al. (2003) was used.

2.2 Greenhouse analysis

2.2.1 Growth of seedlings in greenhouse

A total of 50 weighed and germinated seeds 2-3 days of age were put into plastic bags with about 500 g of non-sterile soil and, as in the previous test, 25 of them were inoculated with a native bacterial culture (CFNEI 156 strain) (TOLEDO et al., 2003) with 10^9 cfu ml^{-1} at the base of the seedlings (DIOUF et al., 2003) and 25 were not (control). Five seedlings were randomly harvested (destructive samples) from each group after 15, 20, 30, 45 and 120 days of growth (CERVANTES et al., 1998), seedlings were separated into roots, shoots and leaves and the length of roots and shoots were measured to the nearest mm and the number of nodules was counted. Each seedling part was also oven dried at 80°C for 48 h and weighed.

2.3 Data analysis

2.3.1 Evaluated variables

a) Total Biomass Production at each harvest time (TBP): The variation in the absolute dry weight of all parts of the plant.

b) Relative growth rate in biomass at each harvest time (RGR): The variation of RGR using model of Hunt and Parson (1974).

c) Resources allocation at each harvest time
- Biomass allocation (leaf, root and shoot weight)
- Root/shoot weight ratio (R/S)
- Root length/dry root weight ratio (RL/RDW).

2.3.2 Statistical analysis

For the parametric statistical analysis, data normalization was done when necessary. To evaluate the effect of inoculation on the total dry weight, dry root, shoot and leaf dry weights and root length, a *t* test was used. To evaluate the dry leaf weight and shoot length, the Kolmogorov-Smirnov test was used because it was not possible to transform the data, as it did not present a normal frequency distribution. To evaluate the effect of inoculation and the time of seedling harvest on RGR, root/shoot weight ratio and root length/root dry weight, an ANOVA factorial analysis (2x5) was used. To compare the distribution of root, shoot and leaf dry weight in each treatment (inoculated and uninoculated), as well as to compare the dry weight rate for each structure (root, shoot and leaves) separately between treatments (inoculated and uninoculated), a goodness-of-fit model (χ^2) was used. All data were analyzed with the STATISTICA (6.0) program.

2.4 Field analysis

To estimate the effect of *S. americanum* inoculation on the initial development (eight months) of *Acacia farnesiana* in a degraded pasture, 120 seedlings cultivated in plastic bags with non-sterile soil from the same location, were introduced into this degraded area when they were 30 days of age with 13 cm average height. Half were inoculated (28 days prior) with *S. americanum* (10^9 cfu ml^{-1}) and half were not inoculated and used as a control with natural symbiosis. The experimental design consists of eight plots of 24 x 24m ($124m^2$) each, randomly placed. In four plots, inoculated seedlings were introduced (15 seedlings in each one) and in another four plots, 15 uninoculated seedlings were planted per plot (60 seedlings per treatment), with a distance between seedlings of 3 x 2m for both treatments. Survival and length of seedlings was measured monthly. The relative growing rate (RGR) was calculated by fitting length measures to a model of Hunt and Parsons (1974). The seedlings were moderately irrigated with around 500ml/ water/plant/week during the dry and hot season to diminish the effect of low soil moisture on the N_2 fixing capacity of *S. americanum* (THRALL et al., 2001).

2.4.1 Statistical analysis

The effect of inoculation on the length of seedlings was evaluated by a t- Student test for independent samples in each data collecting date. The effect of inoculation on relative growing rate (RGR) was evaluated by a non parametric Kolmogorow-Smirnov test. Also, to compare

the effect of time on the RGR, a non parametric Kruskal-Wallis test (was done to substitute the ANOVA). Both non-parametric tests were used because it was not possible to transform the data in order to meet the assumptions of normality.

3 RESULTS AND DISCUSSION

3.1 Greenhouse experiment

3.1.1 Biomass production

Comparing the total dry weight between inoculated and non-inoculated seedlings in non-sterile soil, the inoculation effect was significantly positive in plants harvested at 45 days (t= -2.47453, df = 8, p<0.05) and 120 days (t = -2.69100, df = 8, p<0.05; Figure 1) which coincide with the most nodule appearance in both seedling treatments (in average 8 nodules per plant in inoculated and 4 in the uninoculated seedlings). Luyindula and Karabaranga (1986) in Zaire, also found a positive effect of inoculation with *Rhizobium* on the average dry weight of *Leucaena leucocephala* (675 mg for inoculated and 275 mg for control) when seedlings were 55 days old.
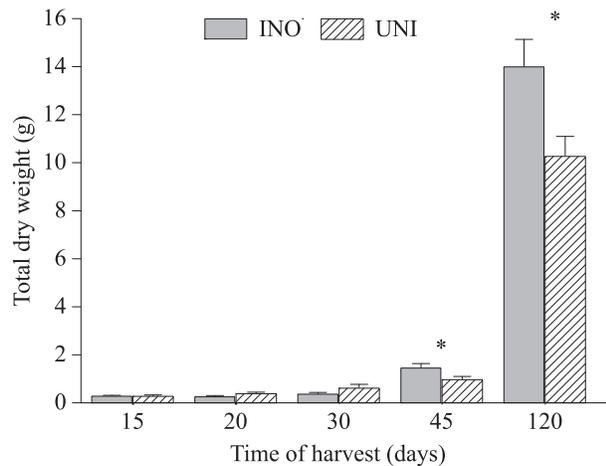
Vertical lines are standard errors. Means are significantly different between treatments when followed by an asterisk (*; P<0.05).

Figure 1 – Effect of *S. americanum* inoculation (INO) on the total dry weight of *A. farnesiana* seedlings compared with the uninoculated control (UNI), as a function of harvest time in a greenhouse experiment.

Figura 1 – Efeito da inoculação de S. americanum (INO) sobre o peso seco total das mudas de A. farnesiana em comparação com o controle não inoculado (UNI), em função da época de coleta em um experimento em casa de vegetação.

However, regarding seedlings structure, the main effect of inoculation was observed on dry weight and length of the shoots, which, in turn, influenced the total dry weight.

In this study, there was also a significant effect of inoculation on shoot (t = -2.54101, df = 8, p<0.05) and root dry weights (t = -2.54101, df = 8, p<0.05) at the last harvest (120 days). But there was no significant effect of inoculation on leaf dry weight. Ojo and Fagada (2002) and Sah et al. (1998) found similar results in the shoot dry weight with inoculated *Leucaena leucocephala* in Niger 48 days after germination and with *Dalbergia sissoo* in Nepal 180 days after germination, respectively. Aryal et al. (1999) also found similar results when studying the effects of inoculation with *Rhizobium* on the length of introduced seedlings in non-sterilized soil; the increase in length over controls was 52.1% for *Albizia procera*, 68.6% for *Albizia lebbeck*, and 95.8% for *Leucaena leucocephala*. It is important to note that in common practice, the length of aerial part is taken as the criteria in greenhouses, to decide when seedlings can be carried to forestation sites. Therefore, any acceleration to plant size reduces its stay in greenhouse and consequently, its production cost as well.

The number of nodules per seedling at last time of harvested was not different between inoculated and uninoculated seedlings (10 and 7 respectively). It is very well know that symbiotic relationship between shrub legumes and rhizobia is rather promiscuous and frequently unspecific (FRIONI et al., 1998; PUEPPKE; BROUGHTON, 1999; THRALL et al., 2001). On the other hand, in cross nodulation in greenhouse experiments, among different species and rhizobia strains (Center for Genomic Sciences, UNAM collection), a positive nodulation of *A. farnesiana* inoculated with *Rhizobium etli* (CFN42[T]), a *Phaseolus vulgaris* nodulant (very abundant in Mesoamerican soils) (SEGOVIA et al., 1993) was found (MARTÍNEZ-ROMERO et al., 1991). At the same time, *S. americanum* is able to nodulate under laboratory conditions, the very same hosts as *R. etli* and *R. tropici* (TOLEDO et al., 2003) and it also nodulates other *Acacia* species (TOLEDO, 2003). However, in this study we found that inoculation had a positive effect on *A. farnesiana* seedlings development in spite of the low difference in number of nodules between inoculated and uninoculated seedlings in a non-sterile soil. Possibly the main reason is that native populations are inefficient in nitrogen fixation and highly competitive in nodule formation (TURK et al., 1993) and a higher efficiency of *S. americanum* inoculated in the *A. farnesiana* seedlings.

3.1.2 Relative growth rate

In average, there was no significant effect of inoculation on the RGR (inoculated, 0.049656 g. $g^{-1}.day^{-1}$; non-inoculated, 0.048178 g. $g^{-1}.day^{-1}$) at different harvest times. In spite of this, inoculation had a significant positive effect in interaction with harvest time after 45 days (F=4.852, df= 3, P<0.05), coinciding with the time when there is a higher presence of nodules in inoculated seedlings. Values cannot be attributed to inoculation during the days previous to nodule formation. In general, the RGR in the greenhouse was lower than that reported by Cervantes et al. (1998) for *A. farnesiana* when using a similar methodology but using a different type of substratum (soil from river bank). Besides, in that study, resident nodulating bacteria, efficient or not, were not considered. Another factor that may affect this RGR difference was the individuals used as seed source. In this study, the trees were located in a degraded secondary forest, which has been constantly perturbed for centuries by humans (CECCON; HERNÁNDEZ, 2009) probably affecting size and quality of seeds (FENNER, 1985). In this study, the average weight of seeds used (0.0728± 0.007g) was lower than that found in Cervantes et al. (1998) (0.083± 0.012 g). According to Leishman et al. (2000) and Leishman and Westoby (1994), seed size plays an important roll in germination and the subsequent development of seedlings.

3.1.3 Resources allocation

No effect of resource allocation patterns due to inoculation was observed, but a large variation at different harvest times was found. Biomass distribution, among different seedling structures throughout harvest times were significantly different for both inoculated and uninoculated seedlings (χ^2=155.849, p=0.000 and χ^2= 156,557, p=0.000 respectively). In the initial harvest (day 15), the leaves contained around 60% of total dry weight of *A. farnesiana* seedlings but, in the final harvest (day 120), they had only 30%. In contrast, in the first harvest (day 15) the roots represented only 11% of total biomass and at the end (day 120), had around 30%. The proportion of shoots increased just 10% with the time of harvest. Regarding the comparison of resources allocation among different structures and different time of harvest between the two treatments (inoculated and uninoculated), no significant effects of inoculation on any plant part were found (root, χ^2= 1.406 p = 0.99; sprout, χ^2= 0,349, p = 0.999 and leaves, χ^2= 0,349, p = 0.999) (Figure 2).
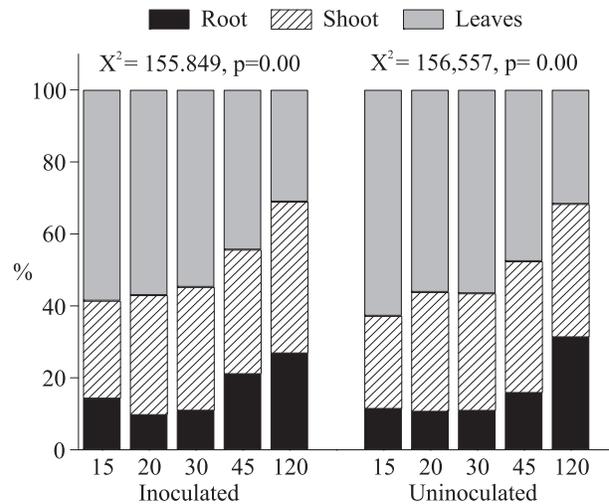

Figure 2 – Effect of harvest time in the dry-weight allocation for different structures of inoculated and uninoculated seedlings of the *Acacia farnesiana* in a greenhouse experiment.

Figura 2 – Efeito do tempo de coleta na alocação do peso seco para diferentes estruturas de mudas de Acacia farnesiana em um experimento em casa de vegetação.

3.1.4 Root/Shoot ratio

The factorial ANOVA showed that there where no effects of inoculation on the root/shoot ratio or in the interaction between inoculation and time of harvest. However, showed significant effects along the harvest times (F=20.10, df=5, P<0.01). The root/shoot ratio, at 120 days (the last time of harvest) was significantly higher than others harvests (Figure 3). The largest resources allocation for roots at the end of the experiment is coincident with the results found by Cervantes et al. (1998) for the same species. The economic interpretation of resource limitations in plants (water is the main limiting resource in SDTF), have contented that the responses of plants to variations in resources supply should involve compensatory changes in root/shoot allocation in order to increase the acquisition of the soil resources which are limiting growth (BLOOM et al., 1985; CHAPIN, 1991).

3.1.5 Root morphology (RL/RDW ratio)

The factorial ANOVA showed that the harvest time had highly significant effects on the RL/RDW ratio (F= 18,036, df=4, P<0.01). However, no significant effect was found in the interaction between inoculation and harvest times. The values for harvest at 15, 20 and 30 days were significantly higher than at 120 days (Figure 3).
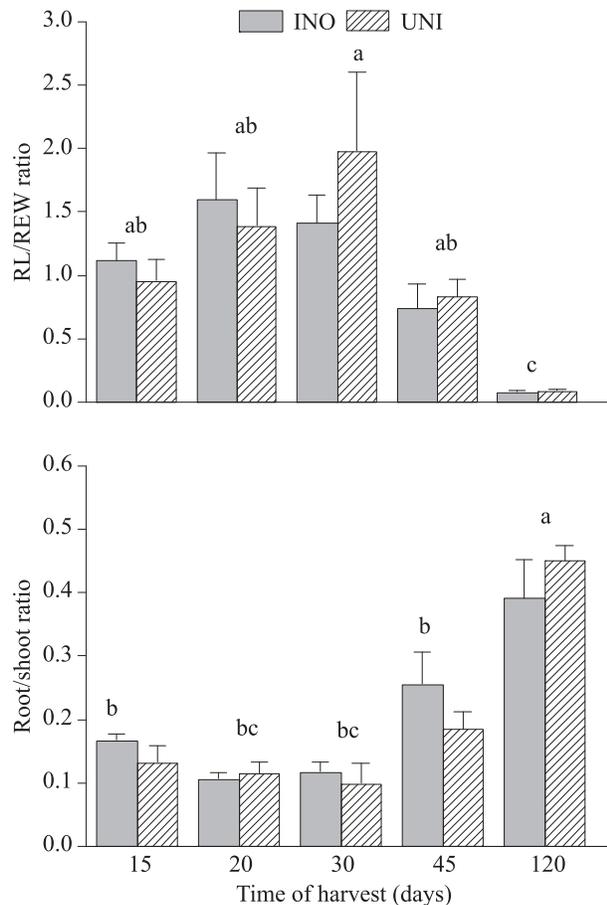
Vertical lines are standard errors. Asterisk (*) means significant differences between inoculated and uninoculated seedlings. Different letters mean significantly differences among the times of harvest (P<0.05).

Figure 3 – Effect of inoculation with *S. americanum* (INO) on root length/ root dry weight ratio (RL/RDW) and the root/shoot ratio (R/S) at different times after inoculation in different times of harvest (15, 20, 30, 45 and 120 days) in *A. farnesiana* compared with the uninoculated control (UNI) in a greenhouse.

Figura 3 – Efeito da inoculação de S. americanum (INO) na relação entre comprimento da raiz/peso seco de raiz (RL/RDW) e a relação entre peso seco de raiz/peso seco da parte aérea em diferentes períodos de coleta (15, 20, 30, 45 e 120 dias) em A. farnesiana comparada com o controle não inoculado (UNI) em uma estufa.

It was in the last period when established nodules were found. This significant decreasing trend for root length/ root dry weight ratio (RL/RDW) observed towards the end of the experiment that means that roots were becoming thicker with the time.

All the allocation resources results together, were similar to those found by Huante et al. (1995) with this species, also in a poor nutrient environment. This suggests that *A. farnesiana* species may be adapted to nutrient-limited environments, since it has been established that plant species adapted to limited resources environments always show low growth shoot rates, high R/S values, and thicker, deeper and longer-lived roots (CHAPIN, 1980; GRIME, 1979). Due to the marked dry season and nutrient poor soils in the SDTF constraining seedling establishment (CECCON et al., 2003, 2004), *A. farnesiana* could strongly indicated for the restoration and fuelwood production programs mainly in degraded SDTF.

3.2 Field experiment

The inoculation with *S. americanum* had a significant impact on the *A. farnesiana* seedling length over seven of the eight months they were in the field, according to the *t*-test (Figure 4). This results could be compared to that found by Foroughbakhch et al. (1987) in *Acacia farnesiana* seedlings after 12 months inoculation (50 and 30 cm respectively) in semi-arid zones of northeastern Mexico. Similarly, inoculation had a significant effect on the average RGR length by the Kolmogorov-Smirnov test (K-S, p<0.05) in spite of its short age/size (13.0 cm average and 30 days) at introduction time. Enhanced growth after inoculation is a remarkable aspect of plants growing in degraded pasture lands, as is the case on this study, because taller seedlings face competition with grasses for light more favorably (NGULUBE, 1989). Also the shadow casted by trees may restrain regrowth of the pasture facilitating the recruitment of other wood species (HOLL, 1999) and so reducing the work need for weeds control. There were also highly significant differences among the months in the RGR length (H =184.19, df =7, P< 0.01) by a Kruskal Wallis test. The lowest RGR was in the month of the introduction of seedlings in the field (adaptation period) at the beginning of the rainy season (June) and in the warmest point of the dry season (April).

No effect to inoculation was observed on the *Acacia farnesiana* seedling survival during both, the rainy season (four mouths) and the dry season (four months) in the field. There were no significant differences in the monthly distribution of the survival proportion between inoculated and uninoculated seedlings by the chi-square goodness-of-fit model ($\chi^2 = 0.052$, p = 1). The average percentage of survival for inoculated and uninoculated seedlings was 97.7% and 100% respectively through out the entire time.
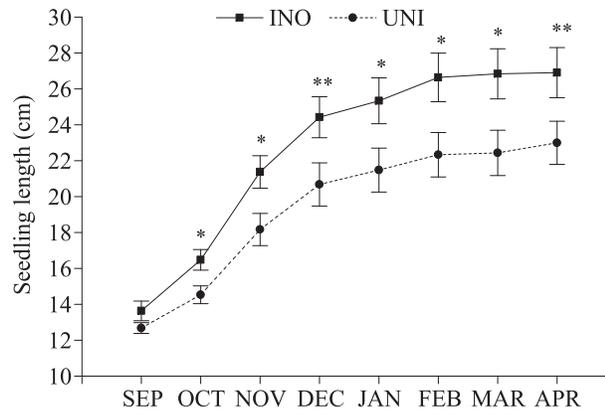
*P<0.05, **P<0.01

Figure 4 – Comparison of the effect of inoculation with *S. americanum* (INO) on seedlings length of *A. farnesiana* seedlings with the uninoculated seedlings (UNI) in a degraded pasture, during 8 months.

Figura 4 – Comparação do efeito da inoculação de S. americanum (INO) no comprimento de plântulas de A. farnesiana com mudas não inoculadas (UNI) em uma pastagem degradada durante 8 meses.

Possibly in this case, the moderate irrigation during the dry and hot season benefited both, inoculated and uninoculated seedlings.

Therefore, inoculation of *A. farnesiana* seedlings with elite native rhizobia strains in greenhouse is highly recommendable as part of the implantation techniques used to establish seedlings in degraded areas for land restoration and fuelwood production. At same time, the re-establishment of important plant-soil interaction in degraded soils can contribute significantly to the development of biodiverse self-regenerating native ecosystems in degraded landscapes (ARONSON et al., 1993; OCHIN; TAILLIEZ, 1985; VIRGINIA, 1986).

4 ACKNOWLEDGEMENTS

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