Scientific Paper

Inoculation of *Canavalia ensiformis* with arbuscular mycorrhizal fungi in the establishment stage

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Abstract

The objective of the study was to evaluate the effect of arbuscular mycorrhizal fungi (AMF) on the forage yield and quality of *Canavalia ensiformis* in the establishment stage. Three AMF species and a control were evaluated, in a randomized block design with four treatments and three repetitions. The evaluated variables were: aerial dry biomass yield, efficiency index, crude protein, NPK extraction, colonization and visual density of the roots. The inoculation with AMF species surpassed the control in all the variables. The highest dry biomass yield values were found in *Funneliformis mosseae*/INCAM-2, and the lowest ones in the control, with significant differences at $p \leq 0,05$ (8,30 and 4,35 t ha^{-1}, respectively). The extraction of N, P_2O_5 and K_2O reached values of 254,73; 32,26; 210,96 and 120,43; 15,03; 77,00 for *F. mosseae* /INCAM-2 and the control, respectively ($p \leq 0,05$). *F. mosseae*/INCAM-2 and *Glomus cubense*/INCAM-4 showed the highest values of colonization and visual density, although the latter did not differ from *Rhizoglomus intraradices*/INCAM-11. It is concluded that the different inoculated AMF strains surpassed the control in the dry matter yield, crude protein and extraction of macronutrients from the soil, although the highest effectiveness in any of these variables was obtained with the inoculation of the strain *F. mosseae* /INCAM-2.

Keywords: inoculation, crude protein, yield

Introduction

Canavalia ensiformis (L.) D. C. is an annual legume native to Mexico, with attributes to produce forage due to its photosynthetic efficiency and accumulation of biomass as available green forage, of high biological value (13-25 % of crude protein and 62 % of digestibility). This species adapts to Cuban conditions, because of its vigorous growth and contributions of atmospheric N incorporated to the soil-plant system, via nitrogen fixation (García-Rubido *et al.*, 2017).

The presence of mycorrhiza-forming fungi in tropical soils is fundamental, because through endosymbiosis with the plant roots not only improves its nutrition, but also allows the plant to adapt to environmental stress conditions (Petipas *et al.*, 2017).

The inclusion of arbuscular mycorrhizal fungi (AMF) in the fertilization systems of pastures and forage crops is an effective way to improve their productivity and nutritional value (Weremijewicz and Seto, 2016); in addition, their use can represent a substantial reduction of agrochemicals, such as fertilizers (Rivera and Fernández, 2003) and phytosanitary products, for which they are acknowledged for having a high potential in the context of sustainable agriculture (Azcón-Aguilar and Barea, 2015).

On the other hand, AMF have been found to decrease the requirements of external critical phosphorus for the annual forage legumes in soils with low content of this element, presumably due to the improvement in the capture of this ion because of a higher exploration by the external fungal hyphae, compared with the non-inoculated plants (Nazeri *et al.*, 2014). That is why it is highly important to study the effect of these beneficial fungi on the yield and nutritional quality of different legumes of agricultural interest.

The objective of this study was to evaluate the effect of AMF on the forage yield and quality of *C. ensiformis* in the establishment stage.

Materials and Methods

Location of the study area. The essay was conducted under field conditions, in experimental areas of the Soil Scientific Basic Unit, located in the Cumanayagua municipality –Cienfuegos province, south central region of Cuba–, whose coordinates are 22º 09' North latitude and 80º 12' West longitude, at 60 m.a.s.l. A total of 123 mm of rainfall, a mean temperature of 25,02 ºC and 76 % of relative humidity were reported during the experimental period (September-December, 2017).

General soil characteristics. The soil is classified as greyish Brown (Hernández-Jiménez *et al.*, 2015). Some characteristics of the experimental area are the following: pH in water 5,68, Norma Cubana ISO-10390 (ONN, 1999a); organic matter 1,78 %, Norma Cubana ISO-51 (ONN, 1999b); P_2O_5 and K_2O 3,62 and 8,35 mg/100 g of soil, Norma Cubana ISO-52 (ONN, 1999c).

Experimental procedure. The species used were: *Funneliformis mosseae*/INCAM-2 (Schü*ß*ler and Walker, 2011), *Glomus cubense*/INCAM-4 (Rodríguez *et al.*, 2011) and *Rhizoglomus intraradices*/INCAM-11 (Sieverding *et al.*, 2014), from the collection of AMF strains of the National Institute of Agricultural Sciences (INCA, for its initials in Spanish).

The design was randomized blocks with three repetitions, and the following treatments: *Funneliformis mosseae*/INCAM-2, *Glomus cubense*/INCAM-4, *Rhizoglomus intraradices*/INCAM-11, and control.

Plots of 8,40 m^2 were established, with a separation between them of 0,5 m. As evaluation unit the two central furrows were taken, discarding the edges, for an area of 2,10 m^2/plot.

The inoculants used were multiplied in a clayey substrate, sterilized in autoclave at 120 ºC for an hour, during three days; and *Brachiaria decumbens* cv. Basilisk was used as host plant. Each inoculant contained 30 spores per gram of substrate of the AMF species to be evaluated, as well as an abundant quantity of rootlet and hypha fragments.

The AMF inoculation was performed by the seed covering method, for which they were submerged in a fluid paste, elaborated through the mixture of a quantity of solid inoculant equivalent to 10 % of their weight and water, in a proportion of 60 mL of water for every 100 g of inoculant (Simó-González *et al.*, 2016).

The trial was conducted without irrigation and no mineral fertilizers were used. In all the treatments seedling emergence occurred between five and seven days after planting. The cutting was manually carried out 69 days after planting, at 5 cm above the soil level, with 100 % of the plants flowered.

At the moment of cutting the aerial leaf biomass was quantified (kg/plot); and a 200-g sample was taken to determine, by weighing (0,1 g), the aerial dry biomass, nitrogen (N), phosphorus (P) and potassium (K).

The aerial dry biomass yield (ADB, t ha^{-1}); extractions of N, P_2O_5 and K_2O (kg ha^{-1}) and crude protein (%), were determined according to the following formulas:

1. $\text{ADB (t ha}^{-1}\text{)} = \dfrac{\underline{\text{GM (kg plot}^{-1}\text{) x \% DM x 10}}}{\text{Calculation area (m}^2\text{)}}$
2. Extraction of N, P_2O_5 and.K_2O (kg ha^{-1}): ADB (t ha^{-1}) x %/element x 10

In the case of P and K, the multiplication was made by 2,29 and by 1,20 to convert them to P_2O_5 and K_2O, respectively.

After the establishment cutting, the roots were sampled in each plot; for such purpose a metallic cylinder of 5 cm diameter and 20 cm height, and the protocol used for determining mycorrhizal structures in pasturelands was followed (Rosales-Jenqui *et al.*, 2017).

The mycorrhizal colonization (% Col) was evaluated, from the clarification and initial staining of the roots (Phillips and Hayman, 1970), with the linear transect technique (Giovannetti and Mosse, 1980); as well as the visual density of the colonization (% VD), according to the scale proposed by Herrera-Peraza *et al.* (2004): zero: absence of AMF; 1: low intensity; 2: 0,5 %; 3: 15,5 %; 4: 35,5 % and 5: 47,5 %. The efficiency index (EI) was calculated, from the dry biomass (DM) yields, according to the formula proposed by Siqueira *et al.* (1987):

$$\text{EI} = \frac{\text{Inoculated DM yield - Control DM yield}}{\text{Control DM yield}} \text{ x } 100$$

Statistical analysis. The results were statistically processed through a double classification ANOVA, after testing the variance homogeneity and normality assumptions; and Duncan's multiple range test was used for mean comparison, with a reliability of 95 %, using the statistical program SPSS for Microsoft Windows version 15.0.

Results and Discussion

The effect of the inoculation of the AMF species on the aerial dry biomass yield of jack bean and the corresponding mycorrhizal efficiency are shown in table 1. There was a significant response

Table 1. Aerial dry biomass yield (ADB) and mycorrizal efficiency index (EI).

Treatment	ADB yield (t ha^{-1})	EI (%)
F. mosseae/INCAM-2	8,30^{a}	90,80
G. cubense/INCAM-4	6,92^{b}	59,00
R. intraradices/INCAM-11	7,80^{a}	79,30
Control	4,35^{c}	-
SE ±	0,100*	

a, b, c: different letters in the same column indicate significant differences at $p \leq 0{,}05$.
* p< 0,05

($p \leq 0{,}05$) to inoculation. The highest yields were obtained with *F. mosseae*/INCAM-2, although it did not differ significantly from *R. intraradices*/INCAM-11, but it did differ from *G. cubense*/INCAM-4. The lowest yield corresponded to the control.

Rivera-Espinosa *et al*. (2017) reported a change in the effectiveness of these species when being inoculated to jack bean, depending on the edaphic environment and with emphasis on the soil pH-H_2O. Under conditions of the nodular ferruginous Gley soils, Acrisol and Ferrasol, with pH-H_2O between 4,7 and 5,8, the highest response was found with the inoculation of *F. mosseae*/INCAM-2, which was considered as efficient species for those pH conditions. This coincides with the results of this study (pH 5,68), although in another soil type.

On the other hand, García-Rubido (2017), when evaluating the response of *C. ensiformis* to the inoculation with *R. intraradices*/INCAM-11, *F. mosseae*/INCAM-2*, G. cubense*/INCAM-4 and *Glomus claroideum*/INCAM-8, in a leached reddish yellowish Ferralitic soil with lower acidity (pH-KCl 5,5 and pH-H_2O 6,2), found the highest response with the inoculation of *G. cubense*/INCAM-4, which corroborates the effectiveness of these AMF strains depending on the edaphic environment and pH.

The efficiency index of the mycorrhiza species varied between 59,0 and 90,8 %. This index should be taken into consideration in any analysis of the fungus/host plant effectiveness, because its shows how yield can be influenced by symbiosis, with regards to the control. The strains with higher efficiency index were *F. mosseae* and *R. intraradices*.

The studies of strain comparison in Cuba proved the existence of high efficient AMF species-soil type specificity (Rivera and Fernández, 2003), which could corroborate the performance, in this soil type, of the different evaluated species.

The species of the *Glomus* genus have a broad range of functional distribution and prevail in high- and moderate-fertility systems, where they are efficient and competitive. The results in Cuba allowed to extend this range to low-and very low-fertility conditions (Rivera and Fernández, 2003); such efficiency prevailed under the conditions of this work, where the strains surpassed the control.

Simó-González *et al*. (2016) reported, in a carbonated loose Brown soil with pH 7,3-7,5, a yield of 6 t ha^{-1} and a high nutrient extraction by the jack bean inoculated with *R. intraradices*/INCAM-11 when planting was performed in the rainy season, which indicates an adaptation of this genus to different soil types.

The crude protein percentage and extraction of macronutrients are shown in table 2. Although there were no significant differences, the crude protein was numerically higher in *F. mosseae*/INCAM-2*, G. cubense*/INCAM-4 and *R. intraradices*/INCAM-11.

The nitrogen and potassium extractions were higher in the inoculation with *F. mosseae*/INCAM-2, which differed statistically from the others; *R. intraradices*/INCAM-11 and *G. cubense*/INCAM-4 followed in order*,* without significant differences between them. Regarding phosphorus, there were no significant differences among the strains. For any of the nutrients the control always showed a significantly lower performance than the one obtained with the inoculation of the species, which, as minimum, doubled the extractions of the former.

AMF improve nutrient extraction from the soil and its efficiency (Bitterlich and Franken, 2016), which brings about higher exports, and this could indicate the need to search for alternatives to return

Table 2. Crude protein content and macronutrient extraction.

Treatment	CP (%)	Macronutrients (kg ha^{-1})		
		N	P_2O_5	K_2O
F. mosseae/*I*NCAM-2	19,16	254,73^a	32,26^a	210,96^a
G. cubense/INCAM-4	18,40	203,70^b	31,46^a	149,20^b
R. intraradices/INCAM-11	17,60	218,43^b	30,43^a	148,60^b
Control	17,31	120,43^c	15,03^b	77,00^c
SE ±	0,076	0,176*	0,132*	0,150*

a, b, c: different letters in the same column indicate significant differences for $p \leq 0,05$.
* $p < 0,05$

them to the soil; however, different authors consider that mycorrhizae act in a guided way in nutrient absorption.

In this regard Rivera and Fernández (2003), in trials conducted in Ferralitic Red and Brown with carbonates soils, found that mycorrhizal symbiosis directly increased the absorption of the three primary elements, through the increase in the concentration of these nutrients; such information indicates that mycorrhization, rather than showing preference for one element or another, behaved as a mechanism that allowed the plants to obtain their nutritional requirements, depending on the nutrient availability in the system.

In addition, it is known that AMF not only improve nutrient extraction in the soil, but they also reduce nutrient losses by leaching (Bender *et al*., 2015); this reinforces the possibility of increasing nutrient extraction by such fungi, as the available ions remain available for the mycelial network in the soil, although the mechanisms that underlie this phenomenon are not fully established (Cavagnaro *et al*., 2015). Nevertheless, it is logical to think that the exploration of a higher soil volume, the extensive networks of external mycelia that are formed, as well as the effective capture of nutrients and the immobilization of diverse ions in the plants and in the fungal tissues, are some of the key mechanisms to reduce the P and N leaching through AMF (Cavagnaro, 2015).

The extraradical mycelium of mycorrhizal fungi has the capacity to take NH_4^+, NO_3^- and organic N from the soil (Pérez-Tienda *et al*., 2014), which could have influenced the differences found in the nitrogen extraction by the plant. Legumes are plants that respond more vigorously to the inoculation with AMF, and a higher association of the hyphae facilitates the increase in nutrient transference (Martín *et al*., 2010). This statement confirms the favorable results reached in the plants inoculated with the mycorrhiza strains with regards to the control, when evaluating the N, P and K extractions.

García-Rubido *et al*. (2017), when studying the inoculation with different strains of mycorrhizal fungi in *C. ensiformis*, in a leached reddish yellowish Ferralitic soil with pH of 5,5 and 1,23 % OM, achieved a significant response, with the highest NPK nutrient contents (264,9 kg N ha^{-1}; 37,6 kg P ha^{-1}; 226,7 kg K ha^{-1}, respectively) with regards to the non-inoculated control.

In a slightly acid clayey soil, with a moderate level in organic matter and low in available phosphorus, Puertas *et al*. (2008) determined the dry matter yield and macronutrient extraction in *C. ensiformis* used as soil cover, and reported a yield of 4,59 t ha^{-1} (lower than the one reached in this work) and an extraction of 173,68; 36,31 and 82,15 kg ha^{-1}, respectively, of N, P_2O_5 and K_2O, lower in nitrogen and potassium, but discreetly higher in P_2O_5.

The ability of AMF to increase the capture of nutrients through the networks of external mycelia offers an interesting strategy to limit the inefficient use of N applied to the crops, as reported by Verzeaux *et al*. (2017). According to these authors, AMF can play an important role in N recycling, by varying the availability of mineral N in the soil through changes in the composition of the microbial community of the rhizosphere, and thus modify the development of denitrification, nitrification and symbiotic diazotrophic or free-living bacteria.

Plana-Llerena *et al*. (2016), when evaluating the forage production based on triticale (x. *Triticosecale* Wittmack) in a lixic ferralic Nitisol soil, with variable nitrogen doses and inoculation with AMF, determined that the last two ones had a direct and significant relation with nutrient absorption. That is, the quantity of nutrients that is recovered in the biomass of the forage DM when AMF are used is higher.

The fungal colonization and visual density of the colonization are shown in figure 1. *F. mosseae*/INCAM-2 and *G. cubense*/INCAM-4 reached the highest rootlet colonization percentage, although the latter did not differ from *R. intraradices*/INCAM-11; the three strains exceeded 50 % of colonization. The control showed 43,12 % of colonization, which indicates the presence of resident mycorrhizae that colonized the roots, although in a lower level.

With regards to the visual density there was no difference among the inoculated strains and exceeded the density of the control; according to the report by Herrera *et al*. (2004) are in the category 2 and 3 (higher than 2 and lower than 15,5 %).

C. ensiformis was evaluated by Ojeda *et al.* (2014) under similar soil conditions, in the animal husbandry watershed El Tablón, and was naturally colonized by mycorrhizae, although in low levels (24-13 %); the visual density was 2,60 and 1,14 % in the first and second sampling, respectively. This seems to indicate the existence of a disturbed soil, where the resident mycorrhizae did not have high incidence on the reference host, for which the authors coincided in the need for testing introduced arbuscular mycorrhiza species.

In an experiment based on a compartmentalized microcosm, it was proven that the marked N transferred from the organic matter to the plants via extraradical hyphae of the fungus resulted in a higher plant biomass, as well as in higher N/P content in the tissues (Thirkell *et al*., 2016). This novel finding suggests that AMF could play an important role not only in improving nutrient capture, but also in diversifying the sources from which they are acquired, as stated by Thirkell *et al.* (2017).

The inclusion of AMF in the fertilization systems for pastures and forage crops is an effective way to improve their yield and nutritional value, because the benefits of these microorganisms in the pastureland agroecosystems are closely linked to the increase of the absorption surface of the roots and, consequently, to the improvement in the efficiency of utilization of nutrients by the plants (Zhang *et al*., 2016).

It is concluded that the different AMF strains inoculated surpassed the control in the dry matter yield, crude protein and extraction of macronutrients from the soil, although the highest effectiveness was found with the inoculation of the strain *F. mosseae*/INCAM-2. Likewise, it was proven that it is feasible to use efficient AMF strains as biological nutrition alternative in *C. ensiformis*.

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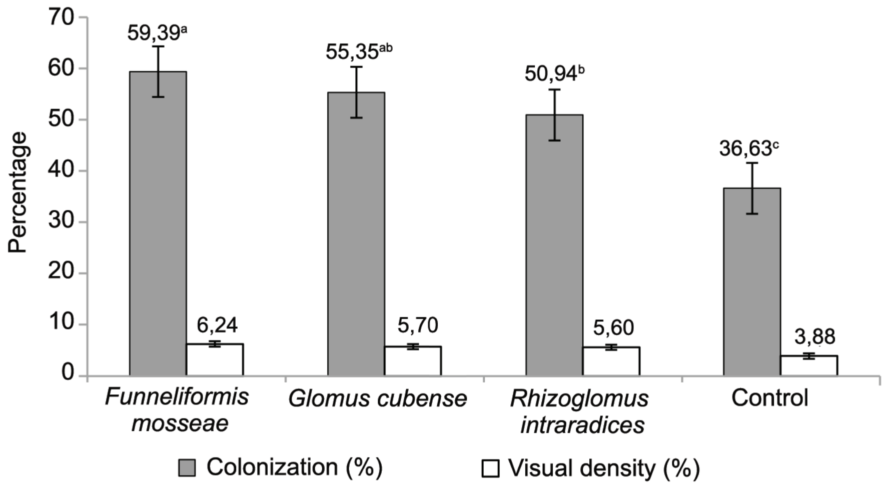

Different letters indicate significant differences for $p \leq 0,05$.

Figure 1. Colonization by the fungus and visual density of the roots.

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