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Benefits of *Canavalia ensiformis*, arbuscular mycorrhizal fungi, and mineral fertilizer management in tobacco production

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Tobacco (*Nicotiana tabacum* L.) has long been vital to Cuban agriculture, with its products renowned for their quality. Cuban tobacco is grown in soils with a long history of continuous farming using traditional fertilization methods characterized by recommended doses of mineral fertilizers. This study aims to improve the nutrition resource strategy in tobacco cultivation to ensure high yields of superior-grade tobacco leaves with adequate quality and increase fertilization efficiency. With this goal, a field experiment evaluated the traditional method of fallow with alternatives of nutrient supply systems for the production of black tobacco in Ultic Paleustalf soils. The experiment utilized *Canavalia ensiformis* (Can) treated with a mycorrhizal inoculum (AMF) based on the *Glomus cubense* strain (INCAM-4) as a preceding green manure, combined with successive mineral fertilizations for tobacco during four growing seasons in a randomized block design with factorial arrangement. Canavalia presented a positive response to mycorrhizal inoculation, significantly increasing dry biomass production (87.34%, 129.96%), mycorrhizal colonization (26.90%, 103.66%), and spore production (26.79%, 52.52%) for Can and Can+AMF treatments respectively. A biplot analysis established a strong relationship between the biomass and mycorrhizal performance of Canavalia and the growth, yield, and mycorrhizal colonization of tobacco. The results indicate that inoculated Canavalia enhances mycorrhizal performance in successional tobacco, with Can+AMF significantly increasing mycorrhization of tobacco roots by (110.06%). Moreover, the combination of Can inoculate with AMF and 75% of the recommended mineral fertilization dose consistently produced the highest tobacco yields (42.06%), growth, and mycorrhizal activity across the four years while maintaining satisfactory combustibility. In this nutrition supply system, variations of the recommended fertilizer dose significantly decreased the percentage of mycorrhizal colonization. After four growing seasons using Can + AMF and Canavalia without inoculations, soil organic matter, and availability of exchangeable calcium, magnesium, and pH increased slightly without decreasing available phosphorus and potassium contents. Consequently, we conclude that *Canavalia ensiformis*, with an inoculum based on the *Glomus cubense* strain and 75% of the recommended dose of mineral fertilizers, provides an enhanced nutrition alternative system for black tobacco production.

KEYWORDS

green manure, AMF inoculation, mineral fertilization, tobacco, nutrition system

1 Introduction

Tobacco is a highly sensitive crop to soils' nutrient deficiencies, and crop production experiences significant yield and quality fluctuations due to nutrient deficiencies or excesses (Tso, 1990; Lisuma et al., 2020). Mineral fertilizers are the most well-known and widely used method to provide crops with immediate availability and high nutrient concentrations. However, its cost is steadily increasing and is becoming unaffordable for small producers. Moreover, inadequate management of mineral fertilizers poses ecological risks, including nutrient imbalances, contamination of water sources, reduced biological activity in the soil, and excessive chemical residues in agricultural products (Kassam et al., 2013; Mishra and Arora, 2016; Hamel and Plenchette, 2017).

Nutrient supply systems are required to meet the nutritional requirements of crops as well as enhance biological mechanisms in the rhizosphere. The combination of mineral and organic fertilizers obtains favorable crop yields of superior quality, concurrently mitigating production expenses (Dai et al., 2021; Sifola et al., 2022). Simultaneously, the synergistic mechanisms maintain or improve the soil's physical, chemical, and biological characteristics (Rivera et al., 2007; Hamel and Plenchette, 2017). The use of leguminous plants contributes nitrogen to the system through biological nitrogen fixation (BNF) (Krishna et al., 2007; Sousa et al., 2016; Palmero et al., 2022; Barbieri et al., 2023). Green manures, crop rotation, and compost offer alternatives for soil management and crop nutrition (Mosquera et al., 2012; Jiang et al., 2022) (Jiang et al., 2022; Li et al., 2024). Their application in agricultural areas leads to improved soil fertility, higher organic matter content, the formation of stable aggregates, enhanced biological activity, and reduction of soil erosion reduced (Cherr et al., 2006; García Rubido et al., 2015) (Asghar and Kataoka, 2021; Voltr et al., 2021). Additionally, Recent studies indicate other beneficial results from a green manure species *Canavalia ensiformis* (Araujo et al., 2024).

Mycorrhizae provide benefits for crop growth by increasing water and nutrient absorption capacity, enhancing resilience to adverse soil conditions, and forming stable aggregates in the soil, which improve tolerance to climatic conditions and pathogens (van der Heijden et al., 2015; Kashyap et al., 2018; Li et al., 2022). Arbuscular mycorrhizal fungi (AMF) establish a symbiosis with the most economically important plant species (Willis et al., 2013), including tobacco (Subhashini, 2013) and leguminous green manures as Canavalia (Rivera et al., 2010). In this context, the use of AMF has been shown to offer several benefits for tobacco, such as inducing tolerance to Tobacco Black Shank (caused by *Phytophthora nicotianae*) (Li et al., 2023), enhancing resistance to water stress (Begum et al., 2022), and improving essential oil production, metabolism, growth and yield (Begum et al., 2021), among others.

Recent fertilization techniques involve the simultaneous use of mycorrhizal inoculants and green manure species like *C. ensiformis*, have demonstrated notable improvements in crop production and soil fertility benefits (Rivera et al., 2020). These methods, particularly when integrated into crop rotation and intercropping systems, have consistently resulted in elevated yields and satisfactory nutritional content across different crops while substantially reducing reliance on mineral fertilizer (Ben-Laouane et al., 2021; Javanmard et al., 2022). Additionally, AMF-inoculated green manure plants have proven effective in stimulating the growth of mycorrhizae associated with economically significant crops, thereby enhancing soil coverage and health (Rayne and Aula, 2020; Dong et al., 2021; Gujre et al., 2021). To the present date, there is a lack of published research regarding these co-management strategies in tobacco cultivation

Considering the low organic matter content and sandy texture of the soils dedicated to tobacco production in San Juan Martinez, Cuba, and the previously mentioned benefits from AMF, green manure, and mineral fertilization combination. This study assesses the potential benefits and feasibility of combining *C. ensiformis* and mycorrhizal inoculants with appropriate mineral fertilization doses for tobacco production. This study aims to establish a more cost-effective and sustainable integrated nutrient supply for tobacco cultivation.

2 Materials and methods

2.1 Site description

The study was conducted from 2018 to 2022 at the Tobacco Experimental Station in Cuba, 22°16'55.2"N and 83°49'19.44"W, 31 meters above sea level. This station is located within the tobacco-rich region of Vuelta Abajo, in San Juan y Martínez, Pinar del Río province. The soil in this area was classified as Ultic Paleustalfs, characterized by a slightly acidic pH and typical exchangeable Ca and Mg levels. Notably, the soil type has low organic matter content and a resident mycorrhizal population, possibly due to continuous cultivation with high doses of mineral fertilizers. Excessive fertilization also accounts for the high levels of available phosphorus and potassium in the soil (Table 1).

The average annual rainfall and monthly temperature during the experiment were typical of the climate in western Cuba, with an annual average rainfall of 1581 mm and an average monthly temperature of 25.1°C. The rainy season typically extends from May to October, accounting for approximately 81% of the annual rainfall, with an average temperature of 26.8°C.

TABLE 2 Treatments studied during the experimental period.

Treatments	Previous crop	Fertilizer Percentage[1] (% RD)
Can+AMF+50% RD	*C. ensiformis* + AMF	50
Can+AMF+75% RD	*C. ensiformis* + AMF	75
Can+AMF+100% RD	*C. ensiformis* + AMF	100
Can+50% RD	*C. ensiformis*	50
Can+75% RD	*C. ensiformis*	75
Can+100% RD	*C. ensiformis*	100
Fallow+50% RD	Fallow	50
Fallow+75% RD	Fallow	75
Fallow+100% RD	Fallow	100

[1]100% RD (Recommended Dose): 120N – 60P_2O_5 – 160K_2O – 30MgO, according to the Technical Instructions of the crop (Espino et al., 2012).

2.2 Experimental design and tobacco management

Field experiments were conducted under conditions that resemble tobacco production environments. The fertilization treatments consisted of two factors. The first factor was the previous crop, where Canavalia with Arbuscular mycorrhizal fungi (AMF), Canavalia, and fallow plots preceded tobacco plantation The first factor was preceding crop, which included Canavalia inoculated with AMF, non-inoculated Canavalia, and fallow plots, which preceded the tobacco planting. The second factor was the macronutrient mineral fertilization doses, resulting in nine treatment combinations (Table 2). The experimental design followed a randomized block arrangement with a factorial A×B design, incorporating the years as the third factor (C). Each treatment had four replicates, with the same plot utilized for succession in different study years. Data from the first season were excluded to standardize the effects of the treatments on both Canavalia and tobacco, and only results from the last three seasons were presented.

Each plot had an area of 22.8 square meters (6 × 3.8 meters). Canavalia was planted in the second half of September with a spacing of 30 cm between plants, 50 cm between rows, and an approximate seeding rate of 70 kg per hectare. Fallow plots were designated as a commercial control, as tobacco growers typically allow fallow vegetation, primarily composed of *Cyperus rotundus* and *Eleusine indica*, with smaller amounts of *Amaranthus* spp., to establish before planting tobacco.

Certified *Canavalia ensiformis* seeds, as per the NRAG 193 (2011) standard, were used in this study. The methodology closely aligns with production conditions and has been tested across a wide range of experiments (Rivera et al., 2023). The certification process considers genetic and mechanical purity, germination capacity, moisture content, and phytosanitary control.

A solid arbuscular mycorrhiza inoculant based on *Glomus cubense* (INCAM-4, DAOM 241198) (Rodríguez et al., 2012), certified by the Mycorrhiza Laboratory of the National Institute of Agricultural Sciences of Cuba. The AMF had a concentration of 25 to 30 spores g^{-1} extracted by the wet sieving and decanting method (Gerdemann and Nicolson, 1963) from 50 g of inoculum.

Mass production of micorrizhal inoculum was prepared by applying *G. cubense*/INCAM-4 with a high degree of purity to seeds of *Urochloa decumbens*, which were subsequently grown in a sterile substrate following the method described by (Fernández et al., 2000). After four months, the substrate enriched with mycorrhizal propagules was extracted. This substrate mixture with mycorrhizal propagules, including mycorrhizal roots with a colonization frequency between 61% and 68%, dried at room temperature in the shade. The spore content was homogenized and adjusted to the commercial concentration of 30 spores g^{-1} (Fernández et al., 2000).

The mycorrhizal seed inoculation used the coating method with 10% of the seed weight (Fernández et al., 2000), equivalent to 10 kg ha^{-1}, preparing a homogeneous mixture of 1 kg of inoculum for every 10 kg of seeds previously moistened with 300 ml of water. Following the coating process, the seeds undergo a one-hour drying period in shaded conditions before sowing. Canavalia sowing and cultivation

TABLE 1 Some initial characteristics of the Ultic Paleustalf soil at 0–20 cm depth (average values and confidence intervals).

Item	pH	P	OM	Ca^{+2}	Mg^{+2}	Na^{+1}	K^{+1}	S	AMF spores in 50g soil
		mg kg^{-1}	g kg^{-1}	cmol kg^{-1}					
CI	5.39 ± 0.16	16.99 ± 0.85	13.6 ± 0.8	3.39 ± 0.45	1.78 ± 0.43	0.10 ± 0.01	0.46 ± 0.05	5.73 ± 0.61	55 ± 5

Chemical determinations (19): pH-KCl potentiometer: soil/solution ratio of 1:2.5; MO (organic matter) Walkey and Black method; P_2O_5 and K_2O extraction with H_2SO_4 0.05 M acid, Exchangeable cations: extraction with NH_4Ac 1 M; S: Base exchange capacity; No. Resident AMF spores (Gerdemann and Nicolson, 1963). CI confidence interval at p<0.05.

were carried out manually. The Canavalia plants were mechanically cut and incorporated into the soil at a depth of 15–20 cm at the onset of flowering, approximately 60–70 days after sowing.

The tobacco variety 'Criollo 98' was transplanted in the third week of December using seedlings from seedbeds. The transplanting was performed manually, with a planting spacing of 30 cm between plants and 76 cm between rows, resulting in a planting density of 38,000 plants per hectare. Each plot consisted of five rows of tobacco. Cultivation and fertilization followed the Technical Instructions for Tobacco Cultivation guidelines in Cuba, as provided by the Tobacco Research Institute (Espino et al., 2012). The recommended dose (RD) of 100% NPKMg fertilizer corresponded to 120 kg per hectare of nitrogen (N), 60 kg per hectare of phosphorus pentoxide (P_2O_5), 160 kg per hectare of potassium oxide (K_2O), and 30 kg per hectare of magnesium oxide (MgO). This fertilizer was applied in two fractions, with 40% 8–10 days after transplanting and the remaining 60% between days 18–20.

Tobacco harvesting started 55 days after transplanting and continued up to 80 days after transplanting. The harvested leaves were dried in a traditional curing house. The residual plant material from the harvest was incorporated into the soil within each plot, and the plot was left fallow until the onset of the rainy season. Then, soil preparation for the subsequent season was initiated. These practices were consistent across all treatments (Table 2).

2.3 Determinations performed on *Canavalia ensiformis*

The sampling procedure for the determinations involving *Canavalia ensiformis*used three consecutive plants selected within each plot approximately 60 days after germination from the central furrow. The sampling included plant roots and rhizospheric soil within the upper 20 cm depth in a sample composed of five sub-samples of 100 g of soil each.

The aboveground biomass (in Mg ha^{-1}) was assessed by separating the leaves and stems from the three selected plants. The fresh weight of each plant organ was measured using a precision balance (0.01 g). Subsequently, a 100 g sample from each organ was taken and dried in an oven at 70°C until a constant weight was achieved. This allowed us to determine each organ's fresh and dry mass and the total dry mass.

Extraction of N, P_2O_5, and K_2O measured the concentration of N, P, and K in the leaves and stems of Canavalia. Nutrients (g kg^{-1}) were determined using samples collected from each plot. Subsequently, the extraction was calculated based on the data for the dry mass of each organ (in Mg ha^{-1}) and the concentration of each element (in g kg^{-1} of N, P, and K), using the following formula:

$$Extraction\ (kg\ ha^{-1}) = \sum [OB_i \cdot MC_i] \cdot fg$$

In this formula: OB represents Organ Biomass; MC represents macronutrient content in each organ; the subscript 'i' corresponds to leaves and stems, respectively; the factor 'fg' was set at 1 for N, 2.29 for P_2O_5, and 1.2 for K_2O.

The assessment of spore counts and the determination of the percentage of mycorrhizal colonization were conducted per plot using rhizospheric soil samples. Spores were quantified using the wet sieving and decanting method (Gerdemann and Nicolson, 1963) in 50 g of soil, while mycorrhizal colonization was assessed through the intercept method and staining with 'blue ink' (Rodríguez Yon et al., 2015) using 200 mg of rootlets extracted from each sample. The quantities of mycelia and infective roots were not determined.

2.4 Tobacco yield and characteristics

Tobacco measurements and observations were conducted on tobacco plants randomly selected from the central furrows of each plot. These assessments were carried out during the harvest of the central leaves, which occurred 70–75 days after transplanting, following the method described by Torrecilla et al. (2012). The measurements included recording the length and width of the central leaf, determining the fresh and dry mass of the central leaf using a gravimetric method with a precision analytical balance (± 0.1 mg), and estimating chlorophyll content using the SPAD-502 instrument (MINOLTA, Spectrum Technologies Inc.) (Espino et al., 2012).

The yield assessment included all the plants within the plot. It was determined as the total yield (kg ha^{-1}), comprising both superior and inferior tobacco leaves grades, following the guidelines outlined in the Technical Instructions for the Collection and Processing of Sun-Cultivated Tobacco (Fernández et al., 2004). Combustion rate determinations followed the procedures and evaluation criteria specified in the document 'Instructions for the Assessment of Combustion Rate in Cuban Tobacco' by Guardiola et al. (2004).

Following the harvest, soil samples were collected from the tobacco plants in each plot. These samples were evaluated for the percentage of mycorrhizal colonization in the roots (Rodríguez Yon et al., 2015), the presence of mycorrhizal spores (Gerdemann and Nicolson, 1963), and the same soil nutrient analyses that were initially conducted (Table 1).

2.5 Statistical procedure

Univariate analysis (ANOVA) with a trifactorial arrangement was performed for the variables evaluated in Canavalia and tobacco. In cases where the highest-order interaction (A × B × C) was significant, further analysis broke down the interaction by assessing the effects of different Canavalia × fertilization combinations for each year. Mean values were calculated using Duncan's test at a $p< 0.05$ significance level. Furthermore, a multivariate Biplot analysis was conducted, integrating the variables from both tobacco and Canavalia. Regression analyses examined the relationship between yearly mycorrhizal colonization percentages and tobacco yield. Additionally, confidence intervals at $p< 0.05$ were calculated for each variable based on the initial and final soil analysis results.

3 Results

The dry biomass of Canavalia (Can) exhibited a positive response ($p < 0.05$) to the inoculation with arbuscular mycorrhizal fungi (AMF) and tobacco mineral fertilization (NPKMg) (Table 3), with an increase of 87.34% for Can and 129.96% for Can+AMF compared to Fallow. The highest dry biomass and macronutrient content ($p < 0.05$) were observed in the Canavalia treatments receiving mycorrhizal inoculations, particularly those treated with 75% and 100% of the recommended dose (RD) compared to the natural vegetation associated with fallow plots.

As expected, Canavalia inoculated with AMF treatments exhibited superior mycorrhizal functioning compared to Canavalia non-inoculated treatments, and all Canavalia treatments were notably higher than those observed in fallow natural. Similarly to the dry biomass and NPK absorption, indicators of mycorrhizal activity compared to fallow vegetation treatments increased in the number of spores by 26.79% for Can and 52.52% for Can+AMF, while for colonization, the increase was 26.90% for Can and 103.66% for Can+AMF.

Canavalia + AMF treatments presented a corresponding increase in spore numbers to AMF inoculation, but differences were not significant ($p < 0.05$) with Can+75% RD. Notably, the highest AMF colonization and number of spores ($p < 0.05$) were associated with the Can+AMF+75% RD treatment, while significant decreases were observed for lower and higher fertilizer doses (Figure 1).

The Biplot analysis indicated that the first two components accounted for a significant percentage of the experimental variance (Figure 2). Specifically, the first component explained 76% of the variance and displayed strong correlation coefficients with all Canavalia and tobacco variables, except for the yield of tobacco leaves of inferior grade, which exhibited high correlation coefficients with the second component.

TABLE 3 Aboveground dry biomass production and nutrient (NPK) uptake by Canavalia and fallow vegetation in the different treatments at the time of Canavalia cutting and fallow.

Treatments	Dry mass (Mg ha^{-1})	Total nutrient absorption (kg ha^{-1})		
	Total	N	P_2O_5	K_2O
Can+AMF +50% RD	4.25 c	131.6 c	18.76 b	98.42 c
Can+AMF +75% RD	6.09 a	209.93 a	27.02 a	140.56 a
Can+AMF +100% RD	6.00 a	194.95 a	25.55 a	132.58 a
Can +50% RD	4.08 c	126.98 c	13.93 c	90.16 c
Can +75% RD	4.06 c	116.83 c	12.88 c	89.53 c
Can +100% RD	5.18 b	164.92 b	17.29 bc	116.76 b
Fallow +50% RD	2.21 d	36.56 d	4.05 d	34.17 d
Fallow +75% RD	2.45 d	38.54 d	4.42 d	40.37 d
Fallow +100% RD	2.44 d	37.91 d	5.42 d	42.93 d
SE X	0.11	7.03	1.05	4.10

Significant interaction Canavalia × fertilization. Means with different letters in the same column differ from each other, according to Duncan's test ($p<0.05$).
AMF: *G. cubense*-INCAM-4.

Furthermore, the Can+AMF+75% RD treatment exhibited the highest values for component 1, followed by the Can+AMF+100% RD treatment, which were considerably distant from the other treatments. Following closely were the Can+AMF+50% RD

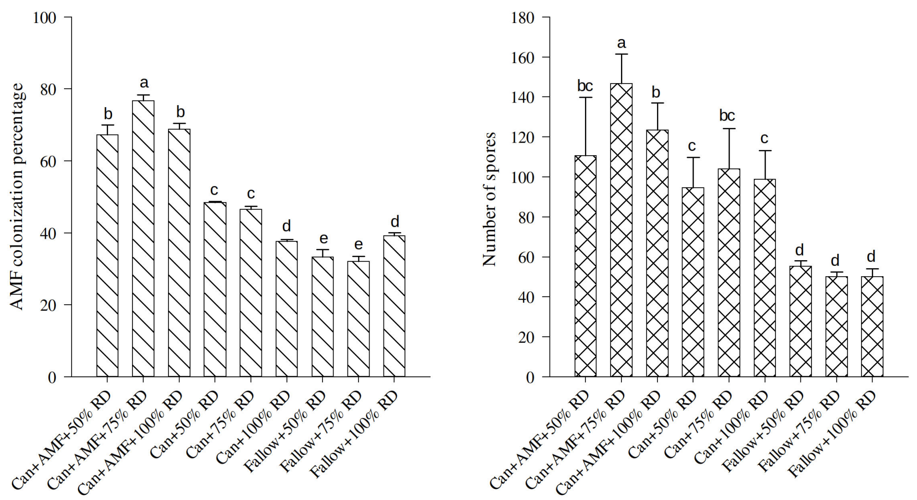

FIGURE 1
Effect of Canavalia-fertilization combinations on indicators of mycorrhizal performance in *C. ensiformis*. Significant Canavalia × fertilization interaction. SE x colonization = 0.69***, and ES x spores = 5.7*. Means with different letters within the same data series differ significantly from each other, as determined by Duncan's test ($p < 0.05$).

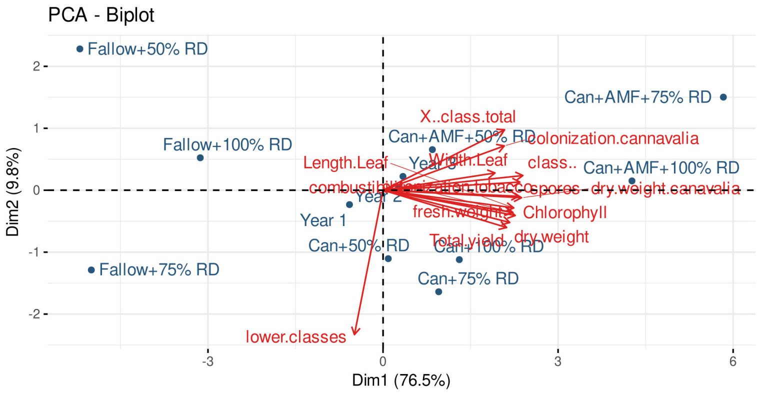

FIGURE 2
Biplot diagram integrating the evaluated variables in tobacco and canavalia.

treatment and the treatments involving Canavalia without inoculation. Among these the latter, the treatment that received 100% RD displayed the most favorable results, indicating a notable response to fertilization.

Canavalia treatments without inoculation were positioned in the first quadrant, which suggests the significant influence of component 2 on them. This association was related to higher values of leaves in inferior grades.

Lastly, treatments that exclusively received mineral fertilization exhibited the lowest values for component 1, with the treatment receiving 100% fertilization achieving the most favorable outcome. The different years showed proximity to each other, indicating minimal variations among them.

The univariate analyses indicated a significant maximum-order interaction (Canavalia × fertilization × years) for dry biomass, yield of superior leaves grade, and mycorrhizal colonization percentage. In the case of fresh biomass, chlorophyll content, total yield, yield in inferior leaves grade, and percentages in superior leaves grade, only the Canavalia × fertilization interaction factors were significant ($p< 0.05$). The leaf length responded solely to the Canavalia and fertilization factors, while the leaf width did not exhibit a response to any of the factors. Nonetheless, the highest values were consistently associated with the Canavalia plus AMF treatments.

In the case of dry biomass, yield of superior leaves grade (Table 4), and fresh total yield (Table 5), the Can+AMF+75% RD treatment presented higher means. Notably, Can+AMF+75% RD yields were higher than the values associated with the Can+AMF +50% RD treatment, and they did not exhibit significant differences when compared to the Can+AMF+100% RD treatment. The superior grade yield increased 42,6% compared to fallow 100% RD from the three seasons. In general, neither Can nor AMF inoculation significantly affected the total yield of tobacco leaves (superior grade + inferior grade). However, the specific yield of superior leaf grade was significantly higher, with an increase of

TABLE 4 Effect of Canavalia and fertilization combinations in each year on dry biomass and yield of Superior tobacco grade (g/plant).

Treatments	Dry Biomass (g ha^{-1})			Yield superior grade (kg ha^{-1})		
	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
Can+AMF+50% RD	35.5 ab	33,0 b	32,9 c	1244.1 bc	1260.5 bc	1213.8 bcd
Can+AMF+75% RD	36,5 a	36,5 a	37,5 a	1507,5 a	1452,5 a	1493.8 a
Can+AMF+100% RD	36.3 ab	35,5 a	36.4 ab	1393 ab	1374.6 abc	1317.8 ab
Can+50% RD	35.6 ab	34.3 ab	34.3 bc	1155,8 c	1214,78 c	1125.4 cd
Can+75% RD	36,7 a	34.9 ab	35.7 ab	1218.7 bc	1259.7 bc	1308.6abc
Can+100% RD	34,1 b	35,6 a	36.6 ab	1191.6 bc	1413.9 ab	1351.9 ab
Fallow+50% RD	23,5 d	26,3 c	24,7 d	1087,8 c	943,0 d	1098.7 d
Fallow+75% RD	23,8 d	26,5 c	24,4 d	838,6d	1068,5 d	1055.4 d
Fallow+100% RD	32,0 c	32,9 b	32,8 b	1058,0 c	966.7d	1110.6 d
SE (A×B×C)		0.605*			49.59***	

Interaction Canavalia × fertilization × years significant. * significance of $p< 0.05$; *** significance at $p< 0.001$. Means with different letters in the same column differ from each other, according to Duncan's test ($p<0.05$).
Partition of Canavalia × fertilization × years (A×B×C) interaction.

TABLE 5 Effect of combinations of Canavalia and fertilization factors on fresh biomass, total and inferior grade leaves yield of dark tobacco.

Treatments	Total yield (kg ha^{-1})	Percentage of superior grade leaves	Inferior grade leaves (kg ha^{-1})	Combustibility (Seconds)
Can+AMF+50% RD	1996,8 b	62,2 bc	757,3 bc	28,50
Can+AMF+75% RD	2173,4 a	68,4 a	688,8 cd	29,67
Can+AMF+100% RD	2128,9 a	64,0 b	766,8 bc	30,00
Can+50% RD	1999,4 b	58,4 c	834,2 ab	28,75
Can+75% RD	2096,6 ab	60,4 bc	901,0 a	27,13
Can+100% RD	2098,6 ab	60,9 bc	820,6 b	29,25
Fallow+50% RD	1821,9 c	57,8 c	673,4 d	25,17
Fallow+75% RD	1893,5 c	52,5 d	906,1 a	26,50
Fallow+100% RD	1716,5 c	61,1 bc	777,0 b	26,67
SE (A×B)	31.62**	1.12**	22.24***	

Significant Canavalia × fertilization interaction** significance at p< 0.01; ***significance at p< 0.001. Unequal letters in each column lead to significant differences between treatments by Duncan's test (p<0.05).

20.49% for Can and 32.82% for Can+AMF compared to fallow treatments (Table 5).

From 2018 to 2022, Tobacco yields from all treatments with Canavalia demonstrated significantly higher values than treatments without Canavalia. Intermediate fertilization doses produced the highest yield values in pre-treated plots with Canavalia. Tobacco yields from Non-inoculated Canavalia treatments presented significant differences (p< 0.05) in contrasting fertilization rates.

On the contrary, yields of the inferior grade leaves from treatments with non-inoculated Canavalia showed the highest values, while the Fallow+50% RD treatment had the lowest values. All treatments exceeded 20 seconds regarding tobacco leaf combustibility, which meets the quality standard (Torrecilla et al., 2012) and is considered excellent.

Regarding leaf length, the highest yield values were observed when Can+AMF+75% RD fertilization (data not shown).

Furthermore, the highest percentages (p< 0.05) of mycorrhizal colonization in tobacco were consistently recorded in the Can +AMF+75% RD treatment across all years (Table 6). Moreover, the mycorrhizal colonization average increased by 110.06% compared to Fallow+100% RD. Treatments with Can+AMF as a precedent presented lower values, although mycorrhizal colonization was significantly higher than those treatments using non-inoculated Canavalia as a precedent, which were higher than fallow preceded tobacco cultivation. Additionally, there was a direct and linear relationship between the percentage of mycorrhizal colonization in tobacco and yield (R^2 = 64%).

At the fourth season, soil analyses (Table 7) indicated that using Canavalia in preparation for tobacco crops, whether inoculated or not with AMF, led to significant increases in the organic matter content, calcium (Ca), magnesium (Mg), and exchangeable potassium (K). Moreover, Canavalia-treated plots exceeded the initial soil conditions (Table 1). There were no significant differences in pH levels, and the available phosphorus (P) contents did not exhibit significant variations relative to the initially high levels.

4 Discussion

This study indicated that Canavalia and tobacco associated with mycorrhizae positively respond to the inoculation of efficient AMF (arbuscular mycorrhizal fungi) strains. Several authors have reported positive effects in both crops, resulting in satisfactory mycorrhizal activity and enhanced potential of Canavalia as a green manure (Martín Alonso et al., 2012; Simó González et al., 2016). Additionally, AMF inoculations had enhanced tobacco yield (Subhashini, 2013).

The results highlight the positive impact of using *Canavalia ensiformis* as a precursor for tobacco cropping (Guerra et al., 2006; Espino et al., 2012), with these benefits further enhanced by inoculating

TABLE 6 Effect of combinations of Canavalia and fertilization factors in each year on mycorrhizal colonization (%).

Treatments	Year 1	Year 2	Year 3
Can+AMF +50% RD	49,65 b	52,22 b	48.54 bc
Can+AMF +75% RD	53,25 a	58,12 a	55,8 a
Can+AMF +100% RD	50.64 ab	51, 25 b	50,31 b
Can+50% RD	40,33 c	41,76 d	44.08 ef
Can+75% RD	49,42 b	48,56 c	41,57 e
Can+100% RD	42,4 c	39,11 d	46.06 cd
Fallow+50% RD	24,61 e	26,44 e	23,57 f
Fallow+75% RD	25,81 e	22,34 f	23,56 f
Fallow+100% RD	29,2 d	28,51 e	19,58 g
SE (A×B×C)		1.17***	

Significant interaction of Canavalia, Fertilization, and Years (*** indicates significance at p< 0.001). Unequal letters in each column indicate significant differences between treatments, as determined by Duncan's test (p<0.05).
Partition of Canavalia × fertilization × years (A×B×C) interaction.

TABLE 7 Effect of Canavalia applications on some soil chemical properties (0–20 cm depth) at the end of the four campaigns.

Treatments	KCl	P_2O_5	OM	Ca^{+2}	Mg^{+2}	K^{+1}
	mg $100g^{-1}$		g kg^{-1}		cmol kg^{-1}	
Canavalia+AMF	5.52 ± 0.23	40.0 ± 1.5	18.7 ± 0.5	4.22 ± 0.37	1.93 ± 0.32	0.49 ± 0.01
Canavalia	5.63 ± 0.25	40.0 ± 1.65	18.2 ± 0.3	4.17 ± 0.27	1.98 ± 0.24	0.49 ± 0.01
Fallow	5.38 ± 0.18	38.0 ± 1.05	14.5 ± 0.3	3.47 ± 0.04	1.54 ± 0.03	0.45 ± 0.01

CI confidence interval at $p<0.05$.

Canavalia. This study recorded that AMF inoculations resulted in higher biomass production, increased nutrient content, and enhanced yields of superior-grade leaves, even with lower fertilizer doses. Furthermore, the Canavalia treatments showed enhanced mycorrhizal activity, reaching colonization values approaching 60%, demonstrating strong mycorrhizal performance, as commonly observed in most crops (Rivera et al., 2023). However, additional benefits associated with mycorrhizal inoculation of Canavalia require further research on soil properties (Rivera et al., 2007).

It's important to note in this study that although non-inoculated Canavalia increased the 'resident' mycorrhiza and thus the mycorrhization of subsequent tobacco crops, it was not completely effective and showed lower performance compared to when Canavalia was inoculated. These results align with the findings of other authors (Sánchez et al., 2009; Rivera et al., 2010), who evaluated several species of non-inoculated green manures in different soil conditions and reported that although mycorrhization of the successional crop increased, it was not fully effective in enhancing crop yield.

Consistent with our findings, several publications have reported the positive impact of *C. ensiformis*, when inoculated with efficient AMF strains. Enhanced yield, nutritional status, mycorrhizal functioning, and fertilizers uptake efficiency for different succeeding crops had been reported (Martín Alonso et al., 2012; Simó González et al., 2016; João et al., 2017). This is in agreement with the effectiveness of *C. ensiformis* as a precursor for the successful mycorrhization of subsequent crops. More previous studies have indicated a lasting effect of the applied inoculant on the first crop of the succession, with intervals of no more than 30 days between the harvest of the inoculated crop and the planting of the succeeding crop (Martín Alonso et al., 2012; Simó González et al., 2016; Espinosa et al., 2018), a similar condition in our study.

While the use of *C. ensiformis* as a precursor offers multiple benefits to the agrosystem, including soil cover and increased organic matter content, soil aggregation, and biological activity (Cherr et al., 2006; Guerra et al., 2006). Canavalia's contribution to the growth and yield of tobacco has been associated with Canavalia's role in providing nitrogen (N_2) and recycling nutrients present in its biomass (Ambrosano et al., 2013; Viola et al., 2013). Green manure nutrients undergo a gradual mineralization process, and in conjunction with mineral fertilization applied to the crop, they provide the tobacco's nutrient requirements. This is especially critical in sandy loam soils, which are prone to nutrient leaching (García Rubido et al., 2015).

The benefits associated with Canavalia are further enhanced with the inoculation of efficient AMF strains. AMF increases the advantages linked to Canavalia growth and nutrient recycling and promotes more efficient biological nitrogen fixation (Larimer et al., 2014; Bulgarelli et al., 2017). Most importantly, AMF inoculations lead to effective mycorrhization of tobacco.

The higher yields with lower fertilizer doses can be attributed to the positive interaction between mycorrhizae and plant nutrition in the presence of organic residues. This interaction has been reported to increase the mineralization of residues by the microbiota in the mycorrhizosphere (Hodge and Storer, 2015; Bukovská et al., 2018). While some authors emphasized the enhancements in nitrogen absorption, once Canavalia biomass decomposes, its various nutrients become available for the crop root system. This is further facilitated by more efficient and rapid nutrient utilization from inoculated Canavalia. More, enhanced mycorrhizae activity, as reported in this study, has also been related to a greater length and number of mycorrhizal hyphae (Hodge and Fitter, 2010; Thirkell et al., 2016; Bukovská et al., 2018), and an increased quantity of mycorrhizal roots (Cheng et al., 2016).

Higher mycorrhizal activity typically results in a reduction of fertilizers. However, when high doses of fertilizers are applied, mycorrhizae growth may decline or be inhibited, and low fertilizer doses may not lead to optimal mycorrhizal functioning (Rivera et al., 2007, Rivera et al, 2020). Specifically, the utilization of the Can+AMF +75% RD treatment yielded the highest mycorrhizal colonization percentages and demonstrated the best overall performance in all variables, except for the yield of inferior-grade leaves (Bilalis et al., 2015). Lower and higher fertilizer doses in the presence of Canavalia inoculated with AMF led to a decrease in mycorrhizal activity, indicating that this dose was optimal for mycorrhizal development and the associated benefits. Higher percentages of superior-grade leaf yields were achieved with this treatment, suggesting improved nutrition and greater physiological efficiency in tobacco plants.

The fallow plot results elucidate the impact of tobacco fertilization on Canavalia's growth and development while endorsing this experimental approach's effectiveness in establishing the treatments' medium-term effects. The increases in soil organic matter and exchangeable cations, along with the sustained availability of phosphorus (P) and potassium (K), are additional benefits derived from this soil management strategy (Chu et al., 2020; Etesami et al., 2021; Shi et al., 2021). These benefits undoubtedly contribute to soil fertility improvement, reassuring that the proposed reduction in fertilizers will not lead to medium-term disadvantages, thereby enhancing agrosystem sustainability.

The high yields achieved, surpassing previous reports for the same tobacco varieties (Espino et al, 2012), in the presence of moderate fertilizer doses, promote the economic and environmental viability of employing Canavalia inoculated with efficient AMF strains as a precursor for tobacco. Consequently, it should be considered an integral component of crop production technology.

5 Conclusions

The use of *C. ensiformis* treated with an inoculum based on an efficient AMF strain (*Glomus cubense*; INCAM-4) as a precursor for tobacco cultivation not only leads to increased biomass production and nutrient availability in Canavalia but also provides an effective means of mycorrhization for the subsequent economic crop. The mycorrhizal activity in tobacco roots, coupled with the enhanced nutrient recycling from the inoculated Canavalia, resulted in greater fertilization efficiency and higher yields of superior grade and total tobacco leaves. In this case, only 75% of the recommended mineral fertilizer dose is required. This is in contrast to the fallow and mineral fertilization employed or when non-inoculated *C. ensiformis* is used. After four years of evaluations, soil properties showed improvements in soil organic matter and exchangeable cations while maintaining adequate available phosphorus levels, demonstrating that the 25% reduction in fertilization does not negatively impact soil fertility. Therefore, incorporating this nutrition strategy into tobacco crop production technology is highly recommended, and further studies should confirm the effects on different edaphoclimatic conditions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

DPdL: Formal analysis, Visualization, Writing – review & editing, Conceptualization, Methodology, Writing – original draft. MGR: Conceptualization, Visualization, Writing – review & editing, Funding acquisition, Investigation, Resources, Writing – original draft. RR: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Writing – original draft. DM-C: Visualization, Writing – review & editing. YG: Visualization, Writing – review & editing, Data curation, Formal analysis.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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