Autochthonous microorganisms as an alternative for biofertilization of *Nicotiana tabacum* L.

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Microorganismos autóctones como alternativa para biofertilização de *Nicotiana tabacum* L.

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Crop production

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Abstract

Tobacco is a crop of economic importance in tropical countries; it generates significant income in small agricultural areas. Within the agrotechnical activities that are carried out on this plant, biofertilization with Autochthonous Microorganisms (AM) represents a viable option for production with low inputs. Therefore, the objective of the research was to determine the biofertilizer effect of AM on black tobacco cv. Havana-2000. The experiment was carried out on a farm located in Los Cayos, Yara, Granma, Cuba, during the period November-February/2021. For this, the yield variables and the components were measured in the field. Statistical processing was performed using ANOVA and principal component analysis. The AM recorded four groups of microbes: bacteria ($7x10^{12}$ CFU.mL^{-1}) *Lactobacillus* sp. and *Rhodopseudomonas* sp., the fungi ($3x10^{11}$ CFU.mL^{-1}) *Trichoderma* sp., *Aspergillus* sp. and *Penicillium* sp., the yeasts ($2x10^{11}$ CFU.mL^{-1}) *Saccharomyces* sp. and *Candida* sp. and the actinomycete *Streptomyces* sp. ($1x10^{10}$ CFU.mL^{-1}). In the field, the AM biostimulated between 15 - 82 % of the yield and the components, achieving the best results at the dose of 36 L.ha^{-1}. Finally, the importance of using AM as an organic alternative for tobacco biofertilization is demonstrated.

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Resumen

El tabaco es un cultivo de importancia económica en países tropicales, genera ingresos significativos en pequeñas áreas agrícolas, dentro de las actividades agrotécnicas que se realizan a esta planta, la biofertilización con Microorganismos Autóctonos (MA) representa una opción viable para la producción con bajos insumos. Por ello, el objetivo de la investigación fue determinar el efecto biofertilizante de MA sobre el tabaco negro cv. Habana-2000. El experimento se realizó en una finca ubicada en Los Cayos, Yara, Granma, Cuba, durante el período noviembre-febrero/2021. Para ello, se midieron las variables del rendimiento y los componentes en campo. El procesamiento estadístico se realizó mediante ANOVA y análisis de componentes principales. Los MA registraron cuatro grupos de microbios: las bacterias ($7x10^{12}$ UFC.mL^{-1}) *Lactobacillus* sp. y *Rhodopseudomonas* sp., los hongos ($3x10^{11}$ UFC.mL^{-1}) *Trichoderma* sp., *Aspergillus* sp. y *Penicillium* sp., las levaduras ($2x10^{11}$ UFC.mL^{-1}) *Saccharomyces*sp. y *Candida*sp. y el actinomiceto *Streptomyces*sp. ($1x10^{10}$ UFC.mL^{-1}). En campo, los MA bioestimularon entre 15-82 % el rendimiento y los componentes, lográndose los mejores resultados a la dosis de 36 L.ha^{-1}. Finalmente, se demuestra la importancia de usar MA como una alternativa orgánica de biofertilización del tabaco.

Palabras clave: bioestimulación, bioensayos, microorganismos, rendimiento.

Resumo

O tabaco é uma cultura de importância econômica em países tropicais, gera renda significativa em pequenas áreas agrícolas, dentro das atividades agrotécnicas que são realizadas nesta planta, a biofertilização com Microorganismos Autóctones (MA) representa uma opção viável para produção com baixos insumos. Portanto, o objetivo da pesquisa foi determinar o efeito biofertilizante do MA em tabaco preto cv. Havana-2000.O experimento foi realizado em uma fazenda localizada em Los Cayos, Yara, Granma, Cuba, durante o período novembro-fevereiro/2021. Para isso, as variáveis de desempenho e os componentes foram medidos em campo. O processamento estatístico foi realizado por ANOVA e análise de componentes principais. O MA registrou quatro grupos de micróbios: bactérias ($7x10^{12}$ UFC.mL^{-1}) *Lactobacillus* sp. e *Rhodopseudomonas* sp., os fungos ($3x10^{11}$ UFC.mL^{-1}) *Trichoderma* sp., *Aspergillus* sp. e *Penicillium* sp., as leveduras ($2x10^{11}$ UFC.mL^{-1}) *Saccharomyces* sp. e *Cândida* sp. e o actinomiceto *Streptomyces* sp. ($1x10^{10}$ UFC.mL^{-1}). No campo, o AM bioestimulou entre 15-82 % a produtividade e os componentes, obtendo os melhores resultados na dose de 36 L.ha^{-1}. Por fim, demonstra-se a importância do uso da MA como alternativa orgânica para a biofertilização do tabaco.

Palavras-chave: bioestimulação, bioensaios, microrganismos, rendimiento.

Introduction

The cultivation of tobacco (*Nicotiana tabacum* L.) is part of the culture of many peoples, as well as, constitutes a crop of economic importance in tropical countries. In Cuba, it represents a strategic line within the economy, since it generates the largest income in the agricultural sector and is considered a cultural tradition. Cuban cigars have a high quality and great acceptance in the international market, due to the exotic organoleptic characteristics of the aromatic leaves (Ceiro-Catasú *et al*., 2021).

Black tobacco is considered native to Cuba, it is identified by having a unique aromatic pattern and its cured leaves show a dark coloration. This type of tobacco is used to obtain layers for the elaboration of Cuban cigars and strong cigarettes. Among the cultivars that are established on the island, Havana-2000 has excellent advantages in terms of its edaphoclimatic adaptability, tolerance to pests and diseases; as well as it has a potential yield of 2.33 t.ha^{-1}, and it adapts well to the forms of covered cultivation, sun-curing (involves simply drying the leaves uncovered in the sun) and with indirect sunlight (Sosa-Sánchez *et al*., 2022).

Historically, autochthonous microorganisms (AM) have been used as biofertilizers in tobacco-producing areas, these promote plant growth, can activate plant defense mechanisms and adaptability to edaphoclimatic conditions (Ceiro-Catasú *et al*., 2023; Reinaldo, 2020; Cables-Labrada and Escalona, 2013). Among the most studied microorganisms are mycorrhizae, bacteria, fungi, yeasts, and actinomycetes, which stand out for practical agronomic use (Ulacio *et al*., 1997).

Currently, AM are considered compatible with sustainable and low-input agriculture, because their active ingredient is made up of autochthonous microbial species of four main groups: bacteria, yeasts, fungi, and actinomycetes, which are characterized by the biofertilizer effect, biostimulant of growth, activator of plant defenses and improvers of physical properties, soil chemistry, and biology (Ramírez *et al*., 2022; Viera-Arroyo, 2020).

In particular, the bacterial species *Lactobacillus plantarum*, *L. casei, Rhodopseudomonas palustris,* and *Rhodobacter sphaeroides* act on the synthesis of amino acids, nucleic acids, bioactive substances, and carbohydrates, as well as in the degradation of soil organic matter and as antagonists of pathogens (Xu *et al*., 2020). Yeasts (*Saccharomyces cerevisiae* and *Candida utilis*) synthesize hormones and enzymes involved in active cell division in plants, while their secretions are helpful substrates for feeding other saprophytic microorganisms (Hernández-Fernández *et al*., 2021; Nadeem *et al*., 2010).

Certain fungal species such as *Aspergillus oryzae*, *Penicillium* sp., *Trichoderma* sp., and *Mucor hiemalis*, are considered key in the transformation and degradation of organic remains, provide nutrients to plants and synthesize secondary metabolites and antibiotics that regulate phytopathogen populations in the soil (Tanya-Morocho and Leiva-Mora, 2019). Actinomycetes (*Streptomyces albus* and *S. griseus*) are involved in the decomposition of soil organic residues and have an antagonistic effect on phytopathogenic species (Mamani *et al*., 2022).

For this reason, autochthonous microbial species are key to the production of biofertilizers at the local level. Meanwhile, certain crop residues contain sufficient nutrients to artificially reproduce microbes of agronomic interest. In tropical agricultural areas, non-commercial fragments of cassava root (*Manihot esculenta* Crantz) remain in the field during harvest and have nutritional importance for the elaboration of AM, due to their adequate contents of carbohydrates, proteins, dry matter, and minerals, which can be used as nutritive substrates for the reproduction of AM (Rivas *et al*., 2022; Ramírez-Marrache *et al*., 2019).

Considering the above, the objective of the research was to determine the biofertilizer effect of the AM on black tobacco cv. Havana-2000 in open field conditions.

Materials and methods

Location of the experiment, type of soil and its preparation

The research was carried out on the farm "La Poderosa", located at coordinates 20°18'33.75"N and 76°49'20.95"W. This farm belongs to the farmer Eurelvis Hernández Mascapa, associated with the Credit and Services Cooperative "José Arteaga" in the town of Los Cayos, Yara municipality, Granma province, Cuba. The research was conducted during the period November-February/2021. The predominant soil was a Fluvisol of medium consistency, with flat relief, without the presence of obstacles, with a loam-clay texture, MO content of 1.3 %, and a pH of 7. The preparation was carried out with animal traction, using the minimum tillage and the following tasks: breaking (25 cm), tiller (20 cm), and furrowing to a depth of 25 cm.

Autochthonous microorganisms and their characteristics

The AM were prepared on the farm with well water of optimum quality (electrical conductivity (EC): ≤0.8). In 200 L of water, three components were incorporated for the formulation: mineralogical (NaCl, 200 g), microbiological (microbial inoculant from the farm, 200 g of mulch + soil at a ratio of 0.5: 1 $^{v}/_{v}$ and nutritious (400 g of nutritious paste, made from the cooking of residues of *Manihot esculenta* root harvest. Once the three components were added to the vessel, they were homogenized, hermetically sealed, and left to ferment in the dark for 72 h at room temperature (25 ± 3 °C).

The AM recorded the following characteristics: pH 6.6, EC: 1.4, and total microbial populations of bacteria $7x10^{12}$ CFU, fungi $3x10^{11}$ CFU, yeasts $2x10^{11}$ CFU, and actinomycetes $1x10^{10}$ CFU; they were quantified by conventional techniques (Da Silva *et al*., 2018). The present genera of bacteria, fungi, yeasts, and actinomycetes were determined in the laboratory of Agricultural Microbiology, University of Granma, Cuba, by morphological characterization (Saif *et al*., 2021; Condori-Pacsi, 2019; Kim *et al*., 2018; Martínez *et al*., 2015; Trujillo and Hernández, 2015; Allende *et al*., 2013; Cardona, 2011; Said *et al*., 1999).

Determination of the biofertilizer effect of the autochthonous microorganisms on yield variables and components on black tobacco plants cv. Havana-2000 in the open field

Planting was done manually on November 22, 2021, and the crop was established at a traditional distance of 0.90 m x 0.30 m. Fertilization with ammonium nitrate at a dose of 100 $kg.ha^{-1}$ was used throughout the experimental area. The cultural practices of identification, hilling, pruning, irrigation, and pest management were carried out according to the Technical Instructions (Espino *et al*., 2012).

Evaluations

They were performed 50 days after transplanting and before the start of harvest, 25 plants were randomly evaluated for each treatment. The variables evaluated were: the number of leaves (CH), measured by direct leaf count for each plant, leaf length (LH in cm), the central leaf (thin center) was measured from the petiole to the apex with a millimeter ruler, leaf width (AH in cm), measured by the central part of the leaf, and yield (R in $t.ha^{-1}$) was obtained on a dry basis for each treatment and was estimated per hectare.

Treatments, experimental design, and statistical processing

The AM were applied ten days after transplantation at a rate of 0, 12, 24, and 36 $L.ha^{-1}$, these doses were considered the treatments of the experiment. A 4x4 randomized block design (four blocks x treatments) was used. The blocks had an area of 90 m^2 with 333 plants each, subdivided into four plots (22.5 m^2) where the treatments were established. An ANOVA and Tukey's test, p≤0.05 were used, while a principal component analysis was performed among the group of variables studied. The data set met the Shapiro-Wilks normality test, processed with the InfoStat package (StatSoft, 2017).

Results and discussion

Microbiological characterization of autochthonous microorganisms

Four groups of microbes were evidenced in the AM. The bacteria were the most abundant $7x10^{12}$ $CFU.mL^{-1}$, with the registration of two genera *Lactobacillus* sp. and *Rhodopseudomonas* sp., followed by fungi with $3x10^{11}$ $CFU.mL^{-1}$, with three genera *Trichoderma* sp., *Aspergillus* sp. and *Penicillium* sp. Yeasts $2x10^{11}$$CFU.mL^{-1}$ were counted, with two genera *Saccharomyces* sp. and *Candida* sp., while the lowest amount of microbes corresponded to the actinomycete *Streptomyces* sp., with $1x10^{10}$ $CFU.mL^{-1}$. Among the bacteria, *Lactobacillus* sp., presented the largest population (63 %), compared to *Rhodopseudomonas* sp. (37 %). *Trichoderma* sp. (46 %) was the most abundant fungus. *Saccharomyces* sp. (88 %) was the yeast with the highest presence within the AM and *Streptomyces* sp. (100 %) was the only actinomycete counted (table 1).

Table 1. Microbiological populations characterization contained in the MA.

Microorganisms	Genera present	Total populations ($CFU.mL^{-1}$)	Percentage (%)
Bacteria	*Lactobacillus* sp. (Lactic acid) *Rhodopseudomonas* sp. (Photosynthetic)	$7x10^{12}$	63 37
Fungi	*Trichoderma* sp. *Aspergillus* sp. *Penicillium* sp.	$3x10^{11}$	46 40 14
Yeasts	*Saccharomyces* sp. *Candida* sp.	$2x10^{11}$	88 12
Actinomycetes	*Streptomyces* sp.	$1x10^{10}$	100

The mixed culture of these beneficial microbes under optimal conditions of water quality, electrical conductivity, pH, nutrients, and mineralogical content, constitute the main basis for multiplication and exponential growth, which was achieved in the present research. Tlais *et al*. (2023) reported that AM are characterized by the synthesis of secondary metabolites with an effect on biostimulation of plant growth and yield. The foregoing indicates that microorganisms and substances derived from their metabolism constituted the main bioactive ingredients of AM (Pereira *et al*., 2019).

Determination of yield and its components in black tobacco plants cv. Havana-2000 treated with autochthonous microorganisms in field conditions

The analysis of the yield variables and their components showed significant differences between the treatments (Tukey, p≤0.05). The largest number of leaves was achieved with the dose of 36 $L.ha^{-1}$ (7.95), followed by the dose of 24 $L.ha^{-1}$ (7.15) and the lowest values were obtained in the treatment with the lowest dose (6.35) and in T0, where the biofertilizer was not applied (5.95). The length of leaves showed significant differences between treatments (Tukey, p≤0.05). The longest length was achieved with the dose of 36 $L.ha^{-1}$ (33.97 cm), followed by 24 $L.ha^{-1}$ (28.81 cm) and 12 $L.ha^{-1}$ (26.21 cm), while the lowest value was recorded at T0 (20.87 cm) (table 2).

The width of leaves showed significant differences between treatments (Tukey, p≤0.05). The highest width was achieved with the dose of 36 L.ha-1 (19.87 cm), followed by 24 L.ha-1 (16.01 cm) and 12 L.ha-1 (14.25 cm), while the lowest value (10.95 cm) was recorded at T0. Agricultural yield showed significant differences between treatments (Tukey, p≤0.05). The highest value was achieved with 36 L.ha-1 (0.78 t.ha-1), followed by the dose of 24 L.ha-1 (0.66 t.ha-1) and 12 L.ha-1 (0.59 t.ha-1), while the lowest value was recorded at T0 (0.51 t.ha-1) (table 2).

Table 2. Quantity, length, width of leaves and yield in tobacco plants cv. Havana-2000 treated with MA under field conditions.

Treatments (L.ha-1)	CH	LH (cm)	AH (cm)	R (t.ha-1)
0	5.95 a	20.87 a	10.95 a	0.51 a
12	6.35 a	26.21 b	14.25 b	0.59 b
24	7.15 b	28.81 c	16.01 c	0.66 c
36	7.95 c	33.97 d	19.87 d	0.78 d
CV	**6.15**	**6.02**	**7.45**	**9.23**

CH: leaves number, LH (cm): leaves length, AH (cm): leaves width, R (t.ha-1): yields, CV: variation coefficient.

There was evidence of biostimulation of the number of leaves between 6.72 and 33.61 % with AM compared to T0. This result is in the range recorded by Calero-Hurtado *et al*., (2019), who reached 16.66% biostimulation in tobacco cv. SS-2006 with the combined application of AM and the Biobras-16 biostimulant at 64 days after transplantation. Taking into account that the leaves constitute the agricultural product of tobacco, it is considered that their quantity, length, width, and mass, are the most important variables on yield (Jiménez, 2021). Calero-Hurtado *et al*., (2019) showed a greater length of the central leaves of tobacco cv. SS 2006 with the combined treatment AM and the Biobras-16 biostimulant, reaching a biostimulation of 11.32 %.

On the other hand, it was demonstrated biostimulation of the width of leaves between 30.13-81.46 % with AM compared to T0. In this regard, the width of the central leaves of tobacco cv. SS 2006 with a combined treatment of AM and the Biobras-16 biostimulant, achieved a biostimulation of 15.32 % (Calero-Hurtado *et al*., 2019). This value was lower than that recorded in the present study. In this regard, although it was not possible to verify scientific results on the agricultural yield of tobacco with the use of AM, it was known that an agroecological alternative based on green manures (*Zea mays* L., *Vigna unguiculata* (L.) Walp, *Canavalia ensiformis* (L.), *Sesamum indicum* L. and *Sorghum vulgare* Pers., managed to boost the yield between 15-20 % of the cv tobacco. Havana-92, on a differentiated eutric fluvisol soil, in areas of the "Camilo Cienfuegos" Agricultural Production Cooperative, located in the town of Bueycito, Buey Arriba municipality, Granma province, Cuba (Nieto-Martínez *et al*., 2007).

The greater size observed in length and width of the leaves is related to the antioxidant activity of bacteria (*Lactobacillus* sp. and *Rhodopseudomonas* sp.) on plants. These are characterized by enhancing the processes of photosynthesis and accumulation of carbohydrates such as trehalose and raffinose, these organic compounds improve cellular osmotic regulation and plasma membrane integrity, enabling greater growth and tolerance of plants to stress (Naamala *et al*., 2022; Hsu *et al*., 2021).

The biostimulation of yield and its variables was probably due to the integrated beneficial action of AM which are characterized by being antagonists of phytopathogenic species, by mechanisms of antibiosis, parasitism, and induced systemic resistance. Likewise, the AM increase photosynthesis, the absorption of water and nutrients, aspects closely related to an increase in yield and its components (Tanya-Morocho and Leiva-Mora, 2019).

Trichoderma sp., Aspergillus sp., and *Penicillium* sp. In interaction with yeasts and actinomycetes in the plant rhizosphere induce the production of amino acids, organic acids, hormones, vitamins and sugars, substances used by plants for the biostimulation of growth, development and yield processes (Elnahal *et al*., 2022; Feijoo, 2016). The above, constitute scientific arguments that could influence the biostimulation of performance in black tobacco cv. Havana-2000.

The principal component analysis explained that 99.00 % of the variability in the observations was due to component one, while the sum of the two components revealed 90.60 % of the total variability of the model. It was evidenced that the dose of 36 L.ha-1 achieved the greatest association with the variables plant height (AP) and yield (R), followed by 24 L.ha-1 (figure1).

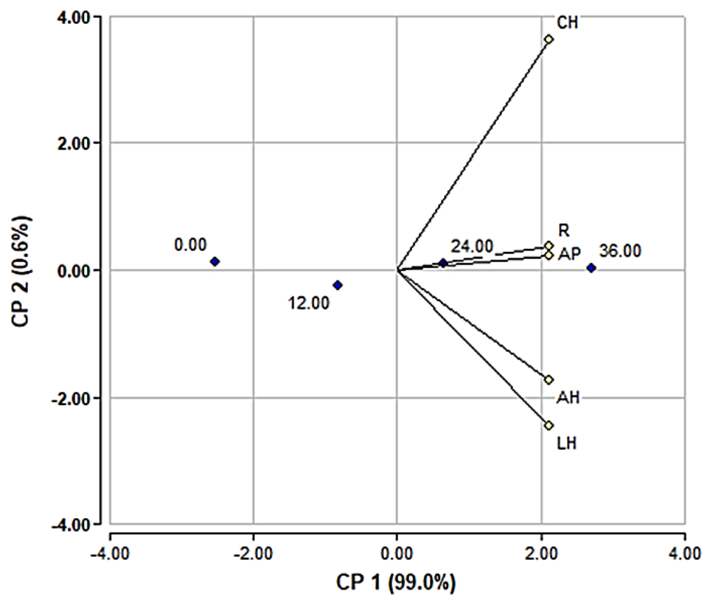

Figure 1. Biplot model between the variables that make up the yield in black tobacco cv. Havana-2000 treated with the autochthonous microorganisms in field conditions. Treatments: 0; 12; 24 and 36 L.ha-1. CH: Number of leaves, AP: Plant height (cm), R: Yield (t.ha-1), AH: Leaf width (cm), and LH: Leaf length (cm).

Ceiro-Catasú *et al*. (2023) recently reported the results of a principal component analysis in *Glycine max* (L.) Merrill, biofertilized with AM in field conditions in eastern Cuba, where they observed that the variables seeds per plant, length of plants, yield, mass of seeds and pods per plant, were associated with the dose of 36 L.ha-1 of AM, while seeds per pod showed a greater association with 24 L.ha-1. Which showed as a tendency, an increase in yield at the highest dose of AM.

Conclusions

The autochthonous microorganisms register four groups of microbes: bacteria (7x10^12 CFU.mL-1) *Lactobacillus* sp. and

Rhodopseudomonas sp., fungi ($3x10^{11}$ CFU.mL^{-1}) *Trichoderma* sp., *Aspergillus* sp., and *Penicillium* sp., yeasts ($2x10^{11}$ CFU.mL^{-1}) *Saccharomyces* sp. and *Candida* sp. and an actinomycete ($1x10^{10}$ CFU.mL^{-1}) *Streptomyces* sp. While, the agricultural yield and components in black tobacco cv. Havana 2000 was boosted between 15-82 % with the AM. This indicates that the use of AM is an organic alternative for crop biofertilization in tropical areas.

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