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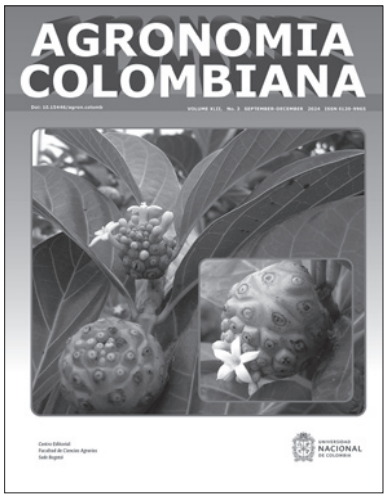

Our cover (photo courtesy of Stanislav Magnitskiy)**:**
Fruits of noni (*Morinda citrifolia* L.) plants

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Protected agriculture in Colombia: A diverse and strategic sector

Agricultura protegida en Colombia: Un sector diverso y estratégico

Human societies demand not only a greater quantity but also an increased variety of agricultural goods. In response to this situation, agricultural researchers have explored various alternatives to meet these demands in the context of constant population growth and environmental deterioration caused in part by agriculture itself. In the most traditional view, the challenge is raised to agricultural communities to produce enough food that would be distributed equitably throughout a population expected to reach 9.8 billion by 2050. It is necessary to emphasize that, according to demographic predictions, global population growth will slow down from that decade on, and in 2100 the human population will be around 10.4 billion. However, this demand is not limited to the production of nutritious, safe, and affordable food. Additionally, more crops are required to provide energy sources (biofuels), raw materials for the pharmaceutical and cosmetic industries, natural fibers, biopolymers, decorative (ornamental) elements, and even inputs for the sports and recreation activities, just to name a few. In Colombia, the agricultural sector also confronts the local and global trade of illicit plant products.

Human creativity has responded to these challenges from different perspectives, implementing various agricultural production systems, most of which share the same bases as for the search for sustainability, although depending on the socioeconomic context and the biophysical conditions of each region. An agricultural production system is understood as a form of organization and administration of resources that enables the generation of agricultural goods. Thus, we can infer that production systems depend on features such as environmental conditions, market characteristics, available resources, and the emphasis placed on sustainability's significance. This creates a wide diversity of agricultural production systems, ranging from traditional open-field crops to those cultivated in protected environments which permit at least partial control of environmental factors.

Protected environments appear to be among of the most important innovations in modern agriculture. A distinctive feature of these systems is the use of structures that protect cultivated plants against biotic and abiotic factors that limit their productivity. These structures are designed to generate favorable microclimatic conditions that, together with the efficient use of water, mineral nutrients, and energy, allow for increased yield. For the latter, we work together with other trends in modern agriculture, such as process automation.

In protected crop systems, the most common structure is a greenhouse, which in the Colombian context is employed to produce ornamental plants, especially cut flowers, such as roses, hydrangeas, carnations, chrysanthemums, and alstroemerias. Greenhouses are also used to produce vegetables (lettuce and tomatoes), potted plants (poinsettias and bromeliads), and other species. In addition, the structures locally called semi-covers provide partial protection against adverse weather conditions during the cultivation of fruit crops, such as passion fruits. Smaller structures, such as macro tunnels, are used to cultivate blueberries, and photoselective nets are employed to provide shading in the propagation areas of woody species. This heterogeneity of structures responds to the inherent variations in the country's geography, as well as to the markets for which the products are intended and the resources available to growers with each system.

Except for the cut flower industry, in other agricultural sectors of Colombia, it is difficult to quantify the use of protective covers. An estimate based on the land area by the Center for Innovation in Colombian Floriculture (Ceniflores) and the Ministry of Agriculture and Rural Development (MADR) indicates that greenhouse-grown plants occupy between 15,000 and 18,000 ha. At first glance, this area seems to be marginal compared with other crops such as coffee (≈850,000 ha), oil palm (≈600,000 ha),

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rice (between 400,000 and 500,000 ha), and sugarcane (≈250,000 ha). However, plants cultivated under protected conditions represent considerable economic value, as the cut flower sector alone is expected to generate export revenues of more than US $ 2 billion by 2023. This illustrates the intensive nature of cultivation in protected environments, in terms of resource use and the need for knowledge generation when creating diverse agricultural products.

Although it is risky to predict the trend of agricultural production in protected environments in Colombia, it is very likely that the cultivation areas will increase in the coming years because of the new species moving from open-field conditions to protected systems. In more consolidated sectors, such as cut flowers, there is already evidence of a technological transition to a more efficient use of resources considering water as a priority, together with the automation of labors such as fertigation. It is important for the academic community to endorse this process. This implies the training of professionals capable of crop production in protected environments as well as the active participation of Agricultural faculties in strategic studies, including a greater use of biological tools and further development of extension programs. These efforts will contribute to increasing efficiency, sustainability, and profitability for growers.

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Clustering of environments in response to the cultivation of black oat genotypes

Agrupación de ambientes en respuesta al cultivo de genotipos de avena negra

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ABSTRACT

The aim of this research was to study the genotype-environment interaction with a focus on decomposing the simple and complex parts of the interaction of black oat genotypes and clustering similar environments. The experiment was conducted at the Federal University of Santa Maria, Campus de Frederico Westphalen/RS (Brazil), in 2018, 2019, 2020, and 2021, using five lines and two cultivars of black oat in a randomized block experimental design. The traits evaluated were total dry mass and seed productivity. After verifying the presence of interaction between genotypes and environments, the interaction was decomposed into simple and complex parts. The UFSMFW 2-07 line stood out in terms of total dry mass and seed productivity. The total dry mass and seed productivity of black oat genotypes were highly influenced by environmental conditions over the four years of cultivation, highlighting the complexity of studying the genotype-environment interaction for more than one trait of interest at the same time.

Key words: *Avena strigosa,* cultivation environment, phenotype, correlation.

RESUMEN

El objetivo de este trabajo fue estudiar la interacción del genotipo y el ambiente con atención en la descomposición de la parte simple y compleja de la interacción de los genotipos de avena negra y la agrupación de ambientes similares. El experimento fue realizado en la Universidade Federal de Santa Maria, Campus de Frederico Westphalen/RS (Brasil), en los años 2018, 2019, 2020 y 2021, con 5 líneas y 2 cultivares de avena negra, en un diseño experimental de bloques al azar. Las características evaluadas fueron masa seca total y producción de semillas. Una vez verificada la presencia de interacción entre genotipos y ambientes, la interacción se descompuso en partes simples y complejas. La línea UFSMFW 2-07 se destacó por masa seca total y producción de semillas. La masa seca total y la producción de las semillas de los genotipos de avena negra estuvieron altamente influenciadas por las condiciones ambientales durante los cuatro años de cultivo, lo que muestra la complejidad de estudiar la interacción del genotipo y el medio ambiente para más de un rasgo de interés al mismo tiempo.

Palabras clave: *Avena strigosa*, ambiente de cultivo, fenotipo, correlación.

Introduction

Black oat (*Avena strigosa* Schreb.) is one of the most cultivated winter cereals in Rio Grande do Sul (Brazil) due to its important agronomic characteristics such as fast growth, rusticity, high forage productivity, and ease of seed production, making it a versatile crop (Debiasi *et al*., 2007). Black oat is an excellent option for soil cover due to its high potential for biomass production; it can also be used in forage production (Leite *et al*., 2012) and has become an important crop in production systems.

With the growing importance of black oats in the market, developing new cultivars has gained significance. The selection and recommendation of more productive genotypes are basic objectives of genetic breeding programs for cultivated species (Cargnin *et al*., 2006). To this end, experiments are carried out where different genotypes of black oat are evaluated in a series of environments, which may be locations or years of cultivation (Olivoto *et al*., 2019).

The phenotype in plants is determined by the genotype and the environment. However, these two effects are not always additive, indicating the presence of the genotype-environment interaction. Genotype-environment interaction refers to the variation in performance of a genotype in different environments (Sharifi *et al*., 2017). The existence of this interaction, depending on its nature (simple or

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complex), directly impacts the process of selecting superior genotypes, as well as the recommendation of cultivars (Cruz *et al*., 2014).

There are several available methods for quantifying the interaction between genotypes and environments, including those based on analysis of variance (Plaisted & Peterson, 1959), simple linear regression (Eberhart & Russell, 1966; Finlay & Wilkinson, 1963) and multiple linear regression (Cruz *et al*., 1989; Verma *et al*., 1978). More recent methods include the Additive Main effect and Multiplicative Interaction method (AMMI), the Genotype plus Genotype and Environment interaction method (GGE), and the Best Linear Unbiased Prediction method (BLUP) via restricted maximum likelihood (REML) or analysis of variance (Souza *et al*., 2021).

The genotype-environment interaction occurs due to changes in the performance of the genotypes from differences in environment. Estimating the magnitude of the interaction and its decomposition into simple and complex parts using the method proposed by Cruz and Castoldi (1991) is a strategy to consider. The decomposition of simple and complex interactions between genotypes and environments was used by Cargnin *et al*. (2006) with wheat, Nunes *et al*. (2006) with corn, Nunes *et al*. (2011) with melon, Pinto *et al*. (2012) with coffee, and Cardoso *et al*. (2019) with cotton.

The interaction of the genotype with the environment occurs when the phenotype undergoes changes because of the environment. The interaction changes due to changes in the proportion of genetic and environmental variation in the expression of the phenotype (Allard, 1999). In this sense, it is possible to decompose the interaction between genotypes and environments and, based on the decomposition, to group similar environments. The aim of this research was to study the genotype-environment interaction with a focus on decomposing the simple and complex parts of the interaction of black oat genotypes and clustering similar environments.

Materials and methods

The experiment was conducted in 2018, 2019, 2020, and 2021 at the Federal University of Santa Maria, Campus of Frederico Westphalen/RS, Brazil (27°23'26" S, 53°25'43" W, 461.3 m a.s.l.). The climate, according to Köppen, is classified as Cfa, humid subtropical, with an average annual precipitation of 2,100 mm (Alvares *et al*., 2013).

Five homozygous lines of black oat (UFSMFW 2-01, UFSMFW 2-02, UFSMFW 2-04, UFSMFW 2-05, and UFSMFW 2-07) and two commercial cultivars (UPFA 21-Moreninha and IPR Cabocla) were tested. A randomized block experimental design was used, with three replicates. Each experimental unit was composed of six rows of 5 m in length, spaced 0.17 m apart, with a sowing density of 300 seeds per m². Sowing in the four years of cultivation was carried out at the end of May. Fertilization was based on soil analysis and the cultural treatments were applied as needed to control weeds, pests, and diseases whenever necessary, following the technical indications for black oat cultivation.

The traits analyzed were a) total dry aerial mass (kg ha^{-1}) measured after cutting an area of 1 m² at full flowering (50% inflorescence), followed by drying in an oven (70°C) to constant mass, and b) seed productivity (kg ha^{-1}) measured by collecting seeds from the plots after physiological maturation.

Meteorological data, including total fortnightly precipitation from May to October for the four years of cultivation (2018, 2019, 2020, and 2021) were collected from the Frederico Westphalen/RS automatic station (A854) and presented graphically.

Statistical analysis

To identify interactions between genotypes and environment for the traits studied, joint analysis of variance (ANOVA) and the F test ($P<0.05$) were performed, using the statistical model below:

$$Y_{ijk} = \mu + G_i + A_j + GA_{ij} + B/A_{jk} + \varepsilon_{ijk} \quad (1)$$

where Y_{ijk} = observation obtained in the plot with *i-th* genotype in the *j-th* block, μ = general mean of the experiment, G_i = effect of the *i-th* genotype considered fixed, A_j effect of the *j-th* environment considered fixed, GA_{ij} = effect of the *i-th* genotype interaction with the *j-th* environment, B/A_{jk} = effect of the *k-th* block within the *j-th* environment, considered random and ε_{ij} = random error effect.

The genotype-environment interaction was decomposed into simple and complex using the methodology proposed by Cruz and Castoldi (1991): simple: $0.5(\sigma_1\ \sigma_2)$, where σ_1 and σ_2 are the genotypic variance for the environments 1 and 2, and complex,

$$(\sqrt{(1-r)^3 Q_1 Q_2}) \quad (2)$$

where *r* is the correlation coefficient between the means of the genotypes from both locations, and *Q*1 and *Q*2 are the mean squares of the genotypes in environments 1 and 2.

Environments were grouped using the average Euclidean distance and the UPGMA clustering method. Data were analyzed using R software, version 4.0.2 (R Core Team, 2020) and the "metan" package (Olivoto & Lúcio, 2020).

Results and discussion

Joint analysis of variance revealed significant effects ($P \leq 0.05$) for genotypes, environment, and their interactions for total dry mass and seed productivity. The coefficients of variation were, respectively, 7.22% and 6.35% for the total dry mass and seed productivity, indicating reliable data. These results demonstrate variation in the response of the genotypes tested over the four years of cultivation. The occurrence of interactions between genotypes and environments makes the selection and recommendation of new cultivars more difficult (Silva *et al*., 2011).

The fluctuation in the response of the genotypes to the years of cultivation can be seen in Figure 1. The year 2019 was the most favorable for total dry mass productivity, with averages exceeding the general average of the trial. The years 2020 and 2021 had averages lower than the general average (Fig. 1) due to the low volume of accumulated precipitation for the period from the second half of August to the second half of October (Fig. 2). The year 2021 was marked by a dry period, followed by another very rainy one, extreme conditions that harmed the growth of the genotypes. Plant phenology is influenced by water availability and air temperature (Oteros *et al*., 2015), which can alter genotype performance and the rankings.

UFSMFW 2-07 line stood out in terms of total dry mass productivity, reaching more than 10,000 kg ha^{-1}. In terms of seed productivity UFSMFW genotypes showed potential, with the UFSMFW 2-07, reaching a seed productivity in 2019 exceeding 4,000 kg ha^{-1}. The highest productivities of total dry mass and of seeds were obtained in 2018 and 2019, while 2020 and 2021 showed lower values due to significant

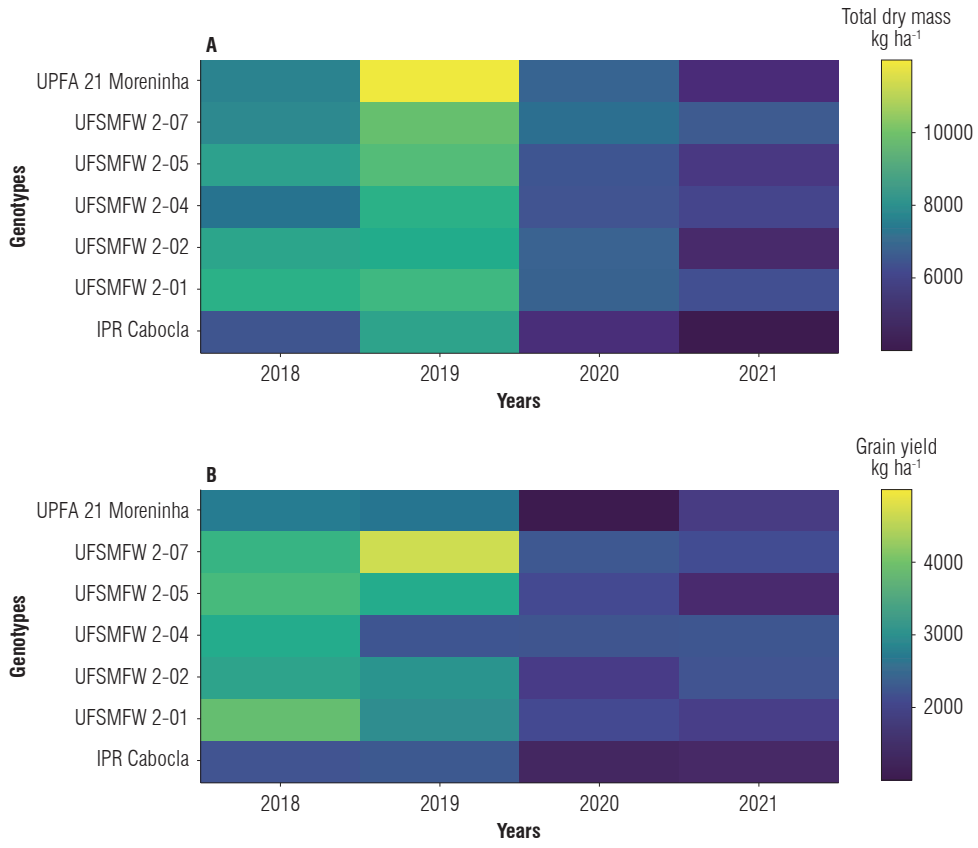

FIGURE 1. Overview of genotype-environment interaction for total dry mass (A) and seed productivity (B) of seven black oat genotypes cultivated in 2018, 2019, 2020, and 2021.

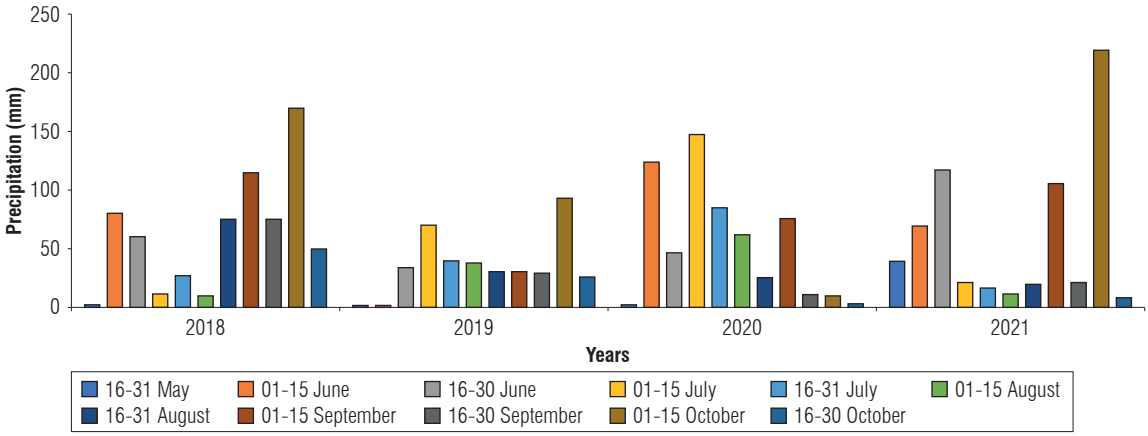

FIGURE 2. Accumulated biweekly precipitation, covering the months of May to October, in 2018, 2019, 2020 and 2021 in the municipality of Frederico Westphalen/RS, Brazil.

drought conditions (Fig. 2). Benin *et al*. (2005) emphasize that the production of white oats is highly influenced by the environment, which results in variable productivity over the years.

Cruz *et al*. (2012) classify genotype-environment interaction into simple and complex parts. The simple part reflects the different responses of genotypes to environmental variations without changes in the ranking of genotypes, while the complex part involves changes in the ranking. When a complex interaction occurs, a top-performing genotype in one environment may perform poorly in another. The fractionation of the interaction revealed a predominance of the complex type of interaction for the total dry mass characteristic, showing the inconsistency of the genotypes in relation to environmental variation, and a predominance of the simple type for the seed productivity characteristic (Tab. 1). Cargnin *et al*. (2006) found a predominance of the complex part of the interaction in wheat for the traits of days from emergence to heading, plant height, grain mass and grain yield.

Complex interaction values greater than 50% hinder genotype selection across environments (Sousa *et al*., 2015). They also indicate the presence of genotypes adapted to specific environments (Costa *et al*., 2015). In the same sense, according to Santos *et al*. (2016), in the presence of a complex interaction, the analysis of the interaction does not provide sufficient information about the performance of the genotypes studied. In view of the above and considering the results presented in Table 1, seed productivity was more influenced by the simple environmental effect, that is, constant conditions from one year to the next.

To simplify the environments according to the fraction of interaction, the environments were grouped using the Unweighted Pair-Group Method Using an Arithmetic Average (UPMGA), based on the correlation between environments for total dry mass and seed productivity following the method of Cruz and Castoldi (1991) (Figs. 3A and 3B, respectively). For total dry mass, the simple interaction showed a similarity between 2018 and 2021, grouped together, while 2019 and 2020 formed two separate groups.

TABLE 1. Pairs of environments, correlation between environments, percentage of the simple part of the interaction (SPI) and complex part of the interaction (CPI), resulting from the decomposition of the interaction between genotypes and environment pairs, according to the methodology of Cruz and Castoldi (1991), for total dry mass and seed productivity in black oat.

Environment pairs	Total dry mass			Seed productivity		
	Correlation	SPI	CPI	Correlation	SPI	CPI
2018 and 2019	0.36	51.87	48.13	0.61	52.59	47.41
2018 and 2020	0.60	36.91	63.09	0.88	83.10	16.90
2018 and 2021	0.39	22.14	77.86	0.56	69.16	30.84
2019 and 2020	0.84	87.96	12.04	0.66	76.80	23.20
2019 and 2021	0.32	48.88	51.12	0.23	66.20	33.80
2020 and 2021	0.71	46.56	53.44	0.61	51.76	48.24
Total	-	49.05	50.95	-	66.59	33.41

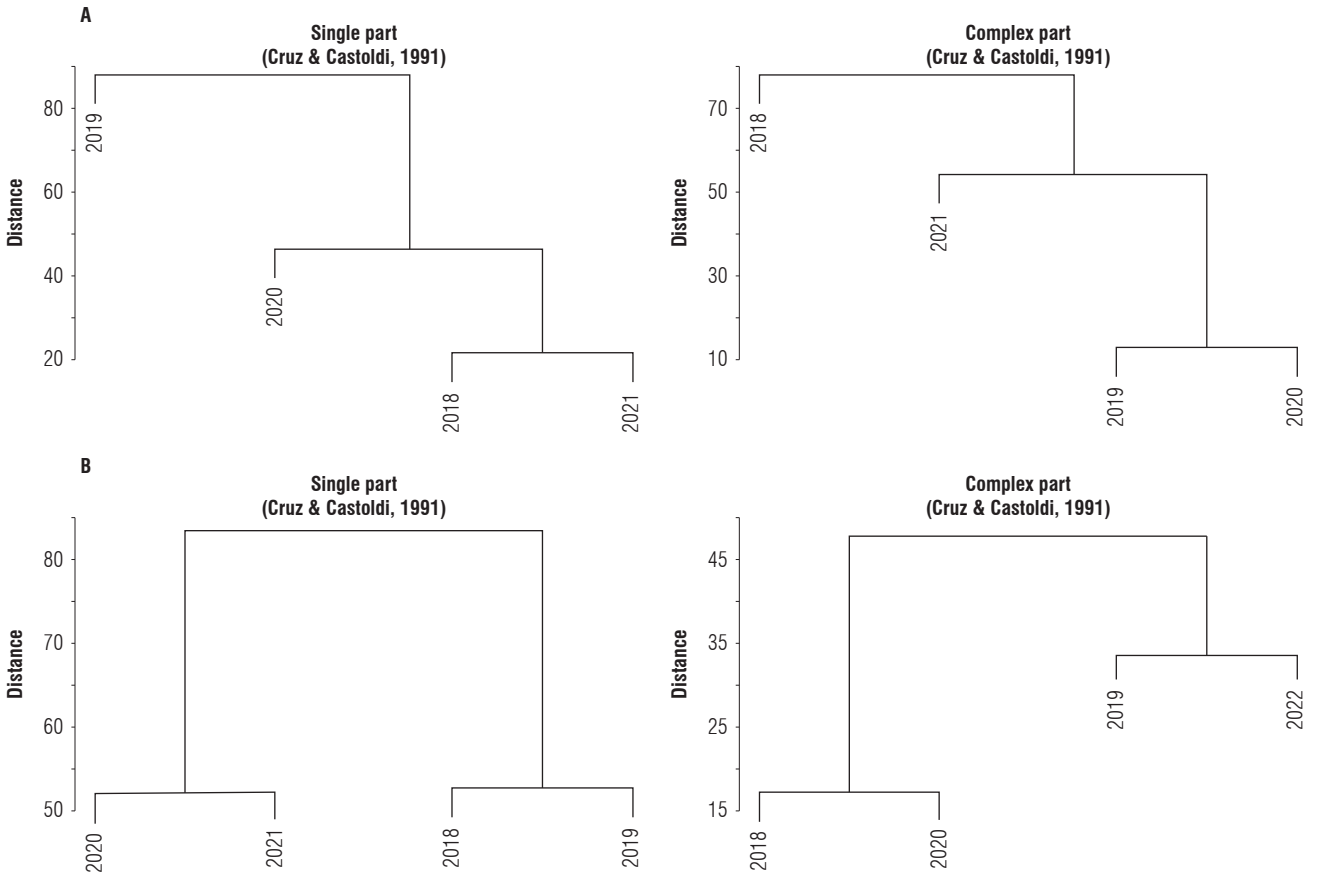

FIGURE 3. Clustering of the years 2018, 2019, 2020 and 2021 using the UPMGA method for total dry mass (A) and seed productivity (B).

In the complex part of the interaction, the clustering revealed similarity for the genotypes of the years 2019 and 2020, while in 2018 and 2021 they formed isolated groups.

For the seed productivity characteristic, the simple part of the interaction revealed the formation of two groups: 2018 and 2021 were in one group, and 2019 and 2020 in another group. When considering precipitation (Fig. 2), similarities were observed in the distribution of rainfall between the years 2018 and 2021 and between 2019 and 2020. As for the complex part, two groups were also formed: one for 2018 and 2020, and another for 2019 and 2021. These results indicate that environments can be similar for one characteristic, but different for another, making genotype-environment interaction studies difficult.

Conclusion

The results indicate variation in the responses of the genotypes across the years of cultivation, with genotype-environment interactions that make it difficult to select new cultivars. The decomposition of the interaction revealed a predominance of the complex part of the interaction for the total dry mass and the simple part of the interaction for the seed productivity trait. The grouping of the environments showed differences in the interactions, highlighting the complexity in the analysis of the effects of the genotype and the environment.

Conflict of interest statement

The authors declare that there are no conflict of interests regarding the publication of this article.

Author's contributions

VSM and LAK: conceptualization, methodology, resources, visualization, data curation, formal analysis, writing - original draft, writing - original draft, review, and editing. DM and TO: conceptualization, data curation, formal analysis, writing - original draft, review, and editing. DFO, CBF, TASL, KMK, and ECMI: conceptualization, and editing. All authors have read and approved the final version of the manuscript.

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Screening for drought tolerance using physiological traits in upland cotton (*Gossypium hirsutum* L.)

Detección de tolerancia a la sequía utilizando rasgos fisiológicos en algodón americano (*Gossypium hirsutum* L.)

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ABSTRACT

Climate change patterns indicate a serious threat to freshwater availability for crops. A selection of drought-tolerant genotypes is essential for breeders. Three key physiological parameters of abiotic stress: relative water content, excised leaf water content, and cell membrane stability were assessed with 68 upland cotton genotypes in Pakistan. The most tolerant and susceptible genotypes were evaluated in a greenhouse under controlled conditions. From the selected genotypes, heat and drought stress-related transcription factors were screened and included the following: GhNAC2, DREB2A, GhABF2, HSC70, HSFA2, GbMPK3, GbMpK17, GhMKK1, APX1, GHSP26, TPS, ANNAT8, GhMPK2, GhMKK3, GhWRKY41, HSPCB, HSP101, HSP3, GhPP2A1, and GbMYB5. Cell membrane stability may be a screening criterion for drought tolerance in cotton under field and greenhouse conditions. Under these conditions, the physiological and molecular analyses revealed that the genotypes CRIS-134, BH-184, and FH-114 were the most tolerant, and the genotypes CIM-240, CIM-446, and FH-900 were susceptible. The selected tolerant varieties can be recommended for cultivation in drought-prone areas. They can be used in future breeding programs for drought tolerance in cotton.

Key words: transcriptional factors, physiological analysis, cell membrane stability, relative water content.

RESUMEN

Los patrones de cambio climático indican una amenaza seria a la disponibilidad de agua dulce para las plantas cultivadas. La selección de genotipos tolerantes a la sequía es esencial para los fitomejoradores. Se evaluaron tres parámetros fisiológicos clave del estrés abiótico: contenido relativo de agua, contenido de agua en hojas extirpadas y estabilidad de la membrana celular, en 68 genotipos de algodón americano "Upland" en Pakistán. Los genotipos más tolerantes y susceptibles se evaluaron en invernadero bajo condiciones controladas. Se evaluaron los factores de transcripción relacionados con el estrés por calor y sequía en los siguientes genotipos seleccionados: GhNAC2, DREB2A, GhABF2, HSC70, HSFA2, GbMPK3, GbMpK17, GhMKK1, APX1, GHSP26, TPS, ANNAT8, GhMPK2, GhMKK3, GhWRKY41, HSPCB, HSP101, HSP3, GhPP2A1 y GbMYB5. La estabilidad de la membrana celular puede utilizarse como criterio de selección para la tolerancia a la sequía en el algodón, tanto en condiciones de campo como de invernadero. En condiciones de campo y de invernadero, el análisis fisiológico y molecular reveló que los genotipos CRIS-134, BH-184 y FH-114 fueron los más tolerantes, y los genotipos CIM-240, CIM-446 y FH-900 fueron susceptibles. Las variedades resistentes seleccionadas pueden recomendarse para el cultivo en áreas propensas a la sequía y pueden utilizarse en futuros programas de mejoramiento para la tolerancia a la sequía en el algodón.

Palabras clave: factores transcripcionales, análisis fisiológico, estabilidad de la membrana celular, contenido relativo de agua.

Introduction

Crop production is affected by several environmental factors brought on by global climate change (Farooq *et al*., 2022; Raza *et al*., 2019). Crops and food security are negatively impacted by climate change and global warming (Jia *et al*., 2022). Rising temperature trends and water scarcity are two significant problems. Drought stress affects the number of crops, mainly those grown in arid and semi-arid regions (Alamri *et al*., 2020; Varshney *et al*., 2021). Reduced precipitation and altered rainfall patterns lead to drought stress worldwide (Cheng *et al*., 2021). Drought means reduced water availability over an extended period (Abdelraheem *et al*., 2015).

Drought stress affects yields and crop development in cotton. The built-in defense mechanisms in crops account for morphological changes such as small, thick leaves, thick cuticles, and waxy and hairy leaf surfaces that reduce water loss through transpiration (Waghmare, 2022). Multiple

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genes and environmental stimuli regulate drought tolerance in plants. Breeding for drought tolerance requires a genetic combination that enables plants to withstand drought stress. Pyramiding different traits into a single genotype requires source population and recurrent selection. Developing drought-tolerant cultivars needs specific genotypes with physiological traits related to drought tolerance. Water deficiency during the fiber growth phase negatively impacts cotton fiber quality, which shortens fiber length. Physiological processes, such as relative water content and excised leaf water loss, were disturbed by dry conditions of soil during the blooming stage (Makamov *et al*., 2023).

Different transcription factors, signaling genes, and functional genes have been identified at the molecular level under abiotic stress in normal plant growth. The cytosolic APX gene has a heat shock element in its promoter region, which enables the production of quick heat responses (Storozhenko *et al*., 1998), drought responses (Smirnoff & Colombé, 1988), salt and ABA responses (Shi *et al*., 2001). The transcription factors DREB (dehydration response element binding proteins) are linked to drought tolerance (Zhang *et al*., 2020). They can be utilized for crop genetic improvement (Niu *et al.,* 2020).

The development, physiology, biochemistry, and reproduction of plants are affected by severe drought, which ultimately reduces agricultural output (Cui *et al*., 2020; Rai *et al*., 2021; Yang *et al*., 2019). The effect, duration, and intensity of drought, plant genetics, and growth stage of plants all play a role in determining plant drought tolerance (Varshney *et al*., 2021).

Cotton is susceptible to environmental changes (Yehia *et al*., 2022). Reduced availability of irrigation water, erratic rainfall patterns, and heat stress are the main risks to cotton production (Ahmed *et al*., 2022). The effects of drought stress on cotton include a reduction in shoot and root length, a blockage of vascular tissues, and a reduction in cell elongation (Mahmood *et al*., 2022). As a result, plants grown under various stress conditions exhibit altered photosynthesis, chlorophyll content, fluorescence, and cell membrane thermostability (Azhar *et al*., 2009). In particular, relative cell injury can be used to assess heat tolerance (Zhang *et al*., 2014).

The water content of plants regulates various physiological and metabolic processes (Mubeen *et al*., 2012). Reducing water content negatively impacts crop plant growth, *i.e*., cotton growth from seedling to fiber maturity (Farooq *et al*., 2009). Saleem *et al*. (2015) indicated that relative water content, excised leaf water loss, and cell membrane stability are variables of quantitative nature in cotton. Cell membrane integrity is an important physiological trait for screening drought-tolerant plants in water limiting conditions (Levitt, 1980). Cellular dehydration negatively affects membranes, increasing their permeability. Various studies on genotypic variations in cell membrane stability have linked these variations to economic yield losses under water stress for many crops (Ashraf *et al*., 1992; Saneoka *et al*., 2004; Tripathy *et al*., 2000). Stomatal closure and decreased activity of photosynthetic enzymes are two coordinated phenomena contributing to decreased photosynthetic activity under water stress (Aranjuelo *et al*., 2011).

Water shortage builds up free solutes in cells, which lower osmotic potential; and due to water loss, the concentration of cellular solution can lower osmotic potential. When cellular water deficit rises above a particular threshold, osmotic adjustment occurs (Singh, 2015). The damage in cellular membranes causes leakage of numerous cellular solutes, including electrolytes. The electrical conductivity of the liquid in which the impacted leaf sample is placed detects electrolyte leakage. The method is applied to determine relative damage or stability of the cells by comparing leakage from stress-affected samples with leakage from control samples. Cell membrane stability is an important parameter to resist dehydration in leaves under stress (Singh, 2015).

Cotton is one of the most important fiber crops in the world, and it is severely impacted by drought stress compared to other crops (Yasmeen *et al*., 2016). Drought stress decreases cellular growth (Turner *et al*., 1986), root and stem growth (Hearn, 1994), number of fiber bolls per plant (Oosterhuis, 2000), relative water content, cell membrane integrity (Khan *et al*., 2011), and cotton yield (Yagmur *et al*., 2014). Both morphological and physiological characteristics, such as cell membrane integrity, excised leaf water loss, and relative water content, contribute to drought resistance. Drought resistance may be improved by plant mechanisms that keep the water content of leaves stable under drought stress (Xoconostle *et al*., 2010). Relative water content measures a plant's ability to retain water, the most crucial defensive characteristic under drought stress (Tahara *et al*., 1990). Similarly, preserving cell membrane integrity is another defensive strategy under drought stress (Bajji *et al*., 2002). Breeding for drought tolerance may be improved by using genetic regulation of these properties.

Plants have acquired different mechanisms to develop tolerance against drought stress (Batool *et al*., 2020; Mahmood *et al*., 2021). They employ avoidance, escape, and tolerance

to resist drought stress (Galindo *et al*., 2018). Phenotypic selection methods are ineffective against complex traits such as drought tolerance with narrow heritability (Abdelraheem *et al*., 2015). It is possible to study the inheritance of drought tolerance through secondary techniques such as selection through secondary traits and correlation estimates (Saeed *et al*., 2011). The third method compares genotypes under stressed and non-stressed environments, assesses germplasm drought resistance and susceptibility, and ranks genotypes (Abdelraheem *et al*., 2015).

This study was aimed at: (i) screening cotton germplasm for drought tolerance, (ii) developing breeding material for cotton, and (iii) investigating the molecular and physiological traits contributing to drought tolerance.

Materials and methods

Experiment 1

We collected a set of 68 cotton genotypes from different cotton research stations in Pakistan, *i.e*., CCRI Multan, CRS Faisalabad, CRS Multan, CRS Vehari, CRS Sakrand, and NIAB for use in the experiment in 2022-23. We grew the genotypes in a three-replicate trail in the field. We planted the experiment in sandy-loam soil, which is standard for cotton growth. The average temperature from germination to the flowering stage was recorded at 25-33°C and the average relative air humidity at 30-40%. We applied recommended local agronomic practices. We calculated physiological traits, including relative water content, excised leaf water loss, and cell membrane stability for all the genotypes under study as follows:

Relative water content (%) of the leaf samples was calculated using the following formula (Clark & Townley-Smith, 1986):

$$\mathrm{RWC} = \left[\frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}}\right] \times 100 \quad (1)$$

Excised leaf water loss (%) was calculated using the following formula (Clarke & McCaig, 1982):

$$\mathrm{ELWL} = \frac{\text{Fresh weight} - \text{Wilted weight}}{\text{Dry weight}} \times 100 \quad (2)$$

Cell membrane stability (%) of the leaf discs as the reciprocal of relative cell injury was calculated using the following formula (Blum & Ebercon, 1981):

$$\mathrm{CMS} = \left[\frac{\left\{1-\left(\frac{T1}{T2}\right)\right\}}{\left\{1-\left(\frac{C1}{C2}\right)\right\}}\right] \times 100 \quad (3)$$

where

T_1 = Stressed sample conductance before autoclaving;

T_2 = Stressed sample conductance after autoclaving;

C_1 = Control sample conductance before autoclaving;

C_2 = Control sample conductance after autoclaving

Experiment 2

We selected a total of 15 tolerant and susceptible genotypes (based on cell membrane stability) grown in a greenhouse in three replicates under controlled environmental conditions. A total of nine plants of each genotype were grown per replicate. Optimum temperature and relative humidity were maintained for cotton's healthy growth under greenhouse conditions. Physiological traits such as RWC, ELWL, and CMS were evaluated as described in Experiment 1.

Molecular analysis

We extracted DNA from 15 selected genotypes using the standard cetyltrimethylammonium bromide (CTAB) method (Doyle, 1990). We used important drought/heat-related transcription factors, such as HSPCB, GHSP26, HSFA2, HSP101, HSP3, DREB1A, DREB2A, TPS, GhNAC2, GbMYB5, GhWRKY41, GhMKK3, GhMPK17, GhMKK1, GhMPK2, APX1, HSC70, ANNAT8, and GhPP2A1 for genotype screening (Saleem *et al*., 2020). We performed PCR analysis using specific primers for all selected genotypes. We separated the amplification products on a 1% agarose gel in 1x TBE buffer stained with ethidium bromide on a 100 bp ladder (Life Technologies Gibco BRL).

Statistical analysis

We calculated an analysis of variance (ANOVA) of all traits (Steel *et al*., 1997). Correlations for all characteristics were calculated to find an association between traits (Kwon & Torre, 1964). We used a t-test to compare RWC, ELWL, and CMS variations between the field and greenhouse data (Usman, 2016).

Results

Experiment 1

Relative water content: The genotypes NIAB-777 (89.19%), BH-184 (84.45%), and CIM-473 (83.79%) had the maximum relative water content in the field conditions. The genotypes CIM-240 (11.26%), CIM-446 (11.65%), and FH-900 (13.24%) showed minimum relative water content in the field (Tab. 1).

Excised leaf water loss: The genotypes CRIS-508 (0.24%), CRIS-9 (0.26%), and FH-114 (0.29%) showed minimum

excised leaf water loss under normal conditions. The genotypes CIM-446 (3.67%), CIM-240 (3.67%), and FH-900 (3.68%) had maximum excised leaf water loss in the field (Tab. 1).

Cell membrane stability: The genotypes BH-184 (80.61%), CRIS-134 (79.12%), and FH-114 (77.14%) had maximum cell membrane stability in the field. The genotypes CIM-446 (46.19%), CIM-240 (47.76%), and FH-900 (48.54%) showed minimum cell membrane stability in the field (Tab. 1).

Experiment 2

Out of 68 genotypes, twelve drought-tolerant and three drought-susceptible genotypes were evaluated in the greenhouse.

Relative water content: The genotypes CRIS-134 (76.16%), CRIS-9 (76.06%), and NIAB-777 (74.29%) showed maximum, whereas the genotypes CIM-240 (44.34%), CIM-446 (46.27%) and FH-900 (47.27%) showed minimum relative water content (Fig. 1A).

TABLE 1. Cotton genotypes selected based on cell membrane stability, relative water content, and excised leaf water loss.

Sr. No.	Genotypes	RWC	ELWL	CMS	Sr. No.	Genotypes	RWC	ELWL	CMS
1	CIM-63	54.58	1.23	56.43	35	FH-900	13.24	3.68	48.54
2	CIM-678	52.70	1.36	52.33	36	FH-118	46.47	1.89	67.50
3	CIM-785	44.33	1.06	51.42	37	CKC-2	63.04	1.75	65.11
4	CIM-343	53.00	1.34	54.13	38	CKC-3	36.82	1.44	64.93
5	CIM-600	57.49	1.67	61.95	39	MNH-552	77.53	0.58	70.35
6	CIM-240	11.26	3.67	47.76	40	MNH-554	58.97	1.17	66.66
7	CIM-109	41.25	1.76	64.83	41	MNH-147	60.28	1.23	67.50
8	CIM-499	70.67	0.31	70.25	42	MNH-1026	77.62	0.63	70.41
9	CIM-70	63.80	2.27	60.71	43	MNH-988	46.83	1.81	62.72
10	CIM-620	56.82	1.45	60.65	44	BH-184	84.45	1.92	80.61
11	CIM-443	65.80	1.27	61.81	45	BH-121	54.84	1.63	62.85
12	CIM-616	55.95	1.56	61.99	46	BH-118	45.69	1.45	66.66
13	CIM-506	59.95	1.52	62.54	47	G-93	61.87	1.36	57.14
14	CIM-446	11.65	3.67	46.19	48	G-105	82.72	2.09	60.00
15	CIM-632	46.26	1.52	56.25	49	AA-802	56.20	2.08	51.51
16	CIM-599	55.98	1.45	60.71	50	SLH-334	40.27	1.48	66.66
17	CIM-707	51.58	1.67	67.50	51	SLH-337	33.36	1.47	57.00
18	CIM-473	83.79	0.77	75.05	52	CRIS-134	75.99	0.96	79.12
19	CIM-482	58.31	1.13	56.25	53	CRIS-533	75.57	2.63	60.60
20	CIM-554	58.94	1.00	64.93	54	CRIS-510	57.40	2.18	53.96
21	CIM-534	57.62	2.45	67.50	55	CRIS-508	80.94	0.24	70.71
22	CIM-598	52.63	1.24	52.72	56	CRIS-121	75.15	0.34	69.81
23	CIM-573	53.54	1.36	60.00	57	CRIS-9	76.43	0.26	71.83
24	CYTO-515	52.50	1.45	59.37	58	CRIS-129	78.98	1.98	73.26
25	NIAB-111	58.50	1.28	62.74	59	SLH-317	60.42	2.24	67.50
26	NIAB-878	46.30	1.36	64.02	60	VH-Gulzar	68.53	2.61	65.00
27	NIAB-112	58.32	1.30	63.33	61	VH-189	46.94	2.37	58.69
28	NIAB-777	89.19	0.89	72.80	62	VH-418	45.27	1.99	54.54
29	BS-20	42.24	1.78	62.67	63	VH-383	44.92	1.65	58.82
30	FH Lalazar	53.77	1.87	67.50	64	VH-351	70.43	2.80	66.02
31	FH-114	70.83	0.29	77.14	65	VH-355	39.38	1.33	51.42
32	FH-444	44.98	1.38	51.81	66	VH-305	63.02	2.02	65.00
33	FH-490	38.36	1.64	60.00	67	VH-402	47.76	1.61	67.54
34	FH-113	62.34	1.24	70.00	68	Sitara-008	47.36	1.60	60.56

RWC – relative water content (%), ELWL – excised leaf water loss (%), and CMS – cell membrane stability (%).

Excised leaf water loss: The genotypes CRIS-129 (0.53%), CIM-499 (0.46%), and FH-114 (0.68%) showed minimum, whereas the genotypes CIM-446 (3.56%), CIM-240 (2.06%) and FH-900 (2.65%) showed maximum excised leaf water loss (Fig. 1B).

Cell membrane stability: The genotypes BH-18 (77.35%), FH-114 (77.21%), and CRIS-134 (76.82%) showed maximum, whereas the genotypes CIM-446 (48.83%), CIM-240 (46.53%) and FH-900 (51.55%) showed minimum cell membrane stability (Fig. 1C).

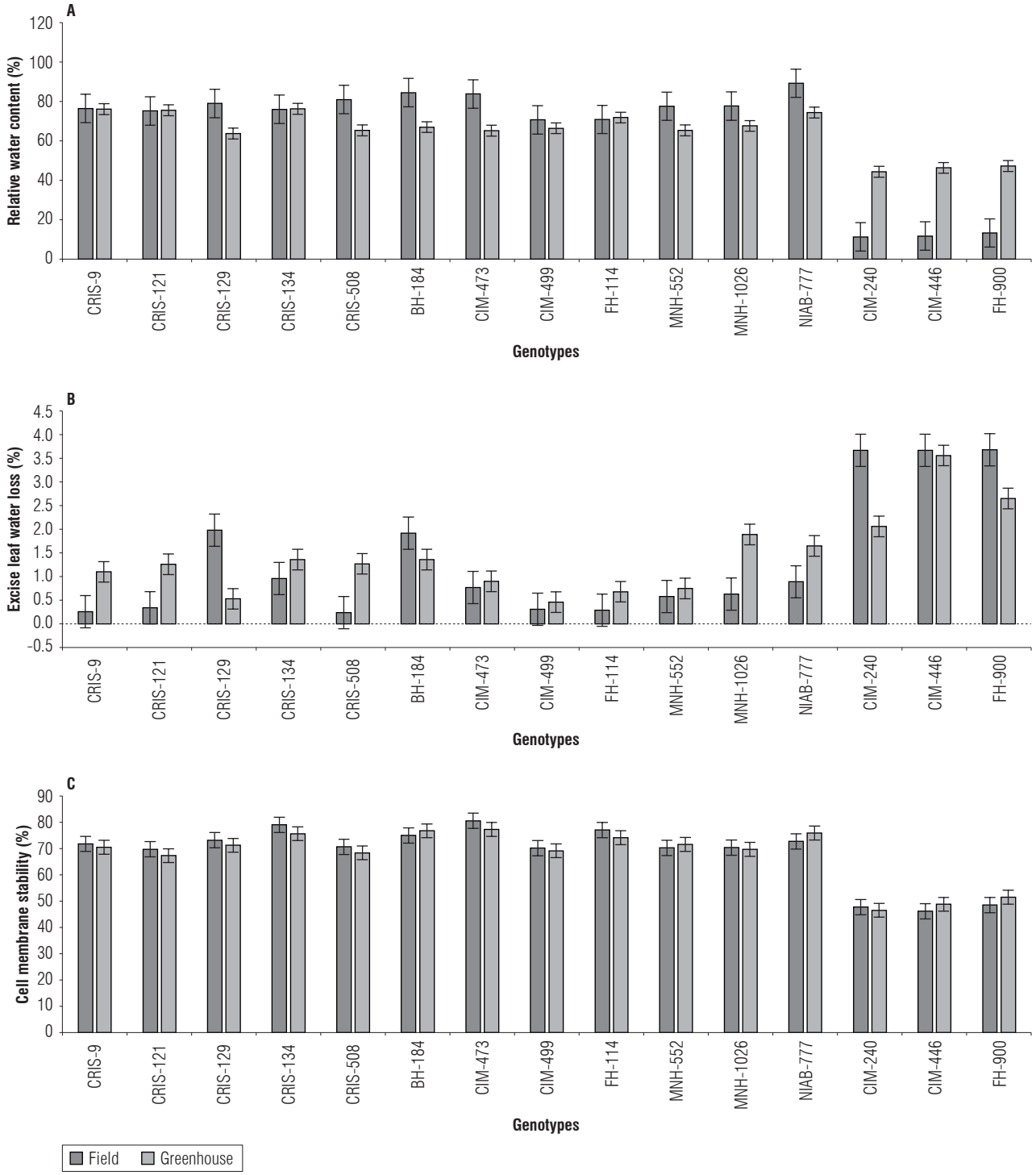

FIGURE 1. Comparative physiological traits in cotton genotypes: A) relative water content (RWC, %), B) excised leaf water loss and (ELWL, %), and C) cell membrane stability (CMS, %) under field and greenhouse. Error bars indicate standard error.

Molecular analysis

A set of 21 transcription factors was related to drought/heat tolerance for screening drought tolerance and susceptibility in selected cotton genotypes. The band sizes of all genes were the same. Drought-tolerant varieties, *i.e.*, CRIS-9, CRIS-121, CRIS-129, CRIS-134, CRIS-508, CIM-473, CIM-499, FH-114, MNH-552 and MNH-1026 had all 21 TFs/gene (Figs. 2 and 3).

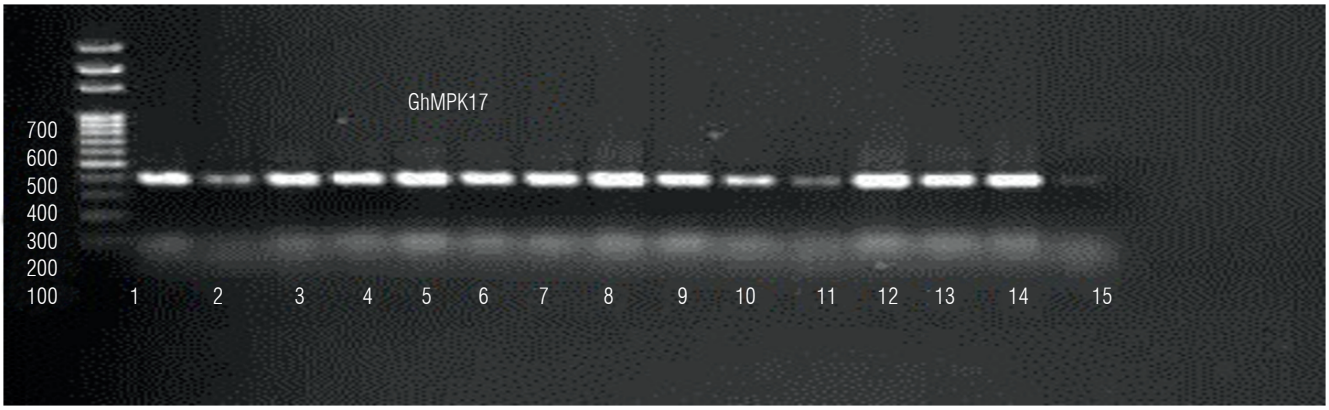

FIGURE 2. Molecular validation of the transcription factor GhMPK17 in selected cotton genotypes.

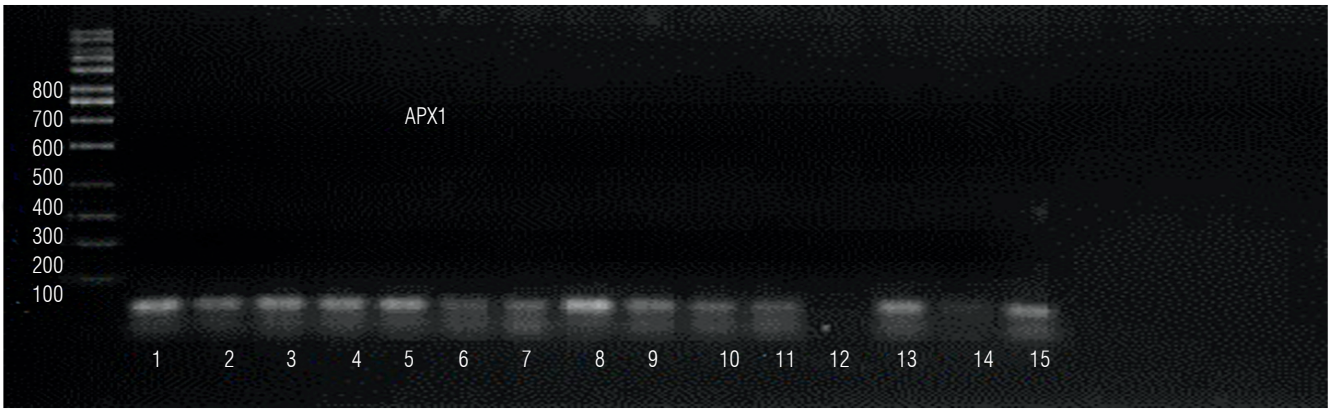

FIGURE 3. Molecular validation of the transcription factor APX1 in selected cotton genotypes.

TABLE 2. Molecular screening of 21 drought/heat-related transcription factors in selected cotton genotypes subjected to drought stress.

Genotype	GhNAC2	DREB2A	GhABF2	HSC70	HSFA2	GbMPK3	GbMpK17	GhMKK1	APX1	GHSP26	GHSP26	TPS	ANNAT8	GhMPK2	GhMKK3	GhWRKY41	HSPCB	HSP101	HSP3	GhPP2A1	GbMYB5
CRIS-9	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CRIS-121	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CRIS-129	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	✓	✓	✓	✓	✓
CRIS-134	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CRIS-508	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
BH-184	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CIM-473	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CIM-499	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
FH-114	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
MNH-552	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
MNH-1026	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
NIAB-777	✓	✓	✓	✓	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CIM-240	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×
CIM-446	✓	✓	✓	✓	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
FH-900	✓	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

The variance (ANOVA) analysis of all physiological traits, *i.e.*, RWC, ELWL, and CMS, was highly significant in both experiments. The correlation study showed that RWC positively correlated with CMS and negatively correlated with ELWL; CMS was negatively correlated with ELWL (Tab. 3).

TABLE 3. Correlations among physiological traits of cotton genotypes.

Variables	RWC	ELWL	CMS
RWC	1		
ELWL	-0.09**	1	
CMS	0.003*	-0.002*	1

RWC – relative water content (%), ELWL – excised leaf water loss (%), and CMS – cell membrane stability (%).

One sample t-test (significance level α=0.05) of the arithmetic mean of all physiological traits (RWC, ELWL, and CMS) was used to evaluate the variations. We found highly significant differences between all the traits studied (Tab. 4).

TABLE 4. Comparative analysis (t-test) among physiological traits under both experiments. Null Hypothesis: mu = 0; Alternative Hyp: mu <> 0. DF = 14.

Variable	Mean	SE	Lower value	Upper value	t-test
RWC	65.18	7.20	49.71	80.64	9.04**
	64.79	2.75	58.88	70.71	23.49**
ELWL	1.34	0.34	0.61	2.07	3.95**
	1.43	0.21	0.96	1.89	6.59**
CMS	68.25	2.90	62.02	74.48	23.50**
	67.64	2.62	62.00	73.28	25.73**

RWC – relative water content (%), ELWL – excised leaf water loss (%), and CMS – cell membrane stability (%).

Discussion

The varieties from the Cotton Research Institute Sakrand were tolerant to drought stress. These varieties have unique morphological traits, such as compact plants, early maturity, small leaves, and small fiber bolls. This study calculated the higher cell membrane stability and relative water content in these varieties. These varieties proved to be morphologically and physiologically drought-tolerant. Smaller bolls in these varieties are associated with more bolls, which keeps these varieties high-yielding genotypes. In this study, CIM-473 (Ullah *et al.*, 2008), CIM-499 (Khan *et al.*, 2009), CRIS-9, CRIS-121, CRIS-129, CRIS-134, CRIS-508 (Keerio *et al.*, 2022), FH-114 (Farooq *et al.*, 2009), BH-184 (Iqbal *et al.*, 2020), MNH-552 (Dahab *et al.*, 2012), MNH-1026 and NIAB-777 (Rehman *et al.*, 2021) were drought tolerant, whereas CIM-240 (Ahmad *et al.*, 2009), CIM-446 Iqbal *et al.*, 2010) and FH-900 (Nasimi *et al.*, 2016) were drought susceptible.

Cotton has moderate drought tolerance at the vegetative stage of growth. It is vulnerable to drought stress during the reproductive phase (Iqbal *et al.*, 2017; Niu *et al.*, 2018). Drought stress is commonly linked to oxidative and osmotic stress, leading to ion inequality, severe changes in cell membrane structure, and other cellular processes in plants (Bernardo *et al.*, 2019). Plants subjected to drought stress have lower relative water content and reduced cell membrane integrity (Hammad & Ali, 2014). Additionally, drought stress depletes lipid membranes, damaging membranes, which become more porous and increase electrolyte leakage (Petrov *et al.*, 2018).

Relative water content, leaf temperature, and osmotic potential are also impacted by drought stress (Fanaei *et al.*, 2012). Similarly, drought stress affects turgidity of cell growth and the development of plant tissues (Reddi & Reddi, 1995), leading to poor cell elongation (Nonami, 1998). The relative water content (RWC) is used to measure the water content in leaves as it reflects the ability of a genotype to survive under water deficit conditions (Silva *et al.*, 2007). A high RWC is preferred to maintain the water balance in a drought-stressed environment. Therefore, greater RWC is used as a base for developing drought-tolerant plants (Rahman *et al.*, 2000). A reduced transpiration rate and less water loss from excised leaves are crucial factors for selecting resistance against drought stress (Rahman *et al.*, 2000). Cell membrane thermostability (CMT) has been extensively used as an indicator of tolerance against water deficit stress, and Ur-Rahman *et al.*, (2004) used cell membrane stability to measure the heat tolerance of cotton.

The physiological and biochemical functions of stress-related transcription factors and genes control crop growth and reproductive development under drought conditions of different plants. GhMKK3 controls stomatal responses (Wang *et al.*, 2016), GbMYB5 helps in plant recovery during drought stress (Chen *et al.*, 2015), GhWRKY41 increases activity of antioxidant enzymes (Chu *et al.*, 2015), and GhMPK17 improves root strength under drought stress (Zhang *et al.*, 2014). This transcription factor set may have significantly impacted the ability of cotton to withstand Pakistan's semi-arid and sub-tropical climate. MAPK, DREB, and APX increase drought tolerance by strengthening physiological mechanisms against drought stress (Hou *et al.*, 2018; Nawaz *et al.*, 2020; Zhang *et al.*, 2020).

Saleem *et al.* (2015) reported a correlation between genes of relative water content and reduced excise leaf water loss in cotton. Relative water content and cell membrane stability sustain higher plant development and better performance

during drought by negatively correlating cell membrane stability with excised leaf water loss. Similar results were recorded in this study. Tolerant genotypes had higher RWC and CMS and lower ELWL values. The selected genotypes can be used as drought-tolerant materials in breeding.

Conclusions

Cell membrane stability was linked to relative water content and excise leaf water loss. This indicates that CMS is a rapid test for selecting cotton genotypes for drought tolerance. Field and greenhouse screening of cotton for drought tolerance showed similar results. The overall results indicate that the genotypes CRIS-134, BH-184, and FH-114 are drought tolerant and can be used for breeding programs.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author´s contributions

NM and AM carried out the research. BMMA and NM prepared the manuscript. SMA supervised the research and reviewed the manuscript. All authors have read and approved the final version of the manuscript.

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Environmental conditions during preharvest influence bioactive compounds in fruits: A review with emphasis on tropical and subtropical species

Las condiciones ambientales en precosecha influencian los compuestos bioactivos en frutos: una revisión, con énfasis en especies tropicales y subtropicales

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ABSTRACT

A healthy diet rich in fruits and vegetables with high contents of bioactive compounds and antioxidants has become an essential habit among the human population, leading to a significant increase in the commercial trade of many fruits, especially of tropical and subtropical origins. The content of phytonutrients in fruits depends on various pre-harvest factors, especially agroclimatic conditions of temperature, light, and air humidity, as well as crop management and fruit maturity stage. Among the essential phytonutrients found in fruits that promote health and prevent diseases are the carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein, etc.), phenolic compounds (flavonoids, phenolic acids, among others), monoterpenes (*i.e.*, limonene), isoprenoids (*i.e.*, lipophilic vitamins), and ascorbic acid. Factors of temperature, light intensity, UV light, and water stress promote the synthesis of phytochemicals in fruits. In contrast, an excess of these factors can either increase or decrease the accumulation of these compounds in fruits. In addition to different abiotic stresses that result from climatic conditions and have inter- and intra-annual variations, the geographical locations, elevation, and genotype influence the content of bioactive compounds in fruits. There is a strong interest in manipulating changes in climate conditions as a factor in fruit quality, including the phytochemical content, while reducing yield losses. This review aimed to explore how preharvest environmental factors affect accumulation of phytochemicals in fruits, which are important for plant resilience and human health, with an emphasis on tropical and subtropical fruit species.

Key words: phytochemicals, human health, carotenoids, phenolics, vitamins, plant stress, temperature, light.

RESUMEN

Una dieta saludable rica en frutas y verduras con altos contenidos en compuestos bioactivos y antioxidantes se ha convertido en un hábito alimenticio muy importante de la población, conllevando a un incremento significativo en la comercialización de muchos frutos, especialmente de origen tropical y subtropical. El contenido de estos fitonutrientes en frutos depende de los factores precosecha, especialmente de las condiciones agroclimáticas como temperatura, luz y humedad del aire, aparte del manejo del cultivo y de la madurez del fruto, entre otros. Dentro de los fitonutrientes más importantes están los carotenoides (α-caroteno, β-caroteno, β-criptoxantina, licopeno, luteína, entre otros), compuestos fenólicos (flavonoides, ácidos fenólicos, entre otros), monoterpenos (por ejemplo, limoneno), isoprenoides (por ejemplo, vitaminas lipofílicas), y el ácido ascórbico. Factores como temperatura, intensidad lumínica, luz UV y el estrés hídrico promueven la biosíntesis de fitoquímicos en los frutos, mientras que el exceso de estos factores puede aumentar o disminuir la acumulación de estos compuestos en los frutos. Aparte de los tipos de estrés abiótico por las condiciones climáticas, con sus variaciones interanuales e intraanuales, también la ubicación geográfica y elevacional y los diferentes genotipos influyen en los contenidos de compuestos bioactivos de los frutos. Existe un gran interés por aprovechar las condiciones del cambio climático como mecanismo para aumentar la calidad de los productos, incluyendo los contenidos de fitoquímicos, reduciendo al tiempo las pérdidas en los rendimientos de los cultivos. El objetivo de esta revisión fue explorar cómo los factores ambientales en precosecha afectan la acumulación de los fitoquímicos en los frutos, los cuales son importantes para la salud humana y la resiliencia de las plantas, con un enfoque en especies tropicales y subtropicales.

Palabras clave: fitoquímicos, salud humana, carotenoides, fenoles, vitaminas, estrés en plantas, temperatura, luz.

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Binomial fruit names in this article

Acerola *Malpighia glabra*	Kiwi *Actinidia deliciosa*	Peach palm *Bactris gasipaes*
Annon *Annona cherimola*	Lemon *Citrus limon*	Persimmon *Diospyros kaki*
Apple *Malus domestica*	Lime *Citrus* x *aurantifolia*	Pitanga *Eugenia uniflora*
Avocado *Persea americana*	Mango *Mangifera indica*	Pineapple *Ananas comosus*
Banana *Musa* sp.	Mangosteen *Garcinia mangostana*	Pitahaya *Selenicereus* sp.
Cantaloupe *Cucumis melo*	Orange *Citrus sinensis*	Plantain *Musa paradisiaca*
Cape gooseberry *Physalis peruviana*	Longan *Dimocarpus longan*	Pomegranate *Punica granatum*
Brazilian guava *Psidium guineense*	Loquat *Eriobotrya japonica*	Rambutan *Nephelium lappaceum*
Cactus pear *Opuntia ficus-indica*	Lulo *Solanum quitoense*	Star fruit *Averrhoa carambola*
Cashew *Anacardium occidentale*	Lychee *Litchi chinensis*	Strawberry *Fragaria* × *ananassa*
Chinese plum *Prunus mume*	Macadamia *Macadamia integrifolia*	Sweet cucumber *Solanum muricatum*
Durian *Durio zibethinus*	Mandarin *Citrus reticulata* / *C. unshiu*	Tangerine *Citrus reticulata*
Goji berry *Lycium barbarum*	Nectarine *Prunus persica* var. nucipersica	Tomato *Solanum lycopersicum*
Grape *Vitis vinifera*	Northern highbush blueberry *Vaccinium corymbosum*	Tree tomato *Solanum betaceum*
Grapefruit *Citrus paradisi*	Papaya *Carica papaya*	Watermelon *Citrullus lanatus*
Guava *Psidium guajava*	Passion fruit *Passiflora edulis*	
Jujube *Ziziphus jujuba*	Peach *Prunus persica*	
	Pear *Pyrus communis*	

Introduction

A diet rich in fresh fruits and vegetables, abundant in antioxidants, vitamins, minerals, and other phytochemicals, has become a crucial habit among the global population due to its health benefits and role in disease prevention (Kaur *et al*., 2017; Sarkar *et al*., 2023; Yahia, 2018; Yan *et al*., 2021). This growing awareness has increased the global trade of fruits and vegetables, particularly those of tropical and subtropical origin. Consequently, interest in the health-promoting properties of these crops has risen, leading to expanded research on their phytochemical composition and strategies to enhance their consumption (Schreiner *et al*., 2013; Yahia, 2018).

The concentration and metabolism of the bioactive compounds in agricultural crops depend on pre-harvest factors such as agroclimatic conditions, genetics, crop management (especially irrigation and fertilization), biochemical processes of fruit maturation and fruit maturity at harvest, and harvest time, as well as on postharvest factors (Mphahlele *et al*., 2014; Thokar *et al*., 2022; Yahia, 2018). Kader (2007) emphasized that environmental factors significantly impact the nutritional value and overall quality of agricultural crops. However, these factors are difficult to control in field conditions. Meanwhile, Lester (2006) concluded that variability in the bioactive compounds in fruits will always exist due to the interactions between genetic factors and environmental conditions on their synthesis and degradation pathways.

Many bioactive phytochemicals are interrelated with plants and their environment. They act as feeding deterrents, protective compounds against abiotic stresses or pathogens, pollinator attractants, signaling molecules or antioxidants (Schreiner & Huyskens-Keil, 2006). Numerous factors influence the phytochemical profile of fruits. In this regard, Nicola and Fontan (2014) highlight the importance of the geographical area (defined by different environmental conditions), available varieties, and applied cultural practices to crops. Lei *et al.* (2007) also emphasize the importance of the fruit developmental stage, and Yan *et al*. (2021) point out the different postharvest management practices.

Tropical fruits are rich in diverse phytonutrients, potentially preventing diseases and extending productive and active life expectancy in humans (Clevidence, 2010). Yahia, García-Solís, *et al*. (2019) classify these fruit and vegetable ingredients into micronutrients (minerals, vitamins), fiber, and a wide range of other bioactive compounds or phytonutrients that, individually or in combination, benefit human health. There are several reasons why these phytonutrients protect human health, primarily through their functions as antioxidants, anticancer agents, and immunomodulators, among others (Yahia, 2018; Yahia *et al*., 2023; Yahia, García-Solís *et al*., 2019).

Fruits, as a source of antioxidants (phenolic compounds, flavonoids, vitamins, etc.), can preserve cellular components against oxidative damage, reducing the risk of some degenerative diseases related to this type of stress (Stafussa *et al*., 2018). In this regard, Schreiner *et al*. (2013) highlight the current trend of incorporating more fruits and vegetables into the diet, especially considering that global fruit and vegetable consumption is not sufficient to meet daily nutritional requirements for good human health and

well-being (Jideani *et al*., 2021). Fruit growers are increasingly aware of the benefits of environmental conditions in promoting the quality and yield of their crops. They are applying technologies to optimize these conditions (Treutter, 2010). However, global warming affects the fruit development cycle by advancing and accelerating flowering and harvest times, which may lead to alterations in fruit quality and yield (García-Pastor *et al*., 2024).

Cervantes *et al*. (2020) point out the complexity of the "fruit quality" concept, which includes organoleptic and functional characteristics highly valued by health-conscious consumers, parameters that can be influenced by environmental changes. Crisosto *et al*. (1997) mention the importance of "orchard quality," which should be maximized through research and understanding of all factors affecting fruit quality. Kyriacou and Rouphael (2018) describe the quality of fruits and vegetables as a dynamic composition of their physicochemical characteristics combined with consumer perception.

The maximal potential of fruit quality can be developed under optimal climatic conditions during cultivation (Fischer *et al*., 2012), when physiological processes such as photosynthesis, translocation of photoassimilates towards sink organs, transpiration, respiration, and other metabolic pathways, which are crucial for both the external and internal quality and postharvest longevity of fruits, are promoted (Ladaniya, 2008). However, adverse conditions can negatively affect fruit quality. Abiotic stress can trigger physiological, biochemical, and molecular alterations through which plants tolerate stressful conditions (Toscano *et al*., 2019). Different types of abiotic stress promote the formation of secondary metabolites, such as phenolic compounds and several other classes that can benefit human health (Toscano *et al*., 2019; Yahia, 2018). Similarly, environmental stresses act as elicitors that induce the biosynthesis of secondary metabolites, commonly known as phytochemicals, many of which are bioactive compounds (Fig. 1) that help plants adapt to stressful conditions and, in turn, may provide significant benefits to human health (Aguirre-Becerra *et al*., 2021; Toscano *et al*., 2019; Yahia, 2018). Among many examples, Fernando *et al*. (2014) report that banana plants counteract oxidative stress from excessive solar radiation and temperature by increasing their antioxidant capacity.

The response of plants to environmental stressors depends on the type of stress, timing, duration of stress, the plant's age and phenological stage, and the various abiotic and biotic factors involved (Ochoa-Velasco *et al*., 2017). Meanwhile, susceptibility to different types of stress can vary significantly among species, varieties, cultivars, and landraces (Días *et al*., 2021; Ochoa-Velasco *et al*., 2017).

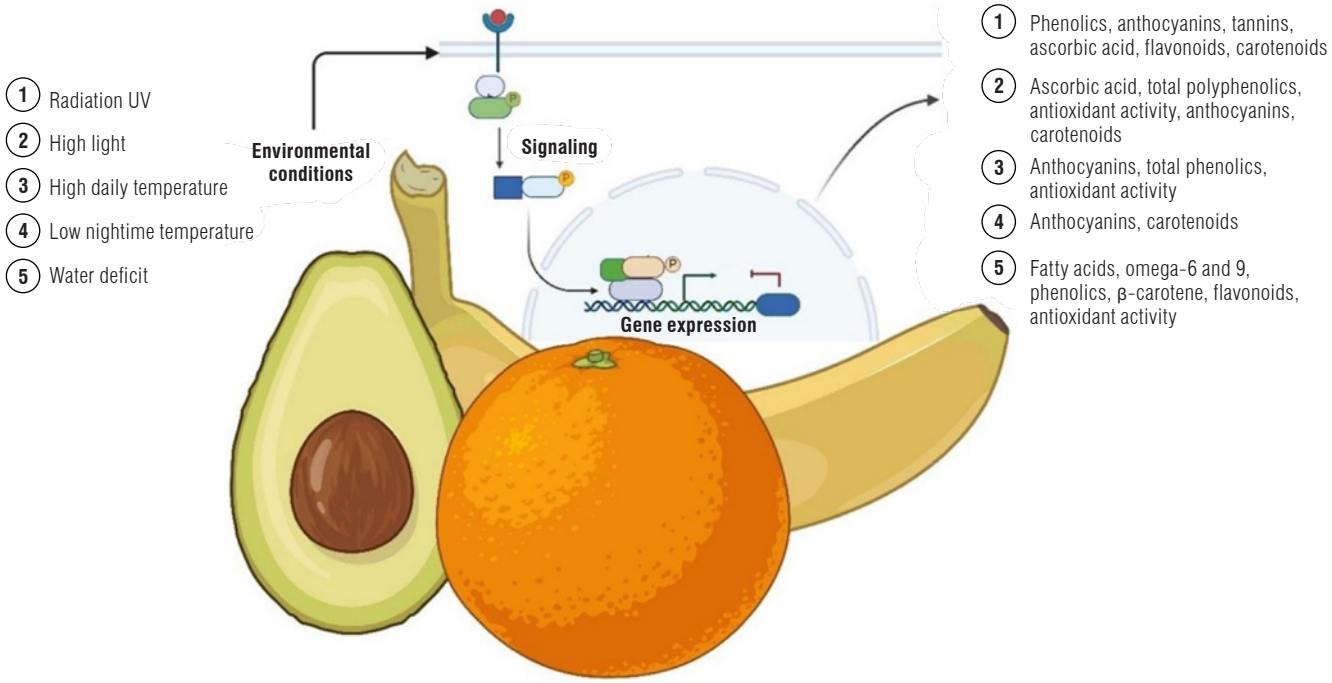

FIGURE 1. Possible role of certain preharvest climatic conditions on increasing contents of bioactive phytochemical compounds in fruits. Superoptimal conditions (that do not cause damage) of temperature, light, UV radiation, and soil water deficit induce the synthesis of various compounds in fruits involved in cell protection and offer health benefits for consumers.

Many environmental effects are currently under study, particularly in harnessing them to increase the content of bioactive phytochemicals in plants (Clevidence, 2010). This literature review explores how preharvest environmental factors such as light, temperature, elevation, water, and carbon dioxide affect fruit phytochemicals, which are essential for plant resilience and human health, focusing on tropical and subtropical species. Additionally, the information in this review can expand knowledge on the impact of climate change on the nutraceutical quality of fruits, providing insights for future research and promoting management practices that modify crop climates to enhance these phytonutrients.

Methodology

The methodology for this literature review consisted of searching for information (articles, book chapters) from various databases following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) guidelines according to Flórez-Velazco *et al*. (2024). The keywords used were "phytonutrients," "preharvest," and "fruit," generating 1,030 results from the past 10 years (2014-2024) in the Google Scholar database (75 publications were initially used), 58 results in ScienceDirect (11 were used), and 601 results in Semantic Scholar (92 were used). Additionally, in a second search using the keywords "climate" and "phytonutrients" in Google Scholar, 260 results were found, 4 of which were used. In a final filter, articles, and book chapters repeated across the three databases or that did not explicitly refer to climatic conditions were excluded. More emphasis was placed on publications about tropical and subtropical fruit species, and, in cases where there were not enough examples, some fruits from temperate zones were included. Due to their importance, some publications from before 2014 were also included.

Bioactive compounds in fruits that benefit human health

The insufficient consumption of fruits and vegetables is one of the five most important factors that pose a risk to human health (Harris *et al*., 2021). According to Zakrevskii (2018), the recommended daily intake ranges from 160 to 460 g for fruits, 240 to 402 g for vegetables, and 25 g for dietary fiber. Currently, there are no specific recommended consumption levels for the different groups of fruits and vegetables (Zakrevskii, 2018). Fruits and vegetables promote protective functions against chronic diseases such as cerebrovascular, cardiovascular, neurological, and ocular diseases, as well as diabetes, cancer, blood-related diseases, hypertension, and strokes (Jideani *et al*., 2021, and references therein; Yahia, 2018).

Due to the wide variety of bioactive compounds that promote health, fruits, and vegetables have a high potential for preventing cancer (Tab. 1) and cardiovascular diseases (Schreiner & Huyskens-Keil, 2006). Fruits contain thousands of phytochemicals (Yahia, 2018; Yahia, García-Solís, *et al*., 2019). Many phytochemicals have antioxidant activity, induce upregulation of detoxification enzymes, cell-to-cell communication, and regulate angiogenesis and apoptosis (Clevidence, 2010).

TABLE 1. Some phytochemicals from tropical and subtropical fruits with cancer-preventive properties (modified from Yahia, García-Solís *et al*. (2019)).

Phytochemical	Fruit species
Dietary fiber	Most fruits
Carotenoids α-carotene β-carotene Lycopene Xanthophylls (β-cryptoxanthin, lutein, zeaxanthin)	Cantaloupe, kiwi, mango, papaya Orange-flesh-fruits (cantaloupe, mango, orange, papaya, persimmon, pineapple, cape gooseberry) Brazilian guava, papaya, watermelon, red grapefruit Cantaloupe
Phenolics Anthocyanidins (cyanidin, malvidin, delphinidin, pelargonidin, peonidin, petunidin) Flavanones (hesperetin, naringenin, eriodictyol) Flavones (luteolin, apigenin, chrysin) Flavonols (kaempferol, myricetin, quercetin, rutin)	Red, blue, and purple fruits *Citrus* (oranges, grapefruits, lemons, limes, tangerines) Guava Berries
Phenolic acids Hydroxycinnamic acids (caffeic acid, ferulic acid, sinapic acid, chlorogenic acid, coumaric acid)	Orange, lemon, grapefruit
Monoterpenes Limonene	*Citrus* (grapefruit, tangerine)
Isoprenoids (lipophilic vitamins) Vitamin E (tocopherols)	Avocado, macadamia

Table 2 shows some tropical and subtropical fruits that contain significant amounts of carotenoids. Carotenoids are notable for having conjugated double bonds in their structure, with antioxidant effects through singlet oxygen quenching and their ability to neutralize peroxyl radicals (Leite *et al*., 2024; Yahia *et al*., 2019). Some of these molecules have been reported to possess antitumor properties (breast and prostate cancer) involving antioxidant functions, immune modulation, and antiproliferative effects (Leite *et al*., 2024; Yahia *et al*., 2018; Yahia, García-Solís *et al*., 2019). The high carotenoid content in many tropical and subtropical fruits, as shown in Table 2, suggests that incorporating them into the diet can significantly contribute to the intake of carotenoids necessary for good health (Phillips *et al*., 2014).

Sarkar *et al*. (2023) reported health benefits (including anticancer, recovery from cardiovascular diseases, antioxidants, anti-inflammatory effects) by consuming tropical fruits lychee, guava, star fruit, tree tomato, rambutan, pepino melon, mangosteen, loquat, durian, persimmon, longan, passion fruit, annon, and pitahaya.

Environmental factors influencing the accumulation of bioactive compounds in fruits

Climatic conditions during the crop growth cycle, especially temperature, light, air and soil composition, and humidity, affect fruit development, such as the duration of cell division, photosynthesis, transpiration, respiration, carbohydrate metabolism and translocation (Burgos *et al*., 2021; Fischer, Parra-Coronado *et al*. 2022; Siddiqui, 2018). These factors include not only the size of the fruits but also their internal and external characteristics and storage potential (Ladaniya, 2008; Siddiqui, 2018). Kader (2007) highlights that light intensity and temperature have a powerful impact on the nutritional quality of fruits and vegetables. The climate is generally interconnected with various factors, especially the geographic location of the orchard and the season, making climate a complex phenomenon (Días *et al*., 2021; Zhao *et al*., 2024). Treutter (2010) notes that the secondary metabolism of plants is highly dependent on the site's environmental conditions.

Environmental stress, especially from drought, high solar radiation incidence (ultraviolet (UV) and photosynthetic active radiation (PAR)), high heat load, and salinity, constitutes the four most detrimental types of stress for agricultural productivity (Brito *et al*., 2019), with significant synergistic effects on growth, quality, and fruit production (Fischer, Orduz-Rodriguez *et al*., 2022). To prevent plants from producing an excess of reactive oxygen species (ROS) and potentially experiencing cell death due to different types of stress, higher plants can activate an antioxidant defense system that includes both non-enzymatic and enzymatic components (Godoy *et al*., 2021). Several classes of phenolic compounds, such as flavonoids,

TABLE 2. Carotenoid content (μg 100 g^{-1} fresh weight-FW) in tropical and subtropical fruits.

Fruit species	Country	α-carotene	β-carotene	β-cryptoxanthin	Lycopene	Lutein	Zeaxanthin	References
Orange	Costa Rica	23.1	41.8	47.3	nd	312	nd	Monge-Rojas and Campos (2011)
Mandarin	Spain	<ld	213	843	<ld	<ld	<ld	Beltrán *et al*. (2012)
Lemon	Spain	<ld	0.4	14.4	nd	2.5	1.2	Olmedilla *et al*. (2005)
Cape gooseberry 'Sudáfrica'	Colombia	3	335	17	nd	nd	nd	Fischer *et al*. (2000)
Cape gooseberry 'Colombia'*	Colombia	-	~115	-	-	~10	-	Etzbach *et al*. (2018)
Loquat	Brazil	nd	38.1	54.8	nd	6.4	nd	Faria *et al*. (2009)
Acerola	Brazil	60	1220	95	nd	115	nd	Porcu *et al*. (2005)
Guava 'Regional Roja'	Colombia	nd	155	nd	2316	7	nd	González *et al*. (2011)
Cactus pear	Mexico	nd	9733	nd	nd	14133	nd	Jaramillo-Flores *et al*. (2003)
Tree tomato	Ecuador	nd	nd	1350	nd	98	59	Mertz *et al*. (2009)
Mango 'Tommy Atkins'	Costa Rica	19.4	838	12.4	27.1	40.9	nd	Ornelas-Paz *et al*. (2008)
Papaya 'Formosa'	Brazil	nd	548.6	3798.6	3137.5	nd	nd	Oliveira *et al*. (2010)
Cashew 'Native'	Costa Rica	109	935	137	nd	56	nd	Monge-Rojas and Campos (2011)
Passion fruit	Brazil	nd	284	24	nd	nd	nd	Wondracek *et al*. (2011)
Pitanga	Brazil	nd	380	1150	1660	100	nd	Burgos *et al*. (2012)
Peach palm 'Colombia'	Costa Rica	120	1590	Nd	nd	nd	nd	Jatunov *et al*. (2010)
Lulo	Ecuador	-	57.93	-	-	-	-	Llerena *et al*. (2019)

nd: no data; <ld: below detection limit. * Expressed in μg g^{-1} dry weight-DW and in all-E isomer.

and several carotenoids play a pivotal role in eliminating excess hydrogen peroxide caused by environmental stress (Hinojosa-Gómez *et al*., 2020). Due to their ecological and physiological relevance, phenolic acids and flavonoids are among plant´s most significant bioactive compounds (Ochoa-Velasco *et al*., 2017). Their content is influenced by climatic and other factors (Cetinkaya *et al*., 2016). Zoratti *et al*. (2014) reported that the genetic origin of plants primarily determines the level of phenolic compounds in their tissues. Still, external factors can qualitatively or quantitatively modify the composition of these phytochemicals.

The accumulation of carotenoids in chromoplasts depends on three climatic factors: temperature, light, and air humidity (Dhunique-Mayer *et al*., 2009). Oranges grown in Mediterranean climate develop a significantly higher content of total carotenoids (up to 10 times more) than those grown in tropical or subtropical regions (Benkeblia *et al*., 2011). In contrast, Dhunique-Mayer *et al*. (2009) observed that the 'Star Ruby' grapefruit produced higher levels of carotenoid lycopene in tropical and subtropical areas than in Mediterranean regions.

Inter-annual and intra-annual variations are observed in fruit´s functional and organoleptic parameters, which also depend significantly on genotype, as Cervantes *et al*. (2020) reported in five strawberry varieties. Among these, 'Sabrina' and 'Cadonga' demonstrated greater inter-annual and intra-annual stability in fruit functional quality. Mean and minimum air temperatures and relative air humidity only partially explained the variation in fruit quality across the five strawberry varieties (Cervantes *et al*., 2020).

Moretti *et al*. (2010), Fischer, Parra-Coronado *et al*. (2022), and Fischer, Melgarejo *et al*. (2022) suggest that, due to climate change, fruit production and quality, especially in tropical and subtropical regions, will have to contend with non-optimal conditions for crop growth such as increased temperature, drought, solar radiation, and CO_2 contents in the air.

Light

Exposure to light depends on the fruit's position on the branch and the tree canopy (Fischer & Parra-Coronado, 2020; Lechaudel & Joas, 2007). The intensity and quality of light significantly influence the contents of many secondary metabolites in plants, often positively affecting the fruit quality (Días *et al*., 2021), as Li and Cheng (2008) find for the level of carotenoids in apple fruit skin. Lester (2006) also highlights that light intensity and quality significantly impact the vitamin content in different fruits.

Additionally, the intensity of light the plant receives affects the level of ascorbic acid produced in fruits (Nicola & Fontan, 2014). Gruda (2019) clarifies that light is a regulatory factor for L-ascorbate (vitamin C) synthesis, a potent antioxidant for plants and animals.

Kim *et al*. (2022) observe in Satsuma mandarins that, as sun damage increases (from mild to severe), total polyphenols and antioxidant activity are more significant compared to fruits that do not suffer from sunburn or have moderate sunburn, while the contents of chlorophyll (*a*, *b*, and total) in the skin and of carotenoids in the pulp decrease with the severity of damage. Meanwhile, 'Keitt' and 'Reynal' mangoes in full sun field-grown conditions with air temperatures reaching 36°C present epidermis temperatures up to 47°C and higher concentrations of total polyphenols and anthocyanins compared to fruits grown under shade (Kagy *et al*., 2024). Additionally, hot water (55°C for 50 min) and hot air (47°C for 20 min) treatments increase heat shock proteins HSP 17.4 two- to six-fold in fruits of both mango varieties exposed to direct sunlight compared to those maintained in the shade (Kagy *et al*., 2024).

In Chinese plum (greengage) fruits, a period of sunshine followed by increased air humidity significantly influences the different biosynthetic pathways of phenylpropanoids (Liu *et al*., 2022). Specifically, radiation, in addition to air humidity and temperature, affects the polyphenol content in these plums during different stages of fruit ripening. In particular, the structures of flavanols and flavanones depend on radiation and temperature (Liu *et al*., 2022).

The effect of ultraviolet (UV) light on fruit quality is undeniable. UV radiation, which is part of the electromagnetic spectrum, can be grouped into three ranges based on wavelength: UV-C, with short wavelengths of high frequency (100-280 nm); UV-B, with medium frequency long wavelengths (280-320 nm), and UV-A, with low-frequency long wavelengths (320-400 nm) (Peng *et al*., 2022). UV light induces a phenomenon known as hormesis, which affects morphological, metabolic, and molecular processes that enhance the phytochemical characteristics of fruits (Pataro *et al*., 2015). UV radiation between 200 and 300 nm is strongly absorbed by phenolic compounds, which protect fruits from this harmful radiation (Felicetti & Schrader, 2008; Oliveira *et al*., 2019), such as in the flavanols that protect against UV light and scavenge free radicals (Zoratti *et al*., 2014).

According to Ávila-Sosa *et al*. (2016), UV light affects plant tissues through three associated pathways: (1) UV

photoreceptors, which control growth and development by influencing the expression of many genes involved in various processes for the production of secondary metabolites in plants; (2) the activation of enzymes such as chalcone synthase, anthocyanidin synthase, and phenylalanine ammonia-lyase, which are involved in the production of secondary metabolites through metabolic pathways of malonic acid, methylerythritol 4-phosphate, shikimic acid, and mevalonic acid; and (3) the production of ROS such as hydroxyl radical, superoxide radical, alkoxy radical, singlet oxygen, and hydrogen peroxide, and antioxidant enzymes such as catalase, superoxide dismutase, and glutathione reductase, among others. Peng *et al*. (2022) characterize the effect of UV-B radiation on plants as the promoter of secondary metabolism, increasing naturally active substances and inducing disease resistance mechanisms.

In fruits, light is a key factor for accumulating some phenolic compounds, including flavonoids, such as anthocyanins, and chlorophyll pigments (Yahia & Carrillo-López, 2019). Since UV-B radiation increases the concentrations of flavonoids such as quercetin and kaempferol in kale (Zhang *et al*., 2003), whilst Clevidence (2010) suggests that a reduction in flavonoid content is an undesirable consequence of growing vegetables in greenhouses, which are covered to protect against UV radiation. Gil *et al*. (2015) also recommend that farmers cultivate crops in open fields rather than in greenhouses to increase phytochemical levels in fruits, where bioactive compounds are typically lower due to the combined effect of low light intensity and high temperatures. However, Fischer, Orduz-Rodriguez *et al*. (2022) state that high levels of UV-B radiation can cause harmful effects at morphological, physiological, and biochemical levels in plant tissues (Brito *et al*., 2019).

In various fruit crops, such as watermelon, blueberry, papaya, mango, guava, and mandarins, UV-B or UV-C radiation has been used to increase the content of secondary metabolites and antioxidant capacity in fruits (Ochoa-Velasco *et al*., 2017, and references therein). In tomatoes, both UV-C and UV-B increase the concentration of phenolic compounds such as flavonoids, lycopene, and β-carotene, as well as the antioxidant capacity (Bravo *et al*., 2012; Liu *et al*., 2011; Pérez *et al*., 2009). Peng *et al*. (2022) note that UV-B and UV-A radiation are typically applied for several hours to days. UV-C radiation is a method where effective doses can be achieved in a shorter time, from 10 s to a few minutes.

Light influences the expression of genes related to pigment biosynthesis and the control of light signaling pathways (Azari *et al*., 2010; Qiu *et al*., 2023). According to Benkeblia *et al*. (2011), purple or bright red skin pigments in tropical fruits may indicate the presence of anthocyanins, with many genes related to the flavonoids that are co-regulated to enhance anthocyanin synthesis, which depends on environmental and other factors (Shi *et al*., 2023). Color is essential to consumers, who associate it with the taste of various fruits. For this reason, plant breeders strive to develop varieties with striking colors and high anthocyanin content in some fruits (Benkeblia *et al*., 2011).

It is important to note that the effects of solar radiation do not only depend on its intensity but also, in many cases, are specific to fruit variety or species and its developmental stage, as reported Bernjak and Cristl (2020) and Zoratti *et al*. (2015) regarding the stimulation of flavonoid synthesis in fruits. On the other hand, pruning or eliminating the leaves that cover the fruits allows for greater light interception, increasing fruit size, color, and quality. However, fruit growers should consider the type of fruits (including the thickness and other properties of the epidermis) and avoid exposing fruits for an excessive time close to harvest, especially in very elevated locations with intense UV light (Fischer, Orduz-Rodríguez *et al*., 2022). In peach (cv. TA-170), with 50% and 75% pruning of branches, maintains significantly higher concentrations of ascorbic acid in the fruits after harvest compared to non-pruned trees, with the higher pruning percentage having a more significant effect on the concentration of ascorbic acid (Choudhury *et al*., 2021).

In several crops, fruit pre-harvest bagging is used to protect against diseases and pests and also from sunburn due to high solar radiation found at high elevations or during extended summer periods (Fischer, Orduz-Rodríguez *et al*., 2022). Usually, this high radiation is combined with excessive air temperatures and low relative humidity or, in many cases, with a deficient number of leaves covering the fruits (Baiea *et al*., 2018). In the case of "sunburn browning", caused by increased solar radiation and temperatures between 46.0 and 49.8°C, the lesions result from severe degradation of chlorophylls and carotenoids, leading to a bronze-brown-yellowish coloration (Muñoz & Munné-Bosch, 2018). It should be considered that light also accompanies high temperatures caused by light intensity.

Results regarding the effect of bagging on fruit phytochemicals are variable. Hossain *et al*. (2020) report higher vitamin C content in unbagged mangoes (29.7 mg 100 g^{-1}) as compared with bagged fruits. Lima *et al*. (2013) observe similar results in peach varieties, where bagged fruits had lower levels of vitamin C, phenolics, and organic acids. On

the other hand, bagging mangoes with brown and white paper, UV-selective plastic bags (transparent to UV light) or muslin fabric effectively increases the level of β-carotene compared to the non-bagged fruits (Islam *et al*., 2017).

For varieties with intense color, such as red fruits, removing the bag from the fruits before harvest is essential. This is the case with the 'Mantianhong' pear (*Pyrus pyrifolia*), which develops its red color quickly, where the key regulator PyMYB10 promotes anthocyanin synthesis in response to light (Qian *et al*., 2013).

Similarly, the color and material of shade netting affect fruit quality (Fischer, Orduz-Rodríguez *et al*., 2022; Siddiqui, 2018). According to Tinyane *et al*. (2018), this is mediated by photoreceptors such as phytochromes, cryptochromes, and phototropins, which involve the upregulation of specific genes during fruit development, such as those involved in the synthesis of phenolic compounds. UVB is also sensed by the receptor UV Resistance Locus 8 (UVR8) cellular component, and this radiation may enhance the synthesis of phenolics and terpenoids in plants (Miao *et al*., 2020; Qaderi *et al*., 2023).

Temperature

Crops develop within an optimum temperature range that guarantees, among other growing conditions, maximum yield until growth is limited (Hewett, 2006). Tropical and subtropical fruit trees are exposed to cold and freezing when grown in temperate climates, except if grown under protected conditions in this geographic location (Hewett, 2006). Temperature changes during fruit growth and development significantly affect fruit composition and quality, especially their phytochemical contents (Yahia, 2018). Crop environment temperature greatly influences fruit production, color, aroma, and carbohydrate biosynthesis through its effect on photosynthesis and, consequently, on maturation and ripening processes (Yahía, Gardea-Béjar *et al*., 2019b). Temperature should always be within the range for the cultivated species to ensure optimal photosynthesis, avoiding a more significant respiratory loss of carbohydrates, so night temperatures should be lower, such as under conditions that provide tropical altitudes (Flórez-Velasco *et al*., 2024).

Aril coloration in seeds of pomegranate fruits change inversely with seasonal temperature (Borochov-Neori *et al*., 2011). For apples, nighttime temperatures around 10°C stimulate red coloration in the fruit skin due to anthocyanin accumulation (Musacchi & Serra, 2018), especially when combined with daytime temperatures of around 18 to 24°C that promote fruit growth, depending on the species and variety (Fischer & Orduz-Rodríguez, 2012). Consequently, high nighttime temperatures in the weeks before harvest decrease the accumulation of red pigments in apple fruit skin (Treutter, 2010).

In some varieties of grapes, lower temperatures than the optimal range can increase the content of phenolic compounds (Yahia, Gardea-Béjar *et al*., 2019). For grape berries, cool nights enhance coloration (Fischer *et al*., 2016), being delphinidin an essential precursor of the anthocyanin pigments of this berry, which synthesis is promoted by low temperatures (Yahia, Gardea-Béjar *et al.,* 2019). The enhancement of fruit coloration by night temperatures lower than daytime temperatures is because these conditions activate the gene expression of chalcone synthase. This enzyme participates in the synthesis of anthocyanins (*i.e*., cyanidin, pelargonidin, naringenin, and malvidin, etc.) in fruits of oranges, grapefruits, grapes, red apples, and strawberries (Yahia, Gardea-Béjar *et al*., 2019).

Fruit epidermis temperatures can rise significantly during the day under clear skies (Konno & Sugiura, 2024) in the skin of Satsuma mandarins and apples, with temperatures at least 15°C higher than the average air temperature in Tsukuba, Japan. High temperatures in strawberries (25-30°C per d) increase the content of anthocyanins and total phenolics and enhanced antioxidant activity in the fruits (Wang *et al*., 2006). Toscano *et al*. (2019) report that elevated temperatures can increase antioxidant concentrations, which help protect the cell membrane from breakdown and peroxidation. In turn, Wahid *et al*. (2007) indicate that plants under heat stress may increase the contents of bioactive compounds such as betaine, proline, and sugar alcohols related to stabilizing enzymes/proteins and the membrane bilayer structure of plant tissues. High temperatures directly affect plant metabolism and influence enzymatic activities (Yahia, Gardea-Béjar *et al*., 2019). Pivotal processes affected by heat stress are phenylpropanoid synthesis pathways and photosynthesis (Toscana *et al*., 2019). By high-temperature stress, ROS can accumulate, which activates detoxification systems and causes buildup by preserving the cell membrane from peroxidation and breakdown (Toscana *et al*., 2019).

Due to changes in the microclimate caused by the production system, such as plastic mulch with row covers, Fan *et al*. (2017) find a significant increase in total phenol content and overall antioxidant capacity in the strawberry variety 'SJ8976-1' compared to the standard matted row system (wide rows with straw). Fan *et al*. (2017) attribute this effect

mainly to the increase in the surrounding temperature of the soil and plants (Fischer, Cleves-Leguizamo *et al*., 2022). With multivariate techniques, such as principal component analysis and heat maps, Moreno-Medina *et al.* (2024) find a close relationship between the accumulation of phenolic compounds and the photosystem II (PSII) of photosynthesis, which favors the adaptation of Andean blackberries to adverse conditions of low temperature and high radiation in Colombian highlands.

Altitude

Each fruit species is adapted to an elevation range that matches its ecophysiological demands in the tropics and subtropics. It is known that, as elevation increases, solar radiation (especially UV) increases, while temperature, partial gas pressure (CO_2, O_2, N_2), precipitation, and water vapor decrease (Benavides *et al*., 2017; Fischer, Parra-Coronado *et al*., 2022), depending on the microclimatic conditions, because interestingly, in Colombian páramos, the relative humidity is close to 100%. Adapting crop varieties to higher elevations depends, in particular, on their photosynthetic performance and reduced susceptibility to photoinhibition (Fischer, Parra-Coronado *et al*., 2022). Additionally, the adaptation of fruit species to elevational conditions depends on anatomical, morphological, and biochemical characteristics, mainly in fruits and leaves (Fischer *et al*., 2024).

Species and cultivars of fruit plants originating from inner tropical regions, especially Andean fruit species from higher elevations, are noted for their better tolerance to UV-B radiation according to Caldwell *et al*. (1980). With increasing elevation, the level of antioxidants, often phenolics, increases in fruits, particularly in the epidermis, in response to increased UV light (Fischer, Parra-Coronado *et al*., 2022). In the fruit skin of apples growing between 300 and 1,200 m a.s.l. in the Caucasus, Voronkov *et al*. (2019) find increased concentrations of phenolic compounds, many potent antioxidants protecting fruits against excess UV radiation. Similarly, in Italy, the content of phenolic compounds, such as ellagic acid and flavanols in 'Elsanta' strawberries, is higher at 1,500 m a.s.l. than at lower elevations (Andreotti *et al*., 2014). In addition, in pomegranate, Mphahlele *et al.* (2014) conclude that elevation may adjust certain climatic factors that significantly affect the biosynthetic pathway of phenolic compounds.

Oliveira *et al*. (2019) find an increase in anthocyanins and tannins in the skin of 'Syrah' grapes with increasing elevation, while Karagiannis *et al*. (2016) observe an increase in anthocyanins, total phenolics, carotenoids, flavonoids, and antioxidant capacity in the skin of 'June Gold' peaches. Similarly, rising elevation results in stronger red coloration in the skin of pomegranate fruits (Al-Kalbani *et al*., 2021) and strawberries (Pérez de Camacaro *et al*., 2017) due to increased levels of anthocyanins. Additionally, in mandarin pulp (Susanto *et al*., 2013), orange (Ayer & Shrestha, 2018), and strawberry (Pérez de Camacaro *et al*., 2017), there is an increase in vitamin C levels at higher elevations. In contrast, in cape gooseberry, which is enclosed in an enlarged calyx, β-carotene concentration decreases with higher elevation (2,690 respect to 2,300 m a.s.l.) (Fischer *et al*., 2000).

The best quality with the highest levels of alkaloids, flavonoids, amino acids, and vitamins in dragon fruit (pitahaya) cv. Jindu1 in Guizhou Province, China, is reached at the highest altitude of 650 m a.s.l., while at the lower altitude (356 m a.s.l.), the fruits have the highest content of phenolic acids (Zhao *et al*., 2024). Likewise, 'Golden Delicious,' 'Royal Delicious,' and 'Red Gold' apples in northern India produce higher total phenolic content and increased overall antioxidant activity at 1,800 m a.s.l. compared to 1,400 m a.s.l. (Kumar *et al*., 2019).

Water

Water is one of the most critical factors influencing fruit crop growth, development, productivity, and quality. Drought is one of the most destructive abiotic stresses for crop productivity (Devin *et al*., 2023; Ochoa-Velasco *et al*., 2017). Water deficit in crops significantly affects the synthesis and accumulation of bioactive compounds in fruits (González-Chavira *et al*., 2018), considering that the stimulation of secondary metabolism in plants also depends on genetic factors and seasonality, as well as the duration and intensity of the water deficit (Ripoll *et al*., 2014).

Moderate water stress or regulated deficit irrigation (RDI) increases some phytochemicals and flavor compounds in fruits (González-Chavira *et al*., 2018). Production of avocado fruits under deficit irrigation conditions in a subtropical Mediterranean climate results in smaller fruit size but increased content of unsaturated fatty acids (oleic acid), omega-6, and omega-3 fatty acids (Durán *et al*., 2021). Navarro *et al.* (2015) observe that moderate water stress increases grapefruit phenolic compounds, β-carotene, and flavonoid contents but decreased lycopene levels. Additionally, in blueberries, a low irrigation regime in plastic tunnels increases the content of total flavanols (by 30%), delphinidin-3-acetyl hexoside (by 54%), and antioxidant activity (by 10%), depending on the cultivation system and genotype (Cardeñosa *et al*., 2016). In goji berries, irrigation deficit (up to 50% of the evapotranspiration) concentrates

most of the health-related metabolites in the fresh fruits. It increases the concentration of total phenolics and carotenoids (+15.5%) and β-carotene (+19.6%) based on dry mass (Breniere *et al*., 2024).

In passion fruit cultivated at 2,260 m a.s.l. during two production cycles in southern Colombia, the second cycle, characterized by high precipitation, elevated relative humidity, and lower solar radiation during the first third of the reproductive period, exhibits a 28% decrease in ascorbic acid content and a 67% reduction in antioxidant capacity compared to the drier preceding cycle (Muñoz-Ordoñez *et al*., 2023). Nectarines grown under long-term regulated irrigation deficit has significantly increase vitamin C content (21-42% in pulp, 20-69% in skin), soluble phenolics (7-11% in pulp, 22-31% in skin), and antioxidant capacity (22-27% in pulp, 8-19% in skin) compared to fruits grown under optimal irrigation (Falagán *et al*., 2015).

Dzomeku *et al*. (2020) find the highest carotenoid levels of three banana cultivars in Ghana during the dry season, which coincides with periods of high UV-B radiation. These authors attribute the high carotenoid content in bananas to increased UV-B radiation under clear sky conditions. Although watermelon yields are reduced by irrigation deficit, lycopene content and fruit quality remain high (Bang *et al*., 2003).

In olives, irrigation deficit regimes produce higher values of phytonutrients and antioxidant capacities, with significant increases in polyphenol content (150-317%), phenylalanine ammonia-lyase (PAL) activity (128-164%), and antioxidant activity (139-292%), depending on the water regime, although these differences tend to decrease as the fruits matured (Machado *et al*., 2013). In contrast, in pomegranates (cvs. Sefri and Wonderful), irrigation deficit at 50% and 70% according to evapotranspiration in a Mediterranean climate in Morocco markedly reduces total phenolic content and antioxidant activity, while an increase in hydrolysable tannin content is observed in both cultivars. However, epicatechin concentrations in both cultivars and caffeic acid in cv. Sefri are not affected by soil water conditions (Adiba *et al*., 2024).

Carbon dioxide concentration in the air

The scenarios of climate change driven by increased atmospheric CO_2 concentration have positive effects on fruit trees, related to enhanced photosynthetic activity, efficient water use, growth, and accumulation of biomass (Fischer, Melgarejo *et al*., 2022), which also affect the production of phytonutrients. Wang *et al*. (2003) observe that increasing CO_2 concentration to 300 or 600 ppm above ambient levels increases flavonoids such as anthocyanin levels in strawberries. Additionally, Wang *et al*. (2003) find that elevated CO_2 concentrations led to higher oxygen-free radical absorbance activity in the fruits. However, Sun *et al*. (2012) observe that increasing CO_2 concentration to 720 mg L^{-1} in growth chambers reduces the total antioxidant capacity of strawberries despite increases in total sugars and fruit dry weight. Loladze *et al*. (2019) report in a meta-analysis that high levels of ambient CO_2 generally decrease carotenoid content by about 15%, and only when plants were stressed by abiotic factors do carotenoid levels increase. Conversely, CO_2 applications in greenhouse vegetable production can increase the edible part´s total flavonoids, phenolics, ascorbic acid, and antioxidant capacity (Dong *et al*., 2018).

Conclusions

Fruit consumption offers numerous health benefits since the fruit's phytochemical contents, including carotenoids, phenolics, monoterpenes, and isoprenoids, prevent various diseases, such as cancer and cardiovascular conditions.

Several critical environmental factors promote the content of phytochemicals in fruits, such as temperature, light quality and intensity, and water deficit. However, excessive levels of these factors can be counterproductive. Besides abiotic stress from climatic conditions, including inter-annual and intra-annual variations, factors such as geographic and elevational location, genotypes, fruit ripening process, and crop management influence the bioactive phytochemical compounds in fruits. Significant interest is in leveraging climate change to enhance product quality while reducing crop yield losses due to adverse conditions.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

HEBL: conceptualization, visualization, writing, and editing. GF: conceptualization, visualization, writing, editing, and supervision. EMY: writing, editing. All authors have read and approved the final version of the manuscript.

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Evaluation of defense gene expression and the virulence factor *Cac1* in the interaction between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum*

Evaluación de la expresión de genes de defensa y del factor de virulencia Cac1 en la interacción *Phaseolus vulgaris* y *Colletotrichum lindemuthianum*

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ABSTRACT

Anthracnose is one of the most limiting diseases in bean cultivation, leading to decreased yield. Mechanisms associated with the induction of the bean defense response during the interaction with *Colletotrichum lindemuthianum* have been studied, but little is known about the expression of certain virulence factors of this fungus during the infection process. The aim of this study was to evaluate specific molecular determinants triggered during the interaction between *C. lindemuthianum* and bean plants. For this purpose, qPCR was used to evaluate changes in the expression of the virulence factor *Cac1* in two isolates of *C. lindemuthianum* (Cl(a) and Cl(b)) with contrasting virulence profiles, and to correlate them with the expression of plant defense genes *PR1*, *PR3*, *PR4*, and *POD* during the early stages post-infection. Molecular ITS analysis showed that both isolates belonged to the Orbiculare clade; however, they clustered differently, a characteristic associated with their distinct virulence profiles. When they were inoculated in bean plants, the Cl(a) isolate was more virulent than the Cl(b) isolate, generating the highest severity value. The Cl(b) isolate induced higher expression of the evaluated plant defense genes than the Cl(a) isolate. However, the virulence factor *Cac1* of *C. lindemuthianum* showed significantly higher expression in Cl(a) than in Cl(b). These results suggest that the Sutagao bean cultivar exhibits a lower expression of defense genes exposed to an isolate of *C. lindemuthianum* expressing the virulence factor *Cac1* in the initial stages of infection.

Key words: Sutagao bean cultivar, anthracnose, *Cac1* gene, plant defense, Orbiculare clade.

RESUMEN

La antracnosis es una de las enfermedades más limitantes del cultivo de frijol, ocasionando disminución del rendimiento. Se han estudiado mecanismos asociados con la inducción de la respuesta de defensa del frijol durante la interacción con *Colletotrichum lindemuthianum*, pero poco se conoce sobre la expresión de algunos factores de virulencia de este hongo durante el proceso de infección. El objetivo de este estudio fue evaluar determinantes moleculares desencadenados durante la interacción entre *C. lindemuthianum* y frijol. Para ello, mediante qPCR se evaluaron cambios en la expresión del factor de virulencia *Cac1* en dos aislados de *C. lindemuthianum* (Cl(a) y Cl(b)) con perfiles de virulencia contrastantes, y se correlacionaron con la expresión de los genes de la planta *PR1*, *PR3*, *PR4* y *POD*, durante estadios tempranos de la infección. Los análisis ITS mostraron que ambos aislados pertenecían al clado Orbiculare, aunque se agruparon de manera diferente, característica asociada con sus diferentes perfiles de virulencia. Al inocularse en plantas de frijol, el aislamiento Cl(a) resultó ser más virulento que el Cl(b), generando la mayor severidad; pero Cl(b) indujo una mayor expresión de los genes de defensa de la planta en comparación con Cl(a). Sin embargo, el factor de virulencia *Cac1* de *C. lindemuthianum* mostró una expresión significativamente mayor en Cl(a) que en Cl(b). Estos resultados sugieren que el cultivo de frijol Sutagao presenta una menor expresión de los genes de defensa cuando se enfrenta a un aislado de *C. lindemuthianum* que expresa el factor de virulencia *Cac1* en las etapas iniciales de la infección.

Palabras clave: cultivar de frijol Sutagao, antracnosis, gen *Cac1*, defensa de plantas, clado Orbiculare.

Introduction

Beans, which are grain legumes, are an important source of protein, especially for populations in the tropical areas of Latin America and East Africa (Schwartz & Pastor-Corrales, 2005). In Colombia, beans are of great importance in the peasant economy because they are traditionally cultivated by small and medium-family producers in the Andean zone and constitute an important source of protein for these populations (Ospina Parra *et al*., 2020). Anthracnose caused by the fungus, *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara in beans (*Phaseolus vulgaris* L.) is considered the most limiting disease in this cultivated species generating substantial losses in yield and a significant reduction in seed quality. Environmental conditions such as air humidity greater than 80% favor the development of infection, especially in susceptible cultivars. The symptoms of this disease in

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bean plants are characterized by necrotic lesions on leaf veins, petioles, stems, and pods (Alvarez-Diaz *et al*., 2022). In leaves, the disease initially appears as necrotic spots on the veins, which extend to form large necrotic lesions that may be accompanied by leaf chlorosis and finally cause leaf death. In pods, lesions are depressed cankers that generate salmon-colored centers, which correspond to the reproductive structures of the pathogen (Pedroza *et al*., 2022).

The infection process of *C. lindemuthianum*, a hemibiotrophic fungus, has been well characterized and divided into an initial biotrophic phase followed by a necrotrophic phase. The biotrophic stage begins 24 h after the arrival of the conidia, the formation of the germ tube and the appressorium, and continues at 48 h with the formation of the vesicle and primary hyphae inside the plant cell (Nabi *et al*., 2024). The transition from the biotrophic to necrotrophic phase occurs at 72 h and is characterized by a morphological change from primary hyphae to secondary hyphae that favors the colonization of the pathogen (Romero *et al*., 2024).

The use of resistant genotypes is the most reliable and cost-effective management strategy for the control of *C. lindemuthianum* because it can reduce yield losses without the negative environmental impact of fungicide application (Alvarez-Diaz *et al*., 2022). However, the wide diversity in virulence associated with the various races of this fungus is the most important reason for the absence of durable resistance to anthracnose in beans (Costa *et al*., 2021). The genetics of this resistance have been studied, revealing that the plant-pathogen interaction between *C. lindemuthianum* and Sutagao cultivar of *P. vulgaris* is specific at race-cultivar level. The race specific resistance is conferred by the presence of resistance genes called *Co* genes that are unique to each genetic pool (Campa *et al*., 2017).

Similarly, studies on the mechanisms associated with the induction of bean plant defense responses during the interaction with *C. lindemuthianum* have demonstrated the importance of pathogenesis-related proteins (PRs) and the activation of salicylic acid (SA) hormone pathways, where *PR1* serving as a marker for the biosynthesis of this hormone. However, the induction of defense mechanisms dependent on jasmonic acid (JA) has also been reported (Alvarez-Diaz *et al*., 2022; Pedroza *et al*., 2022; Shams *et al*., 2020).

The virulence factors of *C. lindemuthianum* in this pathosystem remain largely unexplored. In the genus *Colletotrichum*, the expression of genes involved in the production of cyclic AMP (cAMP) plays an important role in virulence. For instance, *Cac1* codes for an adenylate cyclase in species belonging to the clade Orbiculare and its homologs *CgRhoB* in the Gloeosporioides clade, as well as *ChRgf* and *ChCdc25* in the Higginsianum clade. This adenylate cyclase is involved in the reaction to produce cAMP, which is essential for the activation of protein kinases that regulate conidial germination, appressoria penetration, and invasive fungal growth, which are key determinants of the infection process (Jiang *et al*., 2021). In addition to *Cac1*, annotation of the secretome of the *C. lindemuthianum* infection process in bean revealed the expression of Carbohydrate-Active enzymes (CAZymes), membrane transporters, Candidates to Secreted Effector Proteins (CSEPs), and some extracellular membrane (CFEM) domain proteins implicated in virulence (Romero *et al*., 2024).

Therefore, the aim of this study was to evaluate the expression of some bean defense genes to two different isolates of *C. lindemuthianum* and to evaluate the expression of the virulence factor *Cac1* involved in the kinase-like signaling cascade that regulates fungal morphogenesis and pathogenesis.

Materials and methods

Obtaining isolates of *C. lindemuthianum*

The two isolates of *C. lindemuthianum* were obtained from the Alliance Bioversity International-CIAT microorganism bank and reactivated in Petri dishes containing PDA medium. One of them was named isolate Cl(a) and the other was named isolate Cl(b). Subsequently, a replicate of each was made from hyphal transfer on PDA, which was incubated at 24°C in the dark. To ensure genetic uniformity of the fungal isolates, monosporic cultures were prepared (Ortiz *et al*., 2011).

DNA extraction

Each isolate was grown in Sabouraud Dextrose Broth medium and incubated at 24°C for 21 d in dark conditions. After this time, the grown mycelium was recovered and macerated with liquid nitrogen to subsequently carry out the DNA extraction using the DNeasy Plant Mini Kit from Qiagen®, following the manufacturer's protocol.

Sequencing of the ITS region

To confirm the genus and species of the isolates obtained, amplification of the ITS region was performed using the primers ITS-4 (TCCTCCGCTTATTGATATATGC) and ITS-5 (GGAAGTAAAAGTCGTAACAAGG) (Irinyi *et al*., 2015). Reactions for PCR were brought to a final volume of 20 µl containing 2.0 µl of 10 X Buffer, 0.8 µl of $MgCl_2$ at

50 mM, 0.4 µl of dNTPs at 10 mM, 0.4 µl of each primer at 10 µM, 1 U of Taq Polymerase, 2.0 µl of DNA from each isolate, and 13.8 µl of Milli-Q water. The parameters used for the PCR included a pre-incubation step at 95°C for 2 min, followed by 40 cycles of amplification under a thermal profile of 95°C for 30 s, 54°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. Amplification reactions were run on a Bio-Rad Thermal Cycler C1000. The PCR products were purified and sequenced in the Sequencing and Molecular Analysis Service (SSiGMoL) from the Universidad Nacional de Colombia. The sequences obtained were compared to entries in the NCBI public database using the BLASTn algorithm.

Phylogenetic analysis

Sequences obtained from the ITS region were aligned using the ClustalW algorithm in the CLC Main Workbench v5.5 program (CLCBio). A set of 14 reference sequences of *C. lindemuthianum*, one of *C. orbiculare*, and one of *C. trifolii* were used to represent diversity within the Orbiculare clade (Tab. 1) according to Guevara-Suarez *et al*. (2022). Additionally, the ITS sequences of other *Colletotrichum* clades, such as Acutatum, Gloeosporioides, and Boninense (Tab. 1), were selected as outgroups. Subsequently, Gblocks was performed removing the uninformative ends of the sequences and concatenating the alignments. To construct the phylogenetic tree, the maximum likelihood (ML) method was used together with the Kimura evolution model with gamma distribution (K2+G), and 1,000 bootstrap replicates were considered. The evolution model and tree generation were determined using MEGA v.7.0.

TABLE 1. GenBank accession numbers of the nucleotide sequence of the ITS region of the ribosomal RNA of *C. lindemuthianum* isolates used for phylogenetic analysis.

Colletotrichum species	Strain/Culture collection	GenBank accession number
C. lindemuthianum CI(a)	CI2a	PP545307 (this work)
C. lindemuthianum CI(b)	CI2b	PP545302 (this work)
C. lindemuthianum	L79	KJ956028
C. lindemuthianum	L85	KJ956029
C. lindemuthianum	L83	KJ956030
C. lindemuthianum	L70	KJ956031
C. lindemuthianum	K29	KJ956032
C. lindemuthianum	CBS 144.31	MH855161
C. lindemuthianum	CBS 132.57	JX546806
C. lindemuthianum	CBS 143.31	JX546808
C. lindemuthianum	CBS 152.28	JX546813
C. lindemuthianum	CBS 131.57	JX546805
C. lindemuthianum	CBS 147.31	JX546810
C. lindemuthianum	CBS 150.28	JX546811
C. lindemuthianum	CBS 151.56	JX546812
C. lindemuthianum	CBS 153.28	JX546814
C. orbiculare	CBS 570.97	NR152271
C. trifolii	CBS 158.83	NR152275
C. acutatum	CBS 112996	NR144794
C. gloeosporioides	IMI 356878	NR150754
C. boninense	ICMP 17904 / MAFF 305972	NR165949

Morphological characterization

From each culture of *C. lindemuthianum* grown in PDA, a mycelial disk of 5 mm diameter was transferred to a new culture medium with a punch and incubated at 24°C in the dark for 21 d. After this time, the macroscopic and microscopic characteristics of each of them were evaluated, and aspects such as colony color according to the Pantone® scale colony appearance and presence of sporulation were described. In addition, mycelial growth was measured and the growth rate was calculated with the equation:

$$\text{Mycelial growth rate (mm d}^{-1}) = \frac{MG_2 - MG}{t_2 - t_1} \quad (1)$$

where MG corresponds to mycelial growth and t to time in days.

The assay was conducted twice, with three biological replicates per isolate. The growth rate data were analyzed under the RStudio program (RSTUDIO-2023.09.1-494.EXE) and a t-Student test was performed to establish significant statistical differences between the two isolates.

Plant material and establishment of the experiment

Bean seeds of the Sutagao cultivar were supplied by the legume germplasm bank of the Faculty of Agricultural Sciences at the Universidad Nacional de Colombia. This cultivar results from a cross between the parents G2333 (Mesoamerican origin and resistance differential) and Cabrera (Andean origin). The seeds were placed in growth trays, sown in MKS1 peat substrate, and maintained in the plant propagation greenhouse, at an average temperature in the range of 18-25°C, with a relative humidity of 60-80% and a natural photoperiod of 12 h. In addition, the irrigation of the plants was ensured by applying 10 ml of water per plant two to three times per week.

Fungal biomass propagation

Inoculum growth of each monosporic isolate of *C. lindemuthianum* was carried out following the methodology proposed by Castellanos *et al*. (2011). For this purpose,

previously sterilized fresh bean pods (*Phaseolus vulgaris*) were inoculated with fragments of the fungi grown on PDA for over two weeks to promote the formation of acervuli and conidia, which represent the infective structures of the pathogen. The two isolates, Cl(a) and Cl(b), were incubated on this substrate for 21 d at 24°C in the dark.

Pathogenicity tests

To prepare the inoculum, increased fungal biomass along with the bean pods were macerated and filtered. The conidial suspension obtained was counted in a Neubauer chamber and adjusted to a concentration of 1×10^7 conidia ml^{-1}. Then, 14-d-old bean plants were inoculated by brushing the conidial suspension over both the upper and lower sides of the leaves to ensure that they were completely covered (Pedroza *et al*., 2022). Plants were maintained in relative humidity conditions above 80% to favor the infection process. To assess the pathogenicity potential of the Cl strains, three treatments were implemented: control plants inoculated with sterile distilled water, plants inoculated with Cl(a) isolate, and plants inoculated with Cl(b) isolate. The assay was conducted twice, with 15 biological replicates for each treatment.

Disease severity evaluation

Severity was assessed using the ordinal scale proposed by Van Schoonhoven and Corrales (1987), which assigns scores from one to nine according to the symptoms severity with one indicating no disease and nine indicating leaf death. This evaluation was performed every 4 d, and photographs of disease progression were taken at each evaluation up to 14 dpi. The area under the disease progress curve (AUDPC) was calculated from the severity data using the equation described by Shaner and Finney (1977).

AUDPC data were analyzed using the RStudio program (RSTUDIO-2023.09.1-494.EXE). Analysis of variance (ANOVA) of repeated measures was performed, followed by Tukey's test to determine significant statistical differences between the treatments.

RNA extraction and cDNA synthesis

RNA was extracted from 14-d-old bean plants inoculated using the brushing method with the same conidial suspension used for the pathogenicity test. The infection test was conducted twice with 15 biological replicates. Uninfected bean plants of the same age were used as controls. Three leaves from each test were collected at the early stages of the infection at 24, 48, 72, and 96 h post-inoculation (hpi). Subsequently, RNA pools were created from the extraction of each sample time series. Total RNA was extracted using a CTAB-based protocol with LiCl. Plant tissue was macerated with liquid nitrogen in a sterile mortar until reduced to a fine powder, to which approximately 3.0 ml of extraction buffer (CTAB 2%, Tris-HCl pH 8.0, 100 mM, NaCl 1.4 M, Na_2SO_3 1%, Polyvinylpyrrolidone PVP-40 2%, β-Mercaptoethanol 2% and EDTA pH 8.0, 20 mM) was added. One ml of the homogenized mixture was transferred to a 2 ml tube and placed in a water bath at 65°C for 15 min. Subsequently, after incubation, 1 ml of chloroform-isoamyl alcohol (24:1) was added, mixed, and centrifuged at 10,000 rpm for 20 min at 4°C. After the time elapsed, the aqueous phase (approximately 800 µl) was transferred to a new 2 ml tube, to which 1 ml of chloroform-isoamyl alcohol (24:1) was added. The samples were mixed and centrifuged at 10,000 rpm for 20 min at 4°C. Once the aqueous phase was generated, 800 µl was added to a sterile 1.5 ml tube. The RNA precipitation was performed using 1 ml of 4 M LiCl overnight at 4°C. Afterwards, the samples were centrifuged at 10,000 rpm for 40 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 500 µl of pre-warmed TE-SDS buffer (Tris-HCl pH 8.0, 10 mM; EDTA pH 8.0, 1 mM; SDS 1%) at 37°C. Next, 700 µl pure isopropanol and 200 µl 5 M NaCl were added to each tube. They were mixed by inversion several times and incubated for 1 h at -20°C. Then, they were centrifuged at 10,000 rpm for 15 min at 4°C, the supernatant was discarded, and the pellet was washed with 500 µl of 70% ethanol. Final centrifugation was performed for 5 min at 10,000 rpm at 4°C. The supernatant was discarded, and the pellet was dried for 1 h at room temperature. Finally, it was resuspended in 50 µl of TE buffer (Tris-HCl pH 8.0, 10 mM; EDTA pH 8.0, 1 mM) and quantified using a Thermo Scientific NanoDrop™ Spectrophotometer.

Subsequently, RNA from each treatment was treated with ThermoFisher® DNase I, following the manufacturer's protocol. To verify the absence of DNA in these samples, PCR amplification of the elongation factor (EF1α) of *P. vulgaris* was performed and the absence of amplification of this gene was verified. For this, PCR reactions were brought to a final volume of 20 µl containing 2.0 µl of 10 X Buffer, 0.8 µl of $MgCl_2$ at 50 mM, 0.4 µl of dNTPs at 10 mM, 0.4 µl of each primer (Tab. 2) at 10 µM, 1 U of Taq Polymerase, 2.0 µl of DNase-treated RNA, and 13.8 µl of Milli-Q water. The temperature profile used was 95°C for 10 min, followed by 35 amplification cycles under a thermal profile of 95°C for 1 min, 60°C for 30 s, and 72°C for 2 min. From this treated RNA, cDNA synthesis was performed using ThermoFischer® M-MLV Reverse Transcriptase according to the manufacturer's protocol.

Determination of primer efficiency by qPCR

To determine the amplification efficiency of the bean genes *EF1-α* (elongation factor), *POD* (peroxidase), *PR-1*, *PR-3*, and *PR-4* (antifungal compounds and chitinases) (Tab. 2), serial dilutions of *P. vulgaris* DNA were prepared. The starting concentration was 7.5 ng μl^{-1} leading to dilutions of 0.75, 0.075, 0.0075, 0.00075, 0.000075, and 0.0000075 ng μl^{-1}. Three replicates per dilution were performed. Reactions for qPCR were brought to a final volume of 10 µl containing 5 µl of BlasTaqTM 2 X qPCR Master Mix, 0.2 µl of each primer (Tab. 2) at 10 µM, and 3 µl of *P. vulgaris* DNA. The reactions were performed with a Jena Analytik qTOWER3 thermal cycler with the following parameters used for qPCR: pre-incubation at 95°C for 10 min, followed by 40 cycles of amplification under a thermal profile of 95°C for 1 min, 60°C for 30 s, and 72°C for 2 min. Additionally, one cycle for the dissociation curve (melting curve) was included, increasing the temperature from 60°C to 95°C in 15 s. Three replicates per dilution were performed and SYBR Green was used for detection.

Likewise, serial dilutions of DNA from the two isolates, Cl(a) and Cl(b), were carried out to determine the amplification efficiency of the *C. lindemuthianum clrRNA* (mRNA) and *Cac1* (adenylate cyclase) genes (Tab. 3). The starting concentration was 10.6 ng μl^{-1} leading to dilutions of 1.06, 0.106, 0.0106, 0.00106, 0.000106, and 0.0000106 ng μl^{-1}. Three replicates per dilution were performed. Reactions for qPCR were brought to a final volume of 10 µl containing 5 µl of BlasTaqTM 2 X qPCR Master Mix, 0.2 µl of each primer (Tab. 3) at 10 µM, and 3 µl of *C. lindemuthianum* DNA. The reactions were performed on the Jena Analytik qTOWER3 thermal cycler with the following parameters for qPCR: pre-incubation at 95°C for 2 min, followed by 40 cycles of amplification under a thermal profile of 95°C for 1 min, 60°C for 30 s, and 72°C for 2 min. Additionally, a cycle for the dissociation curve (melting curve) was included, with the temperature increasing from 60 to 95°C in 15 s. Three replicates per dilution were performed, SYBR Green was used for detection.

The efficiency of each primer was calculated using the three Ct values obtained for each dilution of *P. vulgaris* and *C. lindemuthianum* DNA with the following equation:

$$E = \sqrt[-\text{slope}]{\text{logarithm index}} \quad (2)$$

Differential expression analysis

Three replicates of the cDNA were used for each treatment at 24, 48, 48, 72, and 96 hpi for the differential expression analysis of the *POD*, *PR-1*, *PR-3*, and *PR-4* genes, which are related to the plant defense response, using qPCR with the relative quantification method. For this analysis, the crossing point (CP) values of the reference gene (housekeeping gene) *EF1-α* and each of the evaluated

TABLE 2. Sequences of primers for amplification of *EF1-α*, *PR-1*, *PR-3*, *PR-4,* and *POD* genes of *Phaseolus vulgaris*.

Gen	Function	Sequence (5' - 3')	Reference
EF1-α	Elongation factor I	CGGGTATGCTGGTGACTTTT CACGCTTGAGATCCTTGACA	(Mayo *et al*., 2015)
POD	Peroxidase II	TCCTTTTCAGCACTTTCACT AGAAAGCAGTGTTCTTGTGG	(Oliveira *et al*., 2015)
PR-1	Antifungal compound	TGGTCCTAACGGAGGATCAC TGGCTTTTCCAGCTTTGAGT	(Mayo *et al*., 2015)
PR-3	Chitinase	ATTGTTGTGCCAATCCCTTT CACCGCCATACAGTTCAAAA	(Oliveira *et al*., 2015)
PR-4	Chitinase	CGCAGTGAGTGCATATTGT TGTTTGTCACCCTCAAGCAC	(Mayo *et al*., 2015)

TABLE 3. Sequences of primers for amplification of *clrRNA* and *Cac1* genes of *C. lindemuthianum.*

Gen	Function	Sequence (5' - 3')	Reference
clrRNA	rRNA	CCTGTTCGAGCGTCATTTCA CCGGTGCGAGGTGGTATG	(Fontenelle *et al*., 2017)
Cac1	Adenylate cyclase	GCGAGCATAGGTGAAACGTT ATGTTGTTCCTCCGCACGTC	(Romero *et al*., 2024)

defense genes were compared. The qPCR reactions were run to a final volume of 10 µ*l* containing 5 µ*l* of BlasTaqTM 2 X qPCR Master Mix, 0.2 µ*l* of each primer at 10 µ*M* (Tab. 2), and 3 µ*l* of cDNA. The reactions were performed on the Jena Analytik qTOWER3 thermal cycler with the following parameters used for qPCR: pre-incubation at 95°C for 10 min, followed by 40 amplification cycles under a thermal profile of 95°C for 1 min, 60°C for 30 s, and 72°C for 2 min. SYBR Green was used for detection. One cycle for the dissociation curve (melting curve) was included, with the temperature increasing from 60°C to 95°C in 15 s.

Likewise, three replicates of the cDNA were used for each treatment and differential expression analysis of the *C. lindemuthianum* virulence factor *Cac1*. For this analysis, the CP values of the housekeeping genes, *clrRNA*, and *Cac1* were compared. Reactions for qPCR were brought to a final volume of 10 µ*l* containing 5 µ*l* of BlasTaqTM 2 X qPCR Master Mix, 0.2 µ*l* of each primer at 10 µ*M* (Tab. 3), and 3 µ*l* of cDNA. The reactions were performed on the Jena Analytik qTOWER3 thermal cycler with the following parameters used for qPCR: pre-incubation at 95°C for 2 min, followed by 40 amplification cycles under a thermal profile of 95°C for 1 min, 51°C for 30 s, and 72°C for 2 min. SYBR Green was used for detection. One cycle for the dissociation curve (melting curve) was included, with the temperature increasing from 60°C to 95°C in 15 s.

The Pfaffl comparative method (Pfaffl, 2001) was used to calculate the relative expression for each gene evaluated using three repetitions of Ct values obtained for each treatment. The data were analyzed using the RStudio program (RSTUDIO-2023.09.1-494.exe). An analysis of variance (ANOVA) of repeated measures was performed, followed by Tukey's test to establish significant statistical differences between the treatments.

Results

Sequencing of the ITS region

The homology comparison of the ITS region of *C. lindemuthianum* isolates Cl(a) and Cl(b) sequences against the GenBank database showed an identity and coverage percentage greater than 99% with sequences of *C. lindemuthianum* (Tab. 4). Additionally, the results of the phylogenetic analysis performed with the ITS sequences of pathogenic *Colleotrichum* spp. strains from the Orbiculare clade (Guevara-Suarez *et al*., 2022) showed that Cl(a) and Cl(b) isolates grouped differentially in this clade. Isolate Cl(a) was placed in the *C. lindemuthianum* third group reported by Guevara-Suarez *et al*. (2022), unlike isolate Cl(b), which was located in the second group (Damm *et al*., 2013; Guevara-Suarez *et al*., 2022) (Fig. 1).

Morphological characterization of the isolates of *C. lindemuthianum*

The two isolates of *C. lindemuthianum* showed similarities in mycelial color, appearance, and colony shape, with concentric rings on the PDA medium (Tab. 5 and Fig. 2A). However, a distinguishing feature was observed in each, setting them apart. Seven days after sowing, isolate Cl(a) acquired a salmon coloration (Fig. 2A), which correlated with the production of acervuli and conidia, which was confirmed by microscopic visualization (Fig. 2B). In contrast, isolate Cl(b) did not sporulate on the PDA medium, showing only mycelial growth (Fig. 2C). Similarly, isolate Cl(b) formed a colony with radial streaks, which were not observed in isolate Cl(a) (Fig. 2A). The results of mycelial growth rate analysis revealed that the Cl(b) exhibited a significantly higher rate than Cl(a) (Tab. 5). However, the behavior of both isolates Cl(a) and Cl(b) in the bean medium used for the inoculum increase was similar, and both isolates sporulated 7 d after sowing.

TABLE 4. Nucleotide sequence homology of the ITS of Cl(a) and Cl(b) isolates of *C. lindemuthianum*.

Isolate	GenBank accession number	Identity (%)	Coverage (%)	Homology GenBank accession number
Cl(a)	PP545307	99.82	99.00	*C. lindemuthianum* KR012909
Cl(b)	PP545302	99.82	99.00	*C. lindemuthianum* JX546805

TABLE 5. Macroscopic and microscopic characteristics of *C. lindemuthianum* isolates grown on different media and mycelial growth rates.

Isolate	Colony color	Colony aspect	Presence of radial streaks	Sporulation in PDA medium	Sporulation in bean	Mycelial growth rate (mm d^{-1})
Cl(a)	Grey-brown	Cottony	No	Yes	Yes	2.7 a
Cl(b)	Grey-brown	Cottony	Yes	No	Yes	3.9 b

Different letters represent values with significant differences ($P<0.05$) according to the Tukey´s test.

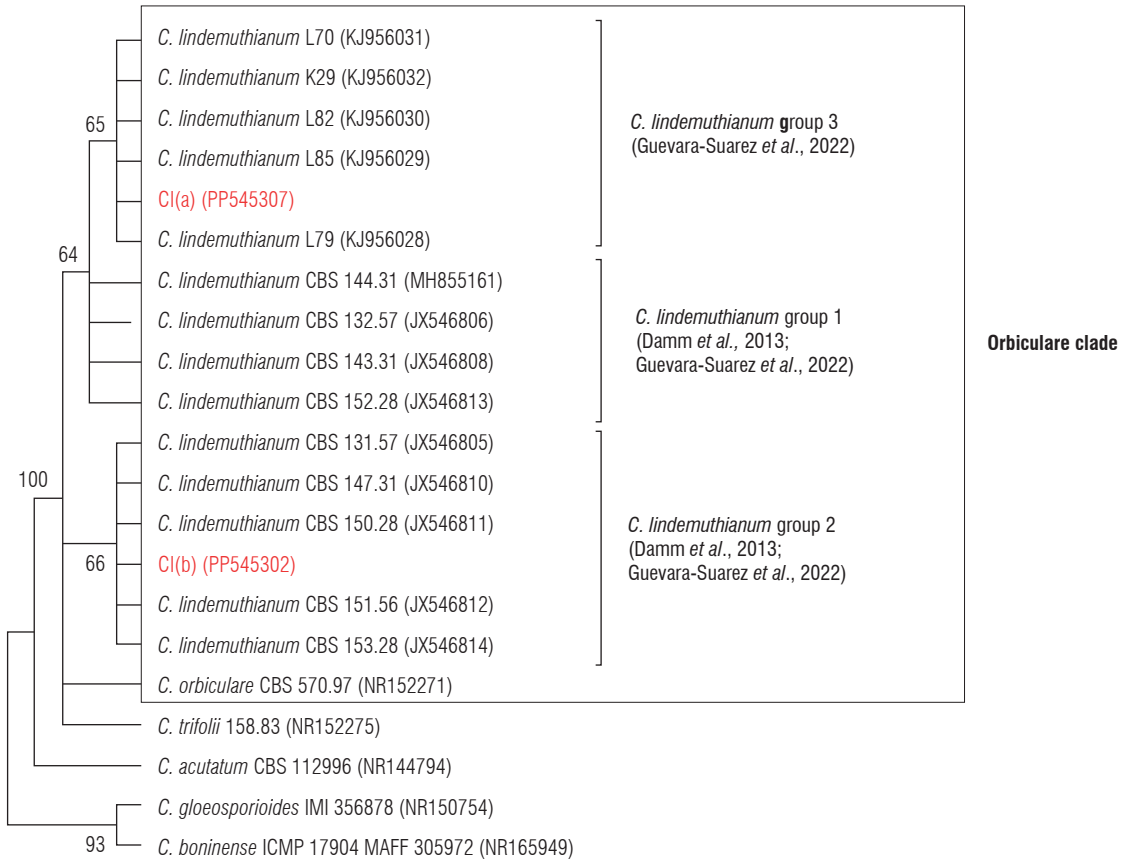

FIGURE 1. Phylogenetic tree based on the ITS region of *C. lindemuthianum* isolates. The maximum likelihood Kimura model with gamma distribution was used, with 1,000 bootstrap replicates. The culture collections of the Orbiculare clade are delimited by the black box. The Orbiculare clade comprises the three groups of *C. lindemuthianum* (Guevara-Suarez *et al*., 2022) as well as strains from *C. orbiculare* and *C. trifolii*. Sequences from specimens belonging to the Acutatum, Gleosporioides, and Boninense clades were used as outgroups. The GenBank accession numbers of the isolates are indicated in the brackets.

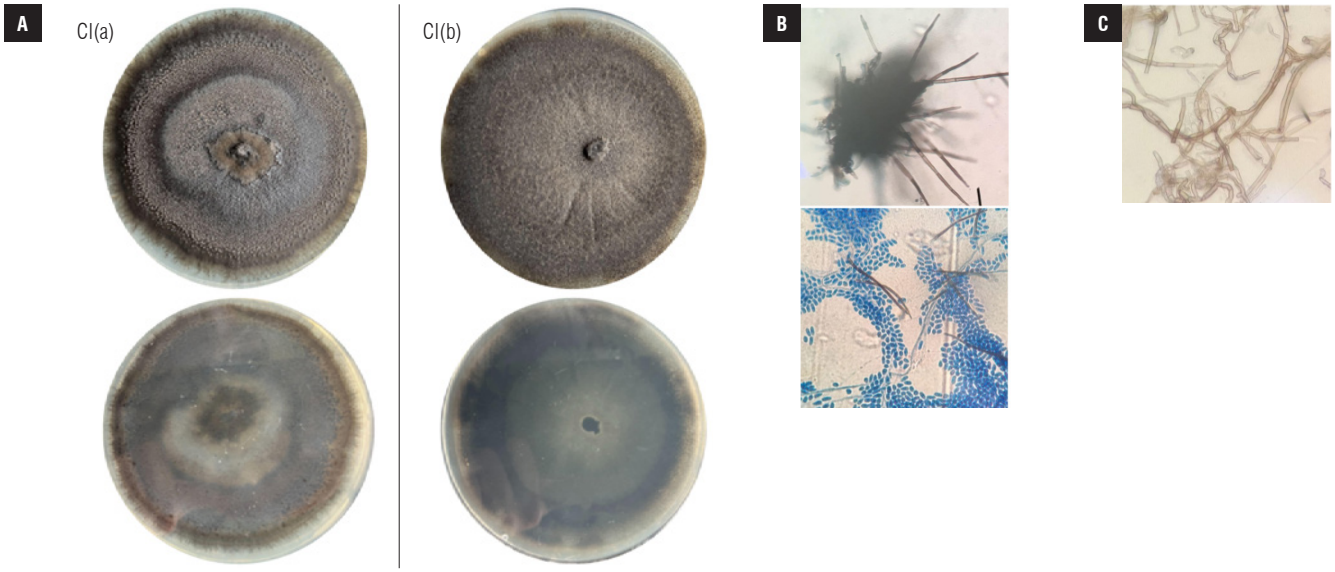

FIGURE 2. Macroscopic and microscopic characteristics of CI(a) and CI(b) isolates of *C. lindemuthianum*. A) Colonies of CI(a) and CI(b) observed 21 d after culture on PDA medium; the images on the left correspond to the front of the colony, and those on the right correspond to the back of the colony; B) Acervuli and conidia of CI(a) observed at 40 X seven days after culture on PDA medium; C) Characteristics of the mycelium of CI(b) observed at 40 X seven days after culture on PDA medium. A Canon EOS Rebel T5 was used to take the photos.

Evaluation of disease severity

The evaluation of disease progression showed that bean plants of the Sutagao cultivar inoculated with isolate Cl(a) had a severity score of 9 (the highest value on the scale) at 14 dpi. The symptoms included severe necrosis and leaf death, which classified the plants as susceptible to this isolate (Fig. 3A). In contrast, plants inoculated with isolate Cl(b) showed a severity score of 4 (intermediate on the scale) and exhibited small necrotic lesions on the underside of the leaf, evident on secondary and primary veins, classifying the plants as having intermediate resistance to this isolate (Fig. 3B).

The plants inoculated with isolate Cl(a) presented significantly higher AUDPC values than the plants inoculated with Cl(b), indicating that isolate Cl(a) is more virulent and produces a greater amount of disease than Cl(b) (Fig. 4). From the early days after inoculation (4 dpi), significant differences were found between the two isolates, which

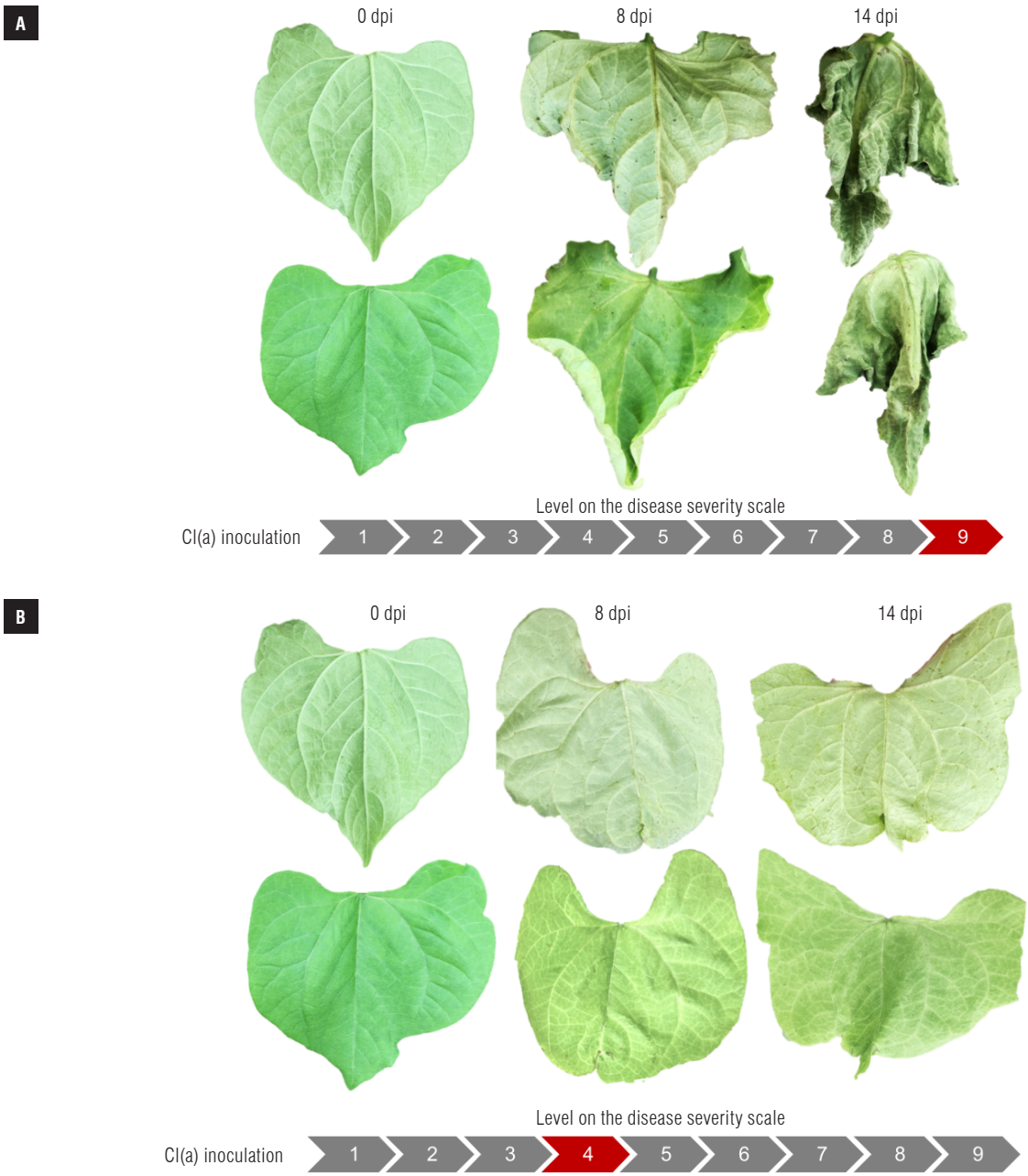

FIGURE 3. Evaluation of symptomatology caused by two isolates of *C. lindemuthianum* on the Sutagao bean cultivar at 0, 8, and 14 dpi. A) Plants inoculated with Cl(a); B) Plants inoculated with Cl(b). The upper images correspond to the underside of the bean leaf, and the lower images show the leaf blades. A Canon EOS Rebel T5 was used to take the photos.

were maintained until the end of the evaluation of disease progression (Fig. 4).

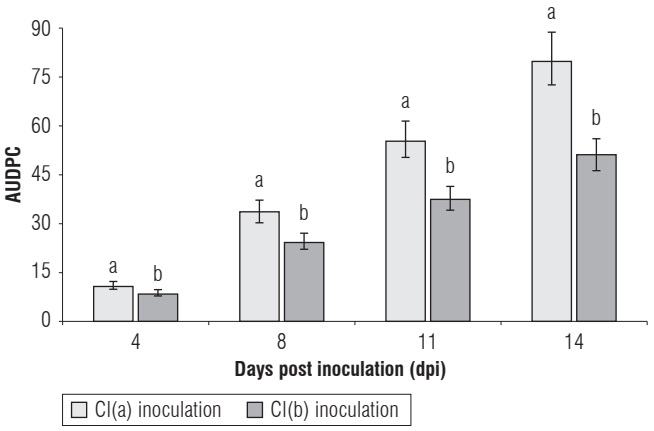

FIGURE 4. Area under the disease progress curve (AUDPC) obtained from inoculation with both isolates of *C. lindemuthianum* Cl(a) and Cl(b) on 14-d-old plants of the Sutagao bean cultivar at different evaluation times. Analysis of variance (ANOVA) of repeated measures was performed followed by the Tukey's test. Bars show the standard error of the mean calculated from 30 individual plants. Different letters represent values with significant differences between treatments ($P<0.05$).

Differential expression analysis associated with plant defense genes

Changes in the expression of bean *POD*, *PR1*, *PR3*, and *PR4* genes showed significant differences between plants inoculated with Cl(a) and Cl(b). Plants inoculated with Cl(b) exhibited significantly higher expression of these genes than plants inoculated with Cl(a) (Fig. 5). The early expression of *PR1* and *PR4* was evident in plants infected with both isolates at 24 hpi (Fig. 5A-B), whereas the expression of *PR3* and *POD* increased later at 48 and 72 hpi (Fig. 5C-D).

Differential expression analysis of *Cac1* gene of *C. lindemuthianum*

The change in the expression of the *C. lindemuthianum* *Cac1* gene in Sutagao bean plants inoculated with Cl(a) and Cl(b) showed significant differences between the two isolates. Virulent isolate Cl(a) showed a significantly higher expression of this fungal virulence factor than the hypovirulent Cl(b) isolate. From the early hours after inoculation (24 hpi), significant differences were found between

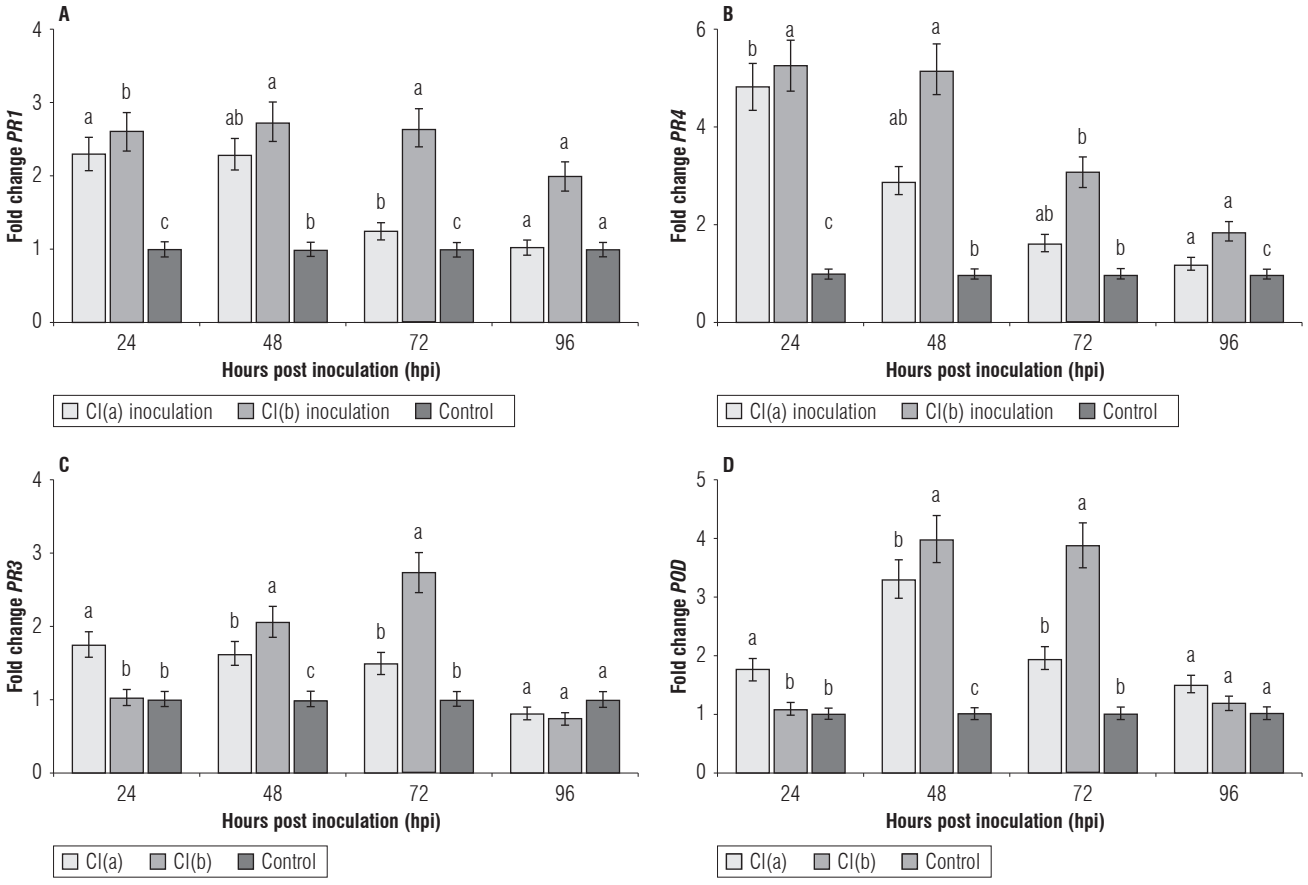

FIGURE 5. Differential expression of bean defense genes in Sutagao plants inoculated with Cl(a) and Cl(b) isolates of *C. lindemuthianum* at different time points. A) Change in *PR1* expression; B) Change in *PR4* expression; C) Change in *PR3* expression; D) Change in *POD* expression. Analysis of variance (ANOVA) of repeated measures was performed followed by the Tukey's test. Bars show the standard error of the mean calculated from 30 individual plants. Different letters represent values with significant differences between treatments ($P<0.05$).

the two isolates, which were maintained until the end of the evaluation period (96 hpi). An increase in *Cac1* expression was detected in isolate Cl(a) from 24 to 72 hpi, with a decrease at 96 hpi. In contrast, *Cac1* expression levels were low for isolate Cl(b) (Fig. 6).

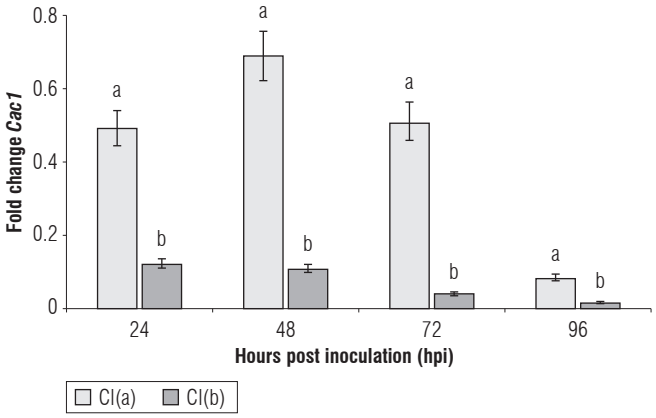

FIGURE 6. Differential expression of the *C. lindemuthianum Cac1* gene in Sutagao bean plants inoculated with Cl(a) and Cl(b) isolates at different time points. Analysis of variance (ANOVA) of repeated measures was performed followed by the Tukey's test. Bars show the standard error of the mean calculated from 30 individual plants. Different letters represent values with significant differences between treatments ($P<0.05$).

Discussion

In this work, we characterized two *C. lindemuthianum* strains morphologically and pathogenically, revealing contrasting results. Isolate Cl(a) acquired salmon coloration conferred by the production of acervuli and conidia on PDA medium and was more virulent than isolate Cl(b), which did not sporulate on PDA medium. Based on the described results the *C. lindemuthianum* isolate Cl(a) was categorized as virulent and Cl(b) was classified as hypovirulent, indicating a correlation between variations in morphological characteristics and virulence. Fu, Shin *et al.* (2022) also associated the morphological characteristics of *Colletotrichum scovillei* with its infective capacity on bell pepper fruits. In that case, the production of larger conidia, a lower germination rate, and the absence of appressoria formation at the tip of the germ tubes, influenced the generation of a successful infection, which was reflected in the absence of anthracnose symptoms in bell pepper fruits (Fu, Shin *et al*., 2022).

The alterations in the fungus morphology, particularly in the infective structures, could be related to the deletion of certain genes or epigenetic changes involved in the regulation of conidial germination and appressorium formation, which are associated with low pathogenicity (Fu, Park *et al*., 2022; Jiang *et al*., 2021; Romero *et al*., 2024; Yamauchi *et al*., 2004). In most plant pathogenic fungi, MAPKs regulate the formation of infection structures that are essential for penetration and colonization within the plant. Among MAPKs, MAP kinase 1 (PMK1) is essential for appressorium formation, plant penetration and infection process (Fu, Park *et al*., 2022). For example, deletion of this gene (*Cspmk1*) in *C. scovillei* resulted in morphologically abnormal conidia, delayed germination and loss of virulence in pepper fruits (Fu, Shin *et al*., 2022).

For molecular identification of *Colletotrichum* at the species level, different markers such as the internal transcribed spacer (ITS) region of ribosomal RNA, actin, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase, and chitin synthase 1 have been recommended (Ruiz-Campos *et al*., 2022). However, in the phylogenetic analyses of *Colletotrichum* spp., the ITS region is an important molecular marker with discriminatory power because it can separate taxa at the species complex level very well, defining the clades of species of this pathogen (Guevara-Suarez *et al*., 2022). *Colletotrichum* species associated with cultivated plants in Colombia, Ecuador, Peru, and Venezuela have been distributed in five clades: Acutatum, Boninense, Gigasporum, Gloeosporioides, and Orbiculare. The last clade includes the species *C. lindemuthianum*, which is restricted to the hosts *Phaseolus vulgaris* and *P. coccineus* (Fabaceae) (Guevara-Suarez *et al*., 2022).

In this study, the ITS marker confirmed that both isolates belong to the species *C. lindemuthianum*, although they presented morphological and pathogenicity differences. At the molecular level, the Cl(a) isolate presented identity with *C. lindemuthianum* accession KR012909, which corresponds to an isolate reported as an endophyte obtained from germinated bean seeds of cultivar CN (Cabeza Negra) in Colombia (Parsa *et al*., 2016). In the phylogenetic analysis, this isolate was placed in the third group of *C. lindemuthianum* within the Orbiculare clade (Guevara-Suarez *et al*., 2022), where there were also sequences of isolates of this fungus from the bean cultivar Cargamanto from the Antioquia Department in Colombia (Fig. 1). In contrast, an unexpected result was found with the isolate Cl(b) which presented an identity with accession JX546805, belonging to group 2 of the Orbiculare clade, where there are ribosomal RNA sequences of bean isolates from the United States, Germany, France, and the Netherlands (Damm *et al*., 2013; Guevara-Suarez *et al*., 2022; Liu *et al*., 2013). Furthermore, the ITS sequences of Cl(a) and Cl(b) did not match other *Colletotrichum* species from the Orbiculare clade, specifically *C. orbiculare* and *C. trifolii*, which are crop pathogens,

or the other five weed-infecting species *C. malvarum*, *C. bidentis*, *C. sidae*, *C. spinosum*, and *C. tebeestii* (Damm *et al*., 2013). Several molecular markers and PCR-based typing strategies (Ansari *et al*., 2004; Mahuku & Riascos, 2004) have been used for the assessment of fungal species variability. However, in this case, an ITS-based characterization allowed relating macroscopic, microscopic and virulence traits, contributing to a comprehensive polyphasic analysis of *C. lindemuthianum* isolates.

The *Colletotrichum* species clustered in the Orbiculare clade also express the *Cac1* gene, which encodes adenylate cyclase, involved in the production of cyclic AMP (cAMP) from ATP. cAMP controls the phosphorylating activity of protein kinase A (PKA) and cooperates with the MAPK cascade, leading to conidial germination, appressorium penetration, and fungal growth (Fu, Park *et al*., 2022; Jiang *et al*., 2021; Romero *et al*., 2024; Yamauchi *et al*., 2004). In this study, the expression of the *Cac1* gene of *C. lindemuthianum* was higher in the virulent isolate Cl(a) with respect to the hypovirulent Cl(b). We hypothesize that the observed morphological changes could result from altered expression or deletions of this gene or other key genes. Homologs to the *Cac1* gene have been detected in several plant pathogenic fungi and are considered essential for a successful infection process, mainly in the early stages (Jiang *et al*., 2021). It has been detected in the colonization process of *Magnaporthe oryzae*, *Fusarium graminearum*, and in different *Colletotrichum* species such as *C. lagenarium*, *C. scovillei*, and *C. lindemuthianum* (Bormann *et al*., 2014; Fu, Park *et al*., 2022; Romero *et al*., 2024; Yamauchi *et al*., 2004; Yin *et al*., 2018; Zhou *et al*., 2012). Romero *et al*. (2024) showed an increase in the expression of *Cac1* in *C. lindemuthianum* race 7 at 24 hpi during the initial stages of infection, although under our conditions, the highest expression was at 48 hpi in Cl(a), demonstrating that the expression of *Cac1* at initial stages is critical for conferring virulence.

Regarding the defense response of Sutagao bean plants exposed to Cl(a) and Cl(b) isolates, we found that plants infected with the virulent isolate Cl(a) presented the lowest expression levels of the genes evaluated. In contrast, plants infected with the hypovirulent isolate Cl(b) exhibited higher expression. Similar results were reported by Alvarez-Diaz *et al*. (2022) in the bean cultivar BAT93, where a higher expression of the defense genes *PR1*, *PR10* and *PR5* was observed at 48 and 72 hpi when exposed to the low-virulence *C. lindemuthianum* strain C531. Contrastingly, a lower expression of these genes was observed when the plants were exposed to the high-virulence *C. lindemuthianum* strain 100 (Alvarez-Diaz *et al*., 2022). Similarly, in a differential expression evaluation of defense genes in different bean cultivars, the lowest expression of *PR1*, *PR3*, *PR4*, and *POD* genes was detected late in the susceptible Sutagao cultivar. However, an earlier expression of these genes was detected in the resistant G2333 bean genotype in the presence of race 7 of *C. lindemuthianum* (Pedroza *et al*., 2022). This behavior was similar to that found in our plants when infected with the hypovirulent isolate Cl(b).

Furthermore, in this work, plants infected with the hypovirulent Cl(b) isolate showed an early increase in *PR1* gene expression at 24 and 48 hpi. This is similar to what was reported by Shams *et al*. (2020) and Alvarez-Diaz *et al*. (2022), who highlighted the importance of this protein in the early defense response of bean against *C. lindemuthianum*. *PR1* is considered a marker of salicylic acid biosynthesis and is positively regulated in the defense process, showing higher expression during an incompatible interaction event in the absence of disease (Alvarez-Diaz *et al*., 2022). *PR1* expression may also be associated with a PTI- and/or ETI-type response, in which the signaling pathway associated with the production of salicylic acid (SA) is activated. SA participates in activating the defense response against biotrophic and hemibiotrophic pathogens such as *C. lindemuthianum* (Peng *et al*., 2021). Early PR1 expression detected in plants inoculated with the hypovirulent Cl(b) between 24 and 48 hpi could correspond to the time when the pathogen was in a biotrophic state in the host (Nabi *et al*., 2024; Romero *et al*., 2024). According to fluorescence microscopy assays reported for the infection process of this fungus, after 24 hpi the conidia fully germinate and penetrate directly to cause infection, and by 48 hpi, it forms a primary hypha inside the cell (Nabi *et al*., 2024; Romero *et al*., 2024). Altogether, these results demonstrate that PR1 is essential in early defense response to *C. lindemuthianum* infection.

Conclusion

P. vulgaris-*C. lindemuthianum* interaction is highly specific and depends on the genetic characteristics of both the host and the pathogen. When bean plants were exposed to a virulent isolate (Cl(a)), they developed the disease very rapidly, leading to plant death. In this scenario, the plants failed to counteract the pathogen infection, which was related to lower expression of the *PR1*, *PR4*, *PR3*, and *POD* genes at 24, 48, and 72 hpi. In this case, the pathogen developed a successful infection, which was also associated with the early expression of the virulence factor *Cac1* during infection (24–48 hpi). This gene regulates the cAMP-dependent kinase-type phosphorylation cascade that allows for the development of infective fungal structures and ensures

disease onset. In contrast, bean plants of the Sutagao cultivar were confronted with a hypovirulent isolate Cl(b) of *C. lindemuthianum*, the disease development was delayed, and the symptoms were less severe. Here, plants showed higher expression of *PR1*, *PR4*, *PR3*, and *POD* genes at 24, 48, and 72 hpi, which counteracted early infection. This was also associated with lower expression of the fungal virulence factor *Cac1* at 24 and 48 hpi, which prevented the early formation of infective structures in the plants.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

Conceptualization: CS and AG; Methodology: CS and AG; Experiments: CS; Data analysis: CS and AG; Writing – original draft preparation: CS; Writing – review and editing: CS and AG; Funding acquisition: AG. All authors reviewed the final version of the manuscript.

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Efficacy of aqueous extracts of neem (*Azadirachta indica*) leaves in the control of date palm Boufaroua (*Oligonychus afrasiaticus*) in Algeria

Eficacia de los extractos acuosos de hojas de neem (*Azadirachta indica*) en el control de Boufaroua (*Oligonychus afrasiaticus*) en la palma datilera en Argelia

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ABSTRACT

In Algeria, the neem plant *Azadirachta indica* (Magnoliopsida: Sapindales: Meliaceae) has insecticidal and miticidal properties. The use of such a natural biopesticide, preserving the environment and human health, is necessary. We conducted this study to evaluate the efficacy of aqueous extracts of *A. indica* leaves in the control of *Oligonychus afrasiaticus* (Arachnida: Trombidiformes: Tetranychidae) (Boufaroua), one of the main pests of the date palm. The results obtained showed a highly significant effect (P<0.001) in the control of this pest for the two doses applied (D1: 100% stock solution of aqueous extracts of neem leaves, D2: 25% dilution of the stock solution). The mortality rate against Boufaroua was 97.61% and 96.36%, respectively, for the two doses used, with no significant difference between them. These highly effective and widely available extracts can be used to control pest populations of date palm such as *O. afrasiaticus*. The results obtained for this biochemical control method are technically effective and economically affordable, given the widespread availability of neem over a large part of southern Algeria. It would be worthwhile extending neem cultivation to all regions where Boufaroua is abundant. This practice would help to protect human health and the environment.

Key words: biological control, biopesticide, mite, Sahara.

RESUMEN

En Argelia, el árbol de neem *Azadirachta indica* (Magnoliopsida: Sapindales: Meliaceae) tiene propiedades insecticidas y acaricidas. Su uso como biopesticida natural que preserva el medio ambiente y la salud humana es necesario. Llevamos a cabo este estudio para evaluar la eficacia de extractos acuosos de hojas de *A. indica* en el control de *Oligonychus afrasiaticus* (Arachnida: Trombidiformes: Tetranychidae) (Boufaroua), una de las principales plagas de la palmera datilera. Los resultados obtenidos mostraron un efecto insecticida altamente significativo (P<0.001) en el control de esta plaga con las dos dosis aplicadas (D1: 100% es la solución madre de extractos acuosos de hojas de neem, D2: diluida al 25% de la solución madre). La tasa de mortalidad al aplicar este producto contra la Boufaroua fue respectivamente de 97,61% y 96,36% para las dos dosis utilizadas, sin que se observaran diferencias significativas entre las dos dosis aplicadas. Estos extractos son eficaces y ampliamente disponibles, por lo que pueden utilizarse en el control de plagas de la palmera datilera tales como *O. afrasiaticus*. Los resultados obtenidos para este método de control bioquímico son técnicamente eficaces y económicamente asequibles, dada la disponibilidad de la planta en gran parte del sur de Argelia. Sería conveniente extender el cultivo de neem a todas las regiones donde abunde la Boufaroua. Esta práctica contribuirá a proteger la salud humana y el medio ambiente.

Palabras clave: control biológico, biopesticida, ácaro, Sahara.

Introduction

One of the most serious pests of date palms is the mite *Oligonychus afrasiaticus* (McGregor), commonly called "Boufaroua". The adult mite, with four pairs of legs and a yellow to pink oval body, measures 0.2 to 0.4 mm long and is practically invisible to the naked eye (INPV, 2009). During its activity, the mite lays eggs on the dates where they are protected by dense white or greyish silky web which is secreted by the adult at the time of oviposition (Bounaga & Djerbi, 1990). In Algeria, the activity increases rapidly in the spring, from May onwards, and becomes important when coinciding with the forming of date bunches (Bahlouli & Talmat, 2017). It feeds by puncturing the fruit epidermis and sucks the contents of the cells. The epidermis of injured fruits becomes rough, wrinkled, pigmented and reddish and dates develop poorly (Munier, 1973). The damage caused by the mite to Algerian palm

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groves was estimated at 30-70% of the date production in 1981 (Guessoum, 1986) and in some cases even wiped out the entire crop (INPV, 2009).

Chemical control remains the main mechanism to reduce the incidence of this pest in palm groves. Since the 1950s, control measures have been the application of sulphur dust mixed with lime or with wood ash at a rate of 1/3 - 2/3, on the bunches and the heart of the palm (N'Diaye & Tourneur, 1972). The effectiveness of sulphur has decreased significantly in recent years in some countries, probably due to the development of resistance in the pest. Other miticides are now used, with the risk of residues in fruits, resistance problems and elimination of beneficials (Gómez Vives & Ferry, 2005). Due to the increasing consumer concern regarding this residual pollution and the toxic effects of many synthetic insecticides, natural alternatives are becoming necessary (Bankole, 2004).

Trials in southern Tunisia using the predatory mite *Neoseiulus californicus* have shown that it is able to effectively control Boufaroua populations, especially when releases are made at the beginning of fruit colonization by the phytophage (Khoualdia *et al*., 2001). Releases of *Stethorus punctillum* (Coleoptera: Coccinellidae) were carried out on date palms infested by *O. afrasiaticus* for the first time in Algeria (Idder & Pintureau, 2007). Their study showed that this ladybug plays an important role in the control of the mites, particularly when the trees are heavily infested.

Several botanical control alternatives have also been developed, including the use of plants with insecticidal, anti-appetent and repellent properties as well as mineral oils. Among these plants, neem (*Azadirachta indica*) (seeds, leaves, and bark) has been used to control populations of insects in several families as well as viral diseases (Déla *et al*., 2014; Kossou *et al*., 2007; Kulimushi Bwanampongo, 2014; Yarou *et al*., 2017).

The objective of this study was to investigate the efficacy of the aqueous extracts of neem leaves in the control of the mite *Oligonychus afrasiaticus*.

Materials and methods

Experimental site

The experiment was carried out in a palm garden located in Sidi Amrane, a commune of Oued Righ, 120 km from El Oued, Algeria. The garden contained 50 palm trees, with 33 Deglet-Nour plants, 13 Degla, three Ghars, and one Tantboucht. The presence of cover crops such as pepper and alfalfa and a few fig trees were noted (Fig. 1).

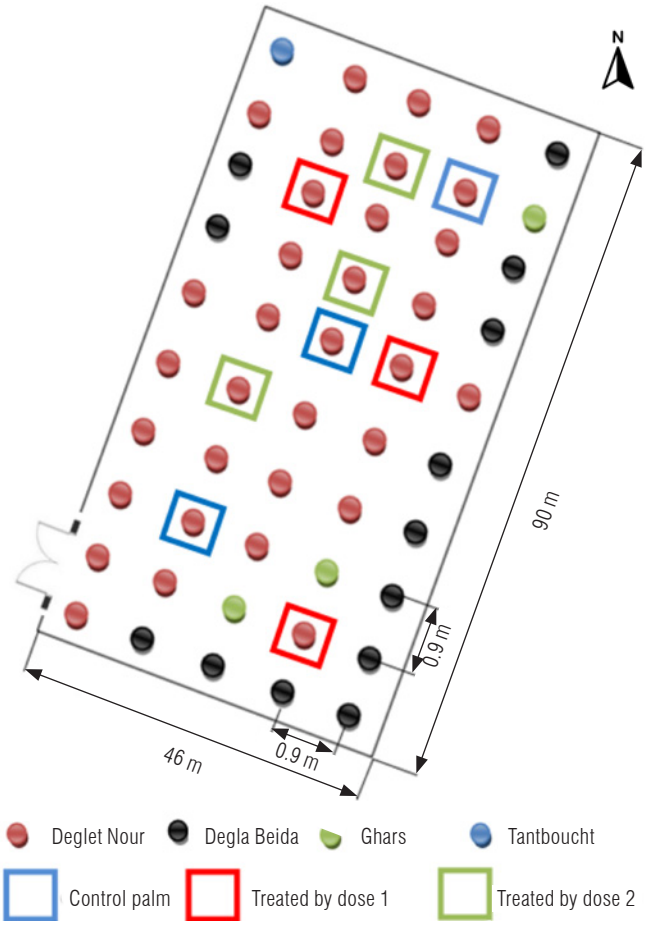

FIGURE 1. Plot diagram and experimental setup of the study site.

The distance between the palms and the rows was 9 m x 9 m. The average height of the date palms was 2 m. The plot was irrigated by submersion with water pumped from the Albian aquifer, a practice authorized by the government and used by all farmers in the Saharan regions given the lack of rainfall. The garden was surrounded by a windbreak composed of dry palms.

The experiment was conducted on the Deglet-Nour variety due to its predominance and economic importance in the study area.

Estimation of the infestation rate of palms by *Oligonychus afrasiaticus*

In a phoenicultural garden, Boufaroua infestation of date palms varies from tree to tree. This degree of contamination can be visually assessed by the amount of webbing present in the date bunches of a date palm.

Initially, for consistency, we identified all Deglet-Nour trees with the same average infestation rate. We took three branches (one external, one internal, and one middle) per bunch for the nine trees selected for our experiment: six were treated with two doses (100%, 25%) and three served as controls. The selected branches, cut with pruning shears, were soaked in labelled bottles filled with 40% alcohol to kill the mites before being brought back to the laboratory for counting.

In the laboratory, the alcohol was filtered through filter paper and then, using a binocular magnifying glass, a total population count was made. This allowed for determining the number of mites per branch was known, then per bunch, which can be extrapolated to the entire palm tree.

Plant selection and preparation of aqueous extracts of neem leaves

The plant material used as an acaricide consisted of fresh neem leaves, collected from trees in Adrar (southwestern Algeria) in July. These leaves were finely ground using an electric mortar grinder.

The crushed material was soaked in water at a rate of 200 g per 1 L of water. The mixture was left to infuse for 24 h under ambient conditions and then filtered through a very fine mesh screen. The filtrate obtained constituted the aqueous extract of neem leaves. Two doses were obtained and applied to the pest *O. afrasiaticus* to determine the insecticidal effect of the extract. The first dose was the stock solution (100%) and the second was at 25%. The dilution was carried out according to the following formula:

D1 x V1 = D2 x V2, where

D1: dosage of the stock solution with 100% neem leaf extract,

D2: dosage of the diluted solution with 25% neem leaf extract,

V1: volume of the stock solution containing 100% neem leaf extract,

V2: volume of the solution diluted to 25% neem leaf extract.

Treatments

Firstly, it would be useful to study the effect of distilled water on Boufaroua. To this end, about 15 bunches of infested dates were sprayed with distilled water. After 48 h, no effect of the water on mite mortality was observed.

Secondly, in the laboratory, we counted the number of spider mites on date branches from the fruit garden under controlled conditions, before and after spraying with water. The effect of water on Boufaroua mortality was practically negligible.

Furthermore, the sparse rainfall in July and August, when mite infestation was at its peak, had no effect on the proliferation of pests.

The application of aqueous extracts of neem leaves was carried out on Deglet-Nour trees with three replicates for each dose. The first application was carried out on 23 July 2019. By spraying, we treated all the bunches of trees (except controls) selected in our sampling.

After one week, a second application was made with a quantity of 4 L per tree. The spraying was done with a 16 L battery-operated backpack sprayer. Three days later, we carried out the final sampling (control and treated trees) in order to evaluate the infestations and the effectiveness of the neem extract.

Socio-economic impact

Such a trial, if it produces significant results, would be worth sharing with the region's palm farmers. These farmers, who generally have very modest incomes, are often looking for alternatives to pesticides, which are relatively expensive, especially when applied to large areas with many trees.

Statistical analysis

The infestation rate of dates was analyzed using: i) a one-factor analysis of variance (ANOVA) (treatment) and ii) a two-factor analysis (degree of infestation of the trees and treatment) in order to compare the infestation rates before and after the treatments. The comparison of means was performed using Fisher's PLSD test to distinguish the effectiveness of the different doses. All analyses were performed with XLSTAT (Version 2009, 1.02).

Results

Infestation of dates by mites *Oligonychus afrasiaticus*

Adult mite population counts carried out on control plants and plants treated with neem leaf extracts (Tab. 1) showed an increase in the number of adult mites on control plants over time, from 195 to 222 on average per branch. This increase appears to be due to climatic factors favorable to mite proliferation and multiplication, such as high heat and low air humidity.

TABLE 1. Infestation of date palms by mites *Oligonychus afrasiaticus* and efficacy of the neem extract applications.

Treatment	Trees studied	Sampled branches per tree	Average number of mites per branch		Percentage of mortality
			Before treatment	After treatment	
Control	3	3	195.56 ± 2.16	222.16 ± 4.83	27
Dose 1 (100%)	3	3	195.56 ± 2.16	4.66 ± 0.040	97.61 ±1.07
Dose 2 (25%)	3	3	195.56 ± 2.16	7.11 ± 0.088	96.36±1.01

Control – distilled water.

TABLE 2. Evaluation of neem extract dose in relation to mite mortality. Applied dose / Fisher's (LSD) test/ Analysis of the differences between the modalities are presented with a 95% confidence interval.

Contrast	Difference	Standardized difference	Critical value	Pr >Diff	Significant
D0 vs D1	190.872	8.473	2.048	< 0.001	Yes
D0 vs D2	188.427	8.365	2.048	< 0.001	Yes
D2 vs D1	2.444	0.100	2.048	0.921	No

D0 – distilled water, D1 – neem leaf extract at 100%, D2– neem leaf extract at 25%.

TABLE 3. Results of the Fisher's PLSD test.

Modality	Mean	Groups
D0	195.538	A
D1	7.111	B
D2	4.667	B

D0 – distilled water, D1 – neem leaf extract at 100%, D2 – neem leaf extract at 25%.

Efficacy of aqueous extracts of neem leaves

Table 1 shows the efficacy of neem leaf extracts in controlling *O. afrasiaticus*. The average number of mites per branch on treated plants decreased from 195 to 4.6 individuals for the first dose of 100% of the stock solution. In addition, 7 individuals remained for the second dose at 25% neem extract, with a very high mortality rate of 96 to 97%.

The analysis of variance of mite mortality rate (Tab. 2) revealed a highly significant difference between the plants treated with the two doses and the control (P<0.001). However, when the averages of the two doses (D1 and D2) were compared, no significant difference was found between them (P=0.921) (Tab. 2). Both doses belonged to the same group B according to Fisher's PLSD test (Tab. 3).

Figures 2A and B show the dates infested with Boufaroua before and after treatments with aqueous extracts of neem leaves.

FIGURE 2. Infested dates A) before treatment, B) three days after treatment.

Discussion

The aqueous extracts of neem leaves significantly reduced the infestation density of date palm mites per plant compared to the control (distilled water), with a very high mortality rate.

The effectiveness of these extracts is thought to be due to the presence of azadirachtin, which is more concentrated in neem seeds (Gauvin *et al*., 2003). Azadirachtin is the most abundant, powerful, and effective compound present in *A. indica* (Morgan, 2009), whose repellent, anti-appetent, fecundity-reducing and larval moult-inhibiting properties have been demonstrated (Lowery *et al*., 1993; Mordue, 2004).

Azadirachtin is a terpenoid that acts as a growth regulator by antagonistically disrupting insect hormones (Banken & Stark, 1997), physiological processes and the hormonal cycle, inducing malformations in the moulting process and preventing normal development, optimal growth, and reproduction (Mordue *et al*., 2005; Morgan, 2009). It also has anti-appetent effects on the natural movement of the intestine, causing paralysis and a decline in target organisms (Juan *et al*., 2000; Senthil-Nathan *et al*., 2004). It is rapidly absorbed by plant tissues, ensuring effective systemic action (Bernard *et al*., 2008).

Our results also show that the effect of the two doses applied of the neem extract (D1, D2) is nearly identical, as they belong to the same homogeneous group B according to Fisher's PLSD test (Tab. 3, Figs. 2A-B). The D2 dose is sufficient to control this pest.

This is confirmed by the results of Looli Boyombe *et al.* (2022), who concluded that the active principle of neem is found in all parts of the tree and that using any part of neem as a pesticide against *Spodoptera frugiperda* can result in a mortality rate of over 50%, even in an uncontrolled natural environment.

According to Biri and Sahli (2022), neem oil also causes several malformations and morphological anomalies in *Drosophila melanogaster* and inhibits adult emergence, which increases significantly as a function of concentration.

Indeed, Fortuné *et al.* (2018), in their study on the effect of *Azadirachta indica*-based aqueous extracts on aphid vectors of green pepper mottle virus, showed the efficacy of these extracts in controlling green pepper mottle virus and its vector (aphids). A similar study conducted on the efficacy of a mixture of medicinal plant extracts (*Gmelina arborea, Eucalyptus citriodora, Azadirachta indica, Hyptis suaveolens, Vernonia amygdalina,* and *Cymbopogon citratus*) in protecting flowering cowpeas against *Megalurothrips sjostedti* (Trybom) revealed that the mixture of these medicinal plants was as effective as the synthetic insecticide treatment such as Decis (Adebayo *et al*., 2007; Oparaeke, 2007).

The results obtained by Mondedji *et al*. (2014) confirm our findings and show that aqueous extracts of neem leaves are highly effective in managing insect pests (*Plutella xylostella*, *Hellula undalis*, and *Lipaphis erysimi*) and improving yields.

Additionally, according to Bélanger and Musabyimana (2005), neem extracts contain chemical substances active against insect pests in fields and stocks.

Moreover, unlike synthetic chemical insecticides, neem's use is safer for the environment because plant extracts are biodegradable and act as fertilizers (Faye, 2010). According to some authors, neem has no harmful effect on beneficial insects (Bélanger & Musabyimana, 2005).

Socio-economic impact

To further validate our results and enhance their credibility, we extended the product applications in the field by directly involving farmers in the process. The experiment was conducted in Ouargla, specifically in the Hassi Ben Abdallah region, where Boufaroua causes significant annual damage. A dozen medium-sized farms were selected to conduct demonstrations in collaboration with their owners. At the end of experiments, all the farmers were completely satisfied with the results. The use of pesticidal plant extracts, such as neem leaves, can increase yields at a lower cost-benefit ratio compared to existing synthetic pesticides on the market, thereby improving farmer incomes. The ease of preparing neem leaf extracts and their effectiveness in controlling *Oligonychus afrasiaticus* were important factors in persuading farmers in the study area to adopt this product. The production cost of this biopesticide is significantly lower than that of a chemical acaricide. It only requires planting a few neem trees in a palm grove to obtain the leaves, and it does not necessitate any special handling or storage equipment – simply storing it in powder form for several months suffices. Neem leaf extracts decompose rapidly in the environment, which limits the risk of environmental pollution and enhances the health quality of the produce grown (Faye, 2010). Moreover, the application of these biopesticides promotes a healthier, more balanced diet.

Conclusion

This study showed the effectiveness of *Azadirachta indica* leaf extracts in controlling *Oligonychus afrasiaticus* in the Oued Righ region of Algeria. The neem leaf treatment was highly effective on Boufaroua and represents an alternative to synthetic pesticides in integrated pest management of date palm.

Given these results, it would be desirable to extend this practice to all date palm growers in the Saharan regions where date palms are abundant.

Neem is well adapted to Saharan conditions. It would be beneficial to consider extending its use and making it accessible to all farmers. Additionally, adopting modern technological resources accessible to farmers would enhance pest control strategies.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

MAI designed the experiment. AA carried out the fieldwork. AA collected the data and redrafted the original draft. HII supervised the work. MAI contributed to the data analysis. MAI revised and wrote the final version of the manuscript. AA translated the final version. All authors reviewed the final version of the manuscript.

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Selection of resistance inducers for managing *Hemileia vastatrix* Berk. & Br. in coffee (*Coffea arabica* L.) seedlings

Selección de inductores de resistencia para el manejo de *Hemileia vastatrix* Berk. & Br. en plántulas de café (*Coffea arabica* L.)

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ABSTRACT

Coffee leaf rust (*Hemileia vastatrix*) is one of the most devastating coffee diseases, causing losses ranging from 23% to 50% of the crop. Chemical applications are the most employed control strategies in countries lacking resistant coffee varieties. In the search for new alternatives for integrated management, an *in vitro* and nursery evaluation protocol was developed for *Coffea arabica* cv. Caturra plants using the following resistance inducers: acibenzolar-*S*-methyl, salicylic acid, potassium phosphite, and Harpin protein. These compounds were tested at three concentrations, across three intervals between product application and pathogen inoculation, and in two response signaling pathways (local or systemic) to assess their effects on the *in vitro* germination of rust urediniospores and disease severity in 6-month-old coffee plants inoculated with the pathogen. In general, all compounds inhibited urediniospore germination and exerted disease control mediated mainly by the concentration of the product with biweekly application intervals and where local responses prevailed more than systemic ones. This study highlights the potential of these compounds as resistance inducers, especially for acibenzolar-*S*-methyl, where we observed the best effects on disease control. Our findings open new avenues for incorporating resistance inducers into integrated disease management programs to complement fungicide applications.

Key words: acibenzolar-*S*-methyl, coffee leaf rust, urediniospores, germination, disease severity.

RESUMEN

La roya del café (*Hemileia vastatrix*) es una de las enfermedades más devastadoras del café, causando pérdidas que van desde el 23% hasta el 50% del cultivo. Las aplicaciones químicas son las estrategias de control más comúnmente empleadas en países que carecen de variedades de café resistentes. En la búsqueda de nuevas alternativas para el manejo integrado, se desarrolló un protocolo de evaluación *in vitro* y en vivero para plantas de *Coffea arabica* cv. Caturra utilizando los siguientes inductores de resistencia: acibenzolar-S-metil, ácido salicílico, fosfito de potasio y proteína Harpin. Estos compuestos se probaron en tres concentraciones, en tres intervalos entre la aplicación del producto y la inoculación del patógeno, y en dos vías de señalización de respuesta (local o sistémica) para evaluar sus efectos sobre la germinación *in vitro* de las urediniosporas de la roya y la severidad de la enfermedad en plantas de café de 6 meses de edad inoculadas con el patógeno. En general, todos los compuestos evaluados inhibieron la germinación de las urediniosporas y ejercieron un control de la enfermedad mediado principalmente por la concentración del producto con intervalos de aplicación quincenal y en donde prevalecieron más las respuestas locales que las sistémicas. Este estudio destaca el potencial de estos compuestos como inductores de resistencia, especialmente del acibenzolar-S-metil, con el que se observaron los mejores efectos en el control de la enfermedad. Los hallazgos abren nuevas vías para incorporar inductores de resistencia en programas de manejo integrado de enfermedades como complemento a las aplicaciones de fungicidas.

Palabras clave: acibenzolar-S-metil, roya del café, urediniosporas, germinación, severidad de la enfermedad.

Introduction

Coffee leaf rust, caused by the fungus *Hemileia vastatrix* Berkeley & Broome (Basidiomycota, Pucciniales), remains the primary disease affecting coffee crops worldwide (Koutouleas *et al*., 2024; Sera *et al*., 2022; Zambolim, 2016). In Colombia, it causes losses exceeding 30% in susceptible varieties of *Coffea arabica* when preventive control measures are not implemented (Rivillas *et al*., 1999). This disease is closely linked to the physiological development of the crop, plant yield, and the region's rainfall distribution and amount (Rivillas *et al*., 2011). Damage from coffee rust is

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caused by a significant reduction in the photosynthetic area due to lesions and generalized defoliation caused by the pathogen (Avelino *et al*., 2015).

Infection of *H. vastatrix* begins with the germination of uredospores on the underside of the leaves, requiring optimal conditions of humidity, temperature, and low or an absence of light intensity (Haddad *et al*., 2009; Hernández-Amasifuen *et al*., 2023). Following germination, appressoria form on the stomata, allowing the penetrating hypha to enter the substomatal chamber. There, the hyphae develop haustoria, enabling intracellular colonization (Gichuru *et al*., 2012; Ramiro *et al*., 2009). During colonization, the hyphae intertwine within the substomatal cavities, eventually forming protosori. These mature into uredos over approximately three weeks, protruding through the stomata to form orange pustules that are visible structures characteristic of the infection on the leaf's underside (Salazar-Navarro *et al*., 2024; Talhinhas *et al*., 2017). Severe attacks result in excessive defoliation, exposing growth nodes on the plant branches and buds to direct radiation, leading to progressive cell death. This continuous cell death and necrosis of growth nodes affect potential plant yields during the current and subsequent harvest seasons (Kushalappa & Eskes, 2019).

Chemical control is a crucial component of integrated coffee rust management, particularly in *Coffea arabica* varieties such as Caturra and Typica, susceptible to the disease. In Colombia, chemical control is applied using cupric compounds or systemic fungicides such as triazoles and strobilurins (Rivillas *et al*., 2011). However, this approach can increase the presence of multiple rust races due to changes in pathogen virulence and dynamics in planting varieties with complete and partial resistance. This leads to new isolates infecting previously resistant materials (Rozo-Peña & Cristancho, 2010). The appearance of new races is often related to increased selective pressure on certain pathogenic races due to improper fungicide application, such as using higher or lower doses than recommended or increasing spraying frequency (Deising *et al*., 2008). Fortunately, until 2012, no rust races resistant to commonly used fungicides, such as triazoles, have been found in Colombia (Cristancho *et al*., 2012).

Induced resistance against plant pathogens represents an alternative disease control method, activating latent defense mechanisms in plants. This resistance can be triggered by biotic agents (plant extracts, microorganisms, or parts of these organisms) or abiotic agents (chemicals) (Cavalcanti *et al*., 2005; Reglinski *et al*., 2023; Resende *et al*., 2006). Induced resistance (IR) is divided into systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Van Loon, 1997). In IR, resistance operates systemically or locally in response to a pathogen causing necrotic lesions (hypersensitivity reaction) or through exogenous application of synthetic compounds primarily mediated by the salicylic acid (SA) metabolic pathway. Unlike SAR, ISR is mediated by jasmonic acid and ethylene metabolism triggered by beneficial or endophytic plant microorganisms or by the exogenous application of synthetic compounds (Flors *et al*., 2024; Pieterse *et al*., 2001). According to Gust *et al*. (2012), initiating plant defense processes against possible infection or colonization by microorganisms requires molecular dialogue between the organisms involved, considering 1) the perception of pathogen-associated molecular patterns (PAMPs) by plant pattern recognition receptors (PRRs) or 2) the perception of microbial pathovar-specific proteins by the receptors of the plant immune system (Akhter *et al*., 2021; Chisholm *et al*., 2006; Dangl & Jones, 2001; Spoel & Dong, 2012).

The success of induced resistance depends not only on the compound itself but also on factors such as 1) the concentration of the molecule, achieving the best effects without affecting the biology of the pathogen, 2) the prolonged duration of the inducing effect, and 3) the type of signaling for the immune response (Hönig *et al*., 2023).

Extensive research has been conducted to find and implement such inducers in integrated coffee disease management. Examples include extracts of rust-infected coffee leaves (Amaral *et al*., 2005, 2007; Barguil *et al*., 2005), suspensions of inactive spores from *H. vastatrix* (Costa *et al*., 2007; Leonel & Barros, 2013), foliar fertilization with phosphites or potassium silicates (Carré-Missio *et al*., 2009, 2012; Costa *et al*., 2007; Lopes *et al*., 2013; Mehta *et al*., 2022; Pereira *et al*., 2009), or the foliar application of exogenous molecules such as ASM (Fernandes *et al*., 2013; Galdeano *et al*., 2010; Ito *et al*., 2024; Marchi *et al*., 2002; Patrício *et al*., 2008), harpin proteins (Chuang *et al*., 2014; de Capdeville *et al*., 2003; Galdeano *et al*., 2010; Sands *et al*., 2022), xanthan gum (Guzzo *et al*., 1993; Hassanisaadi *et al*., 2025), and SA (Berumen *et al*., 2015; McLaughlin *et al*., 2024; Mogollón Ortiz & Castaño Zapata, 2023), etc.

Despite using conventional management strategies, such as the recurrent application of fungicides and the planting of resistant varieties, disease control remains a challenge due to the genetic variability of the pathogen and the favorable environmental conditions for its development. In this context, resistance-inducing products represent a promising alternative within integrated management programs. However, even though these types of exogenous

compounds have been widely studied and evaluated in a wide variety of pathosystems in Colombia, the information on the efficacy of these products in coffee cultivation is limited and poorly verified; this makes it challenging to integrate and adopt them in integrated coffee rust management programs at a national level. Therefore, this research seeks to generate knowledge by evaluating four molecules currently marketed in the country as resistance inducers and their efficiency in controlling coffee rust. The results will contribute to the development of innovative and sustainable strategies for disease management, benefiting producers by reducing the use of agrochemicals and strengthening the resilience of coffee plantations in the face of this crucial phytosanitary problem.

Materials and methods

Four molecules with high potential for induced resistance were selected with documented results for managing and controlling foliar diseases in economically important crops. All evaluation parameters of these molecules were designed according to selection criteria proposed by Steiner and Schönbeck (1995). They classified as inducing agents all compounds that met the following characteristics: 1) absence of direct toxic effects by the inducer on pathogen germination, 2) increase in the magnitude of resistance, regardless of the applied concentration, 3) prolonged duration of the inducing effect between the expression of resistance and plant exposure to the pathogen, and 4) systemic response. The protocol development was proposed in the following two stages:

Effect of the inducer on *H. vastatrix* urediniospore germination

Aqueous solutions of each inducer treatment were prepared at three different doses, considering the concentration of the active ingredient in the commercial product (Tab. 1). The pH of the water used ranged between 7.05 and 7.10. The source products were ASM (BION 50WG®, Syngenta Crop Protection LLC, Greensboro, NC, USA), SA (Re-Leaf®, Stoller International Inc., Houston, TX, USA), Potassium phosphite (PhytoGard Phosphyte®, Stoller International Inc., Houston, TX, USA), and Harpin protein (Messenger®, Plant Health Care, Holly Springs, NC, USA).

Coffee leaves (*Coffea arabica* var. Caturra) were collected from experimental plots at the Naranjal Central Station of Cenicafé (Chinchiná, Caldas) to obtain the rust inoculum. The leaves exhibited characteristic symptoms and signs of the disease. They were free of biological control agents such as *Simplicillium* sp. and *Lecanicillium* sp. mycoparasites of rust uredospores that are visually easy to identify due to the whitish color of the rust pustules. Subsequently, the leaves were stored in paper bags and taken to the Phytopathology Laboratory at the National Research Center of Coffee (Cenicafé), where they were placed for 48 h in moist chambers at 25°C in the dark to preserve the inoculum. Once the lesions showed abundant sporulation, the urediniospores were harvested by scraping the undersides of the leaves with a #22 scalpel blade and then storing them in gelatin capsules at room temperature for no longer than 24 h.

Inoculum solutions were prepared at a rate of 0.3 mg ml^{-1} urediniospores in sterile distilled water, yielding a final concentration of approximately 4000 urediniospores ml^{-1}. These solutions were homogenized by ultrasound for 30 s at 50 Hz and maintained under constant stirring. Ten reading units were subsequently evaluated, each consisting of 4 µl of the inducing compound solution plus 4 µl of the suspension containing homogenized urediniospores. These tests were performed on slides covered with Parafilm® M. Once all the slide covers were prepared, they were placed in moist chambers at a temperature of 22°C in complete darkness. After 4 h, 2 µl of lactophenol cotton blue dye was added to each slide to stop germination and facilitate counting germinated and non-germinated spores using an Axio Lab. A1 stereomicroscope (Zeiss Company, Oberkochen, Germany) located in the Phytopathology Laboratory at Cenicafé. All processes of counting germinated and non-germinated spores were supported by Carl Zeiss® ZEN image acquisition software and processed using an algorithm designed for this purpose in ImageJ® (version 1.49).

TABLE 1. Products and doses used.

Inducer (treatment)	Active ingredient per ml of water		
	Low dose	Recommended dose*	High dose
Acibenzolar-*S*-methyl (ASM)	50 µg	200 µg	800 µg
Salicylic acid (SA)	0.3 µl	1.2 µl	5.0 µl
Potassium phosphite	0.2 µl	0.8 µl	3.3 µl
Harpin	7.5 µg	30 µg	120 µg

*According to the product manufacturer.

Spores were considered germinated when they exhibited a germinative tube measuring more than one-third of the diameter of the urediniospore (Kushalappa & Eskes, 1989). The germination rate variable was calculated by randomly applying the treatment doses to the experimental units (slide mounts) and the doses of each inducer as the treatments. Since each molecule was evaluated separately, a daily evaluation of the absolute control (germination only in distilled water) was necessary to determine the quality of the inoculum. If the germination rate was lower than 50%, the treatment readings were not taken, and the trial was discarded. The percentage of inhibition in germination was obtained by percentage difference, considering the germination rates obtained for the absolute control of each treatment.

Effects of inducers on the control of coffee leaf rust

The coffee plants were obtained from the harvested seeds of self-fertilized *C. arabica* cv. Caturra populations to avoid genetic variability. These plants, located at the Naranjal Central Station of Cenicafé, are highly susceptible to all physiological races of *H. vastatrix*. The seedlings were transplanted into 17 x 23 cm seedbed plastic bags filled with soil supplemented with organic matter and placed in a mesh house under 40% shade, an average temperature of 25°C, and an average relative humidity of 70% until they reached six months of age or developed five pairs of fully expanded leaves. The application of fungicides or other products that could interfere with disease development was restricted. The seedbed was fertilized before treatment application with 2 g of diammonium phosphate (DAP) every two months, and irrigation was scheduled twice weekly.

When the plants reached the required number of leaves (four pairs of fully extended leaves), aqueous solutions of each inducer treatment were prepared at three different concentrations, as shown in Table 1. Each treatment was applied using a Gast® spray pump (Gast Manufacturing Inc., Benton Harbor, MI, USA) at a pressure of 5 psi, with a volume of 0.5 ml/leaf, maintaining a 30 cm distance between the applicator nozzle and the plant. For evaluations of the local and systemic response of the inducers, the second and fourth pairs of leaves were protected with a waterproof plastic cover to avoid direct contact during spraying (systemic response). The first and third pairs of leaves were applied on upper and lower surfaces to ensure uniform distribution (local response). Control treatments were applied using only distilled water, following the same spraying method.

Each treatment had an exposure time interval between product application and pathogen inoculation of 15, 30, and 45 d. Inoculation with *H. vastatrix* was performed on the same day for all intervals to avoid variations. As previously described, an inoculum solution of approximately 4 x 104 spores ml^{-1} was prepared. The inoculation was applied to the first through fourth pairs of leaves from the apex, including those previously protected using a Gast® spray pump at a constant pressure of 5 psi, with a volume of 0.5 ml/leaf, maintaining a 30 cm distance between the applicator nozzle and the plant. Once inoculated, the plants were placed in a dark room for 72 h at 23°C with a relative humidity of 90% or more, maintained by a Bahnson® humidifier (DnB Humidifier Manufacturing Inc., Winston-Salem, NC, USA), to favor pathogen germination and penetration. Afterward, the plants were returned to the mesh house under the same conditions (40% shade, average temperature 25°C, and average relative humidity 70%) until the first symptoms appeared.

The test was completely randomized with a 3x3x2 factorial arrangement (three doses x three-time intervals x two response types). The experimental unit was the seedling or nursery plant. The treatments were the interaction of the three doses (low, recommended, and high), the time intervals (15-, 30-, and 45-d post-inducer application), and the response signaling pathways (local and/or systemic). Each treatment, including the control, had six plants as replicates, each with eight leaves as reading units (n = 48). After 60 d post inoculation (the date on which the control reached maximum pustule development), the evaluation process of each treatment began. To do this, each leaf corresponding to the reading units was removed with the help of a scalpel, trying not to cause disturbance or loss of the generated pustules. The leaves were then placed in previously labeled trays and taken to the Phytopathology laboratory of Cenicafé.

Each treatment was evaluated by recording the percentage of the affected area as disease severity using a UMAX Powerlook® scanner (Model 2100XL). Each image was processed at 500 dpi on a black background with a quadratic area-preserving map. Images were individually processed with ImageJ® (version 1.49) software, calibrated to identify each lesion's minimum and maximum visible spectral ranges to determine the final severity on each reading unit. The percentage of control was obtained by percentage difference, considering the final severity obtained for the

absolute power. The test duration was 6 months and was not repeated over time.

Statistical analysis

For both the *in vitro* germination rate of urediniospores and the disease severity in the leaves, we checked for normal distribution and homogeneity of variance with the Shapiro-Wilk and Levene test. Then, a descriptive analysis of variance (ANOVA) was performed using the statistical arithmetic mean, standard deviation, and coefficient of variation. Variables showing significant differences were subjected to Duncan's means comparison test ($\alpha = 0.05$). Data analysis was performed with a Dunnett's means comparison test ($\alpha = 0.05$) for the germination rate variable since each treatment was individually compared to the absolute control. All statistical analyses were conducted using R version 3.3.2 (R Core Team, 2023).

Results and discussion

Effect of the inducer on *H. vastatrix* urediniospore germination

None of the treatments were statistically equal to the germination rates obtained for the absolute control (without product application) across the three evaluated doses (Fig. 1). On average, the germination rate for the absolute control was 67.9%, followed by potassium phosphite treatment (15.7%), ASM (15.1%), harpin (6.3%), and SA (0%). The treatment with the highest inhibition rate was SA (100%), followed by harpin (90.9%), potassium phosphite (76.4%), and ASM (74.5%).

According to the ANOVA, there were significant differences among treatments in terms of urediniospore germination after 4 h of exposure to the three different doses ($F = 230.9$, $df = 4$, *P-value* <0.0001). None of the evaluated molecules exhibited germination rates close to those of the absolute control (water alone without product application), which ranged from 59.2% to 76.2%. The germination inhibition displayed an inversely proportional relationship between the dose and the urediniospore germination rate. However, this effect did not occur with SA, which prevented spore germination even at the lowest dose, demonstrating the compound's marked direct inhibitory effect (Fig. 2).

Germination at the low dose (0.2 µl ml^{-1}) was only 27.9% germination, followed by the recommended dose (0.8 µl ml^{-1}) with 19.1% germination, and finally, the high dose (3.3 µl ml^{-1}) where germination did not occur. These findings are consistent with Costa *et al.* (2007), who evaluate the biocidal effect of 7.5 µl ml^{-1} potassium phosphite on the *in vitro* germination of *H. vastatrix* urediniospores, reporting 100% inhibition compared to the absolute control, indicating a significant toxic effect of this molecule on pathogen germination. Similarly, Fernandes *et al.* (2013), studying the efficacy of copper and manganese phosphates at a concentration of 5 µl ml^{-1} on the in vitro germination of *H. vastatrix* urediniospores, observes low germination rates of 22.8% and 20.8%.

Likewise, ASM showed adverse effects compared to those obtained in the absolute control. Germination at low doses (50 µg ml^{-1}) was only 29.9%. This was followed by the recommended dose (200 µg ml^{-1}) with 15.4% germination, and finally, the high dose (800 µg ml^{-1}) where germination did not occur. These results differ from those Guzzo *et al.* (2001) reported, who assessed ASM's biocidal effects on urediniospore germination and appressorium formation

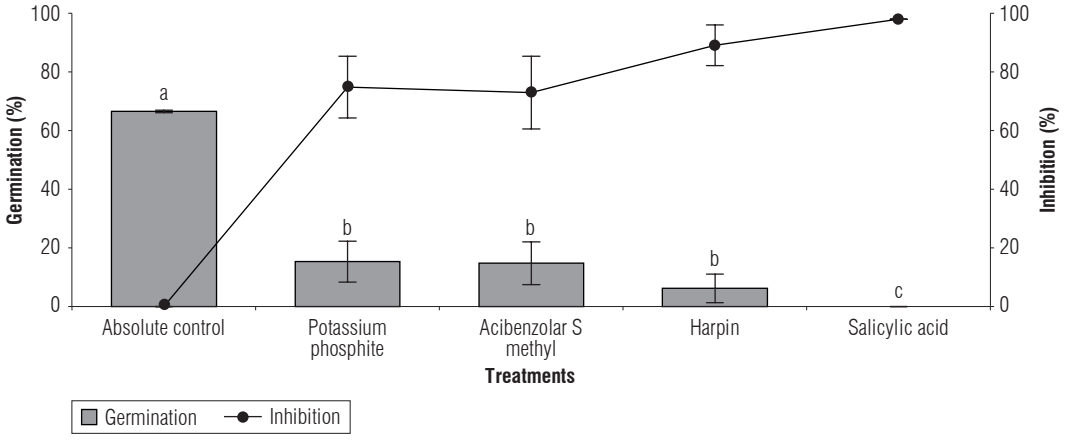

FIGURE 1. The mean germination rate of urediniospores was exposed to three doses of potassium phosphite, Acibenzolar S methyl, harpin, and salicylic acid for 4 h, compared to the absolute control. The vertical lines on the bars and dots indicate 95% confidence intervals (± SE, n = 30). According to Duncan's multiple comparison test at 5%, different letters on the vertical lines indicate significant differences.

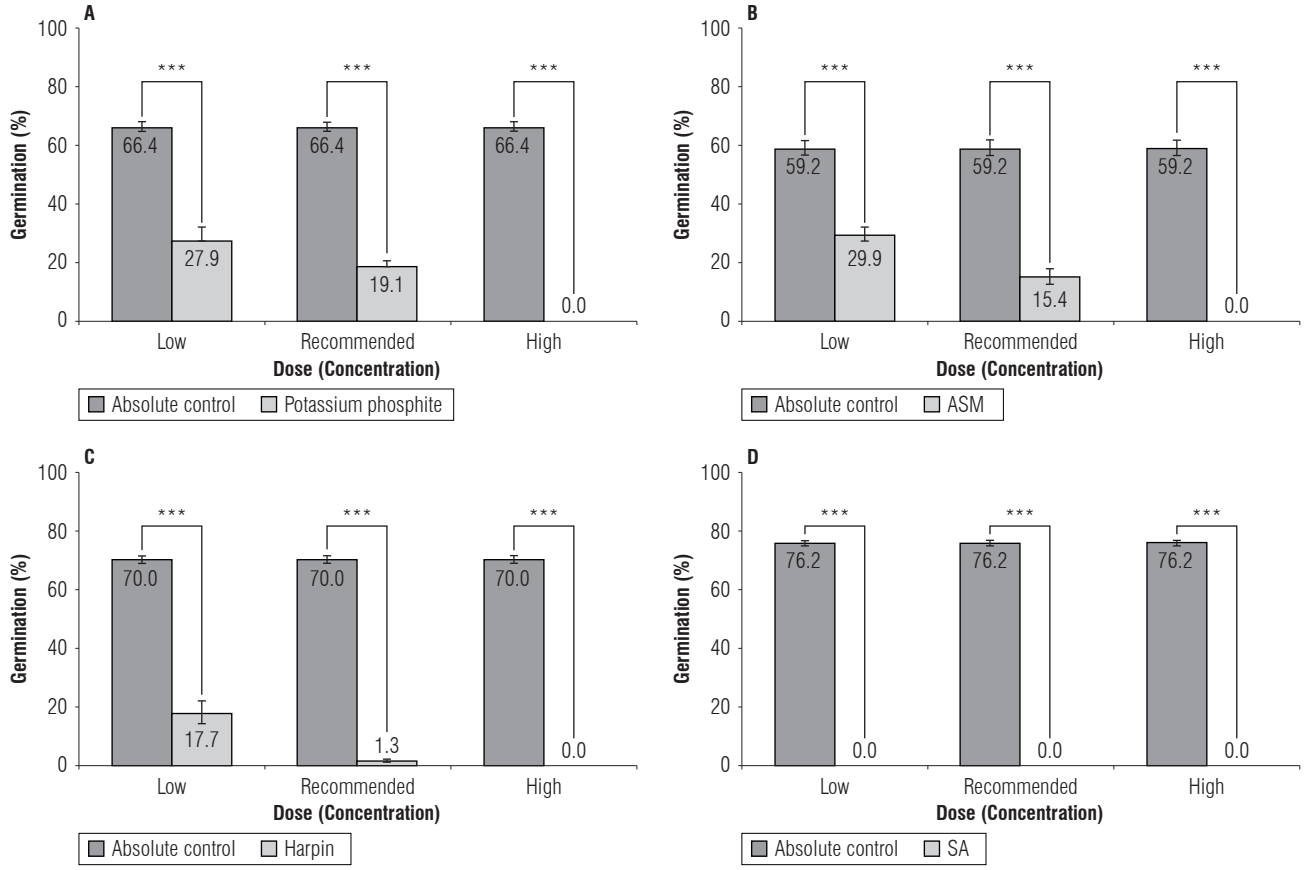

FIGURE 2. The germination rate of urediniospores exposed to three different doses of A) potassium phosphite, B) ASM - Acibenzolar S methyl, C) harpin, and D) SA - Salicylic acid for 4 h. The vertical lines on the bars indicate 95% confidence intervals (± SE, n = 10). Asterisks connected by solid lines indicate the significance levels according to Dunnett's multiple comparison tests at 5% between the dose (light bars) and the absolute control (dark bars), significance $P<0.05$ *, $P<0.01$ **, and $P<0.001$ ***.

in *H. vastatrix* using fluorescence microscopy. Their study found no significant inhibition of germination or appressorium formation across all ASM concentrations tested (10 to 400 µg ml^{-1}). Similarly, Marchi *et al*. (2002) evaluated ASM across different concentrations (1 to 1000 µg ml^{-1}) on coffee leaves. They found no fungitoxic effects on the pathogen.

Harpin also showed adverse effects compared to those obtained in the absolute control. Germination at the low dose (7.5 µg ml^{-1}) was only 17.7%, followed by the recommended dose (30 µg ml^{-1}) with 1.3% germination, and finally at the high dose (120 µg ml^{-1}) where germination did not occur. These findings contrast with those reported by Jesus (2009), who found no significant differences in the toxic effects of harpin on *H. vastatrix* urediniospore germination across a range of concentrations from 250 to 4000 µg ml^{-1}, results that were comparable to the absolute control. However, Galdeano *et al*. (2010) investigated harpin's effects on *Cercospora coffeicola*. They observed a stimulating effect on conidial germination and mycelial growth at concentrations ranging from 7.5 to 120 µg ml^{-1}. These discrepancies suggest that harpin may not exert a direct toxic effect on pathogen development but could instead stimulate growth, a phenomenon whose significance in disease control remains debated. Unlike our study, Jesus (2009) and Galdeano *et al*. (2010) applied harpin directly to leaf surfaces, allowing for absorption over approximately 6 h before pathogen inoculation (*H. vastatrix* and *C. coffeicola*), indicating that the molecule was not in direct simultaneous contact with pathogen spores.

Furthermore, it is essential to consider the pH of the aqueous dilutions used with Harpin treatment, which ranged between 5.40 and 5.57, representing the most acidic conditions in our experiment. Carré-Missio *et al*. (2012) investigated the effects of potassium silicate applications at different concentrations on *H. vastatrix* urediniospore germination across pH values of 5.5, 7.5, and 10.5. They report that at a dose of 8 g L^{-1}, germination inhibition rates are 13.9%, 15.6%, and 20.8%, respectively, indicating

varying effects of the product with spores germinating more effectively under acidic conditions than neutral pH. Finally, SA showed inhibitory effects on germination for all evaluated doses, even at the lowest dose (0.3 µl ml^{-1}).

Although most authors did not report adverse effects on the germination of *H. vastatrix*, our results suggest a toxic effect on the germination processes of the spores, mainly depending on a high concentration of the applied product. Because of this, and in light of our results, it is not possible to ensure that the control of the disease corresponds to the direct activation of the plant's defense mechanisms but to the adverse effects that the molecules have on the germination of the spores, an essential aspect if we consider that this type of research seeks the selection of molecules without direct toxic effects (Steiner & Schönbeck, 1995), due to the implications that this has on the selection pressure of the pre-existing populations of the pathogen or the generation of a more significant number of resistant isolates.

Effects of inducers on the control of coffee leaf rust

According to the ANOVA, there were significant differences among treatments regarding disease control, regardless of the dose, application interval, and type of response signaling, compared to the absolute control ($F = 26.08$, $df = 4$, *P-value* <0.0001). The severity of the absolute control was 11.6%, followed by treatments with potassium phosphite (2.7%), harpin (2.5%), SA (1.6%), and ASM (1.3%). The treatment with the best control was ASM, achieving 88.6% control compared to the absolute control, followed by SA (86.1%), harpin (78.6%), and potassium phosphite (76.6%) (Fig.3).

Regarding the dose effects, only SA and ASM showed significant differences in disease control at high doses. Potassium phosphite, SA, and ASM exhibited substantial differences compared to harpin at recommended doses. In contrast, harpin, SA, and ASM showed significant differences compared to potassium phosphite at low doses. Regarding the effect of application intervals, harpin, SA, and ASM displayed substantial differences at a 15 d interval. Additionally, SA and acibenzolar-S-methyl (ASM) showed significant differences at 30 and 45 d application intervals. Concerning the effect of type of response signaling, SA and ASM showed substantial differences for local signaling. Furthermore, ASM was the only compound showing significant differences compared to the others for systemic signaling (Fig. 4).

Potassium phosphite achieved the best results when applied at the high dose (3.3 µl ml^{-1}), followed by the recommended dose (0.8 µl ml^{-1}), and then the low dose (0.2 µl ml^{-1}), with disease control percentages of 83.4%, 82%, and 64%. Optimal disease control was observed when treatments were applied at a 15-d interval, followed by 30 and 45-d intervals, resulting in control percentages of 82.6%, 75.5%, and 71.2%. The predominant signaling pathway was more local (83.7%) than systemic (69.2%), indicating that disease control is more effective with higher doses applied at intervals not exceeding 15 d, achieving control rates ranging from 80% to 86% (Fig. 5A). These results contrast with Costa *et al.* (2007), who demonstrates effective disease control (57-65%) with 7.5 µl ml^{-1} potassium phosphite applied at intervals close to 30 d. Similarly, Fernandes *et al.* (2013) found that manganese phosphite provided higher disease

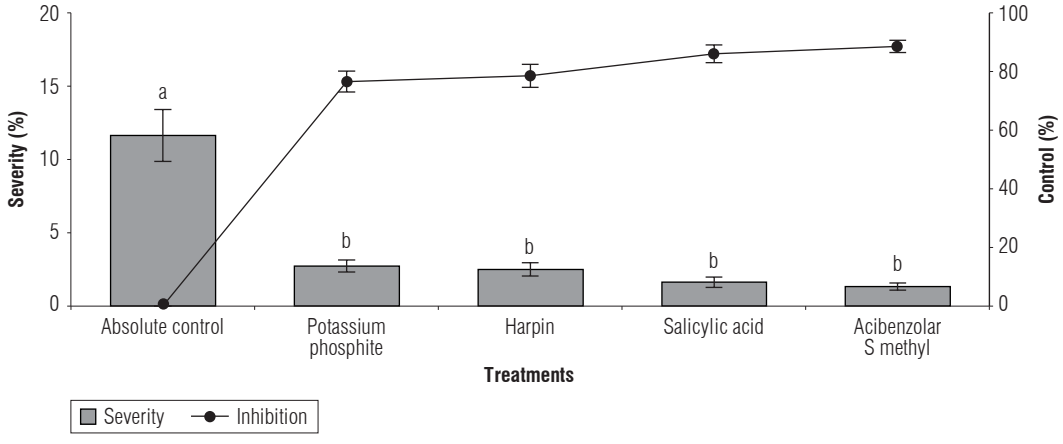

FIGURE 3. Mean severity index at three doses, three different time intervals between product application and pathogen inoculation, and two signaling pathways in the responses to potassium phosphite, harpin, SA, and ASM compared to the absolute control. The vertical lines on the bars indicate 95% confidence intervals (± SE, n = 54). According to Duncan's multiple comparison test at 5%, different letters on the vertical lines indicate significant differences.

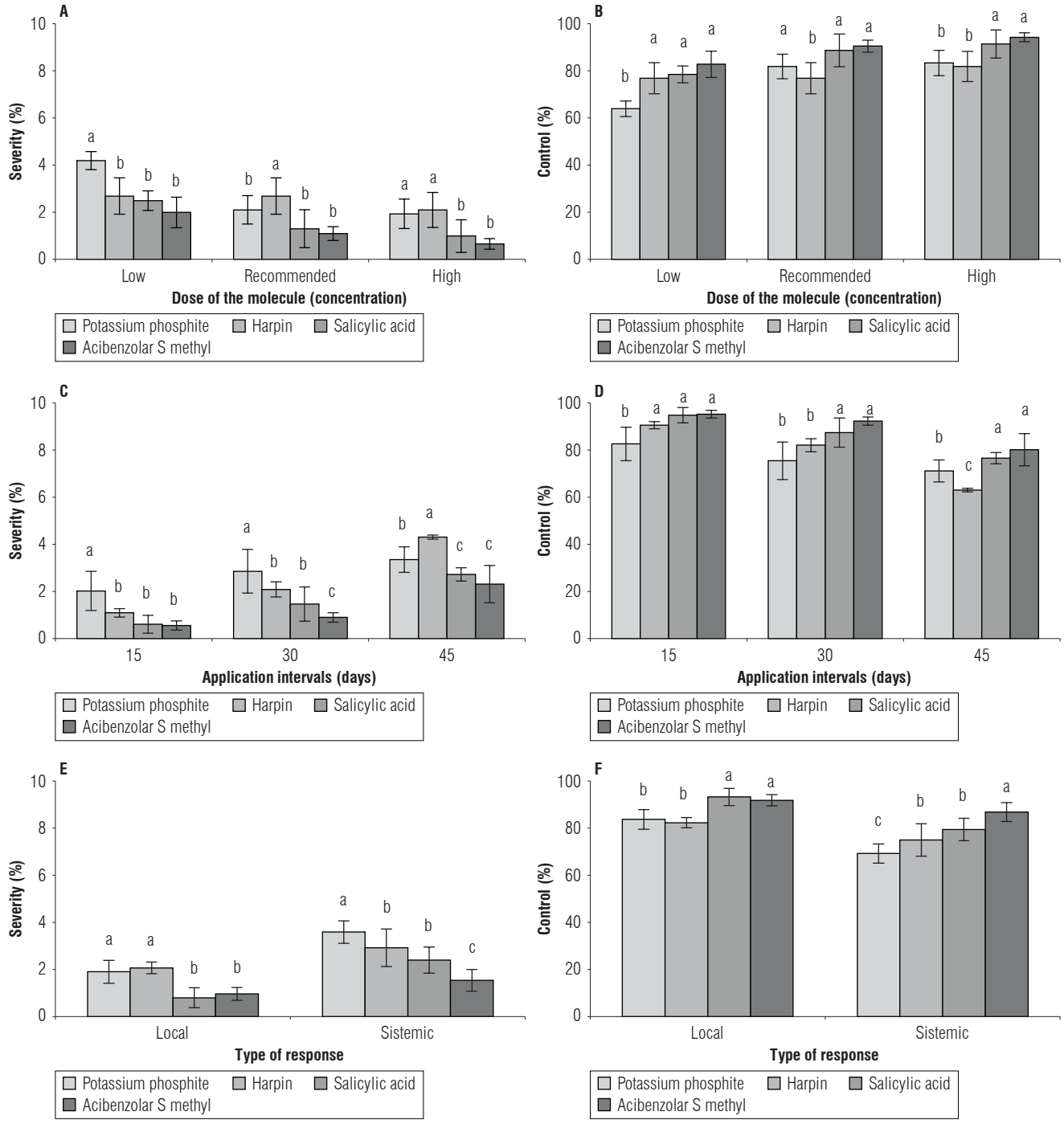

FIGURE 4. Mean severity index (left) and percent control (right) of variables. A and B) The molecule doses, C and D) The time intervals between product application and pathogen inoculation, E and F) The types of responses to potassium phosphite, harpin, SA, and ASM. The vertical lines on the bars indicate 95% confidence intervals (± SE, n = 18). According to Duncan's multiple comparison test at 5%, different letters on the vertical lines indicate significant differences.

control (70%) than copper phosphite (56%) during longer application intervals, including 40 d against coffee rust.

Harpin exhibited the most effective results when applied at the highest dose (120 µg ml^{-1}), followed by the recommended (30 µg ml^{-1}), and then the lowest dose (7.5 µg ml^{-1}), achieving disease control of 81.9%, 76.9%, and 76.9% (Fig. 5B). Optimal disease control outcomes were observed with application intervals of 15 d, followed by 30 and 45 d, resulting in controls of 90.6%, 82.1%, and 63.2%, respectively, with a predominant signaling effect more local (82.3%) than systemic (74.9%).

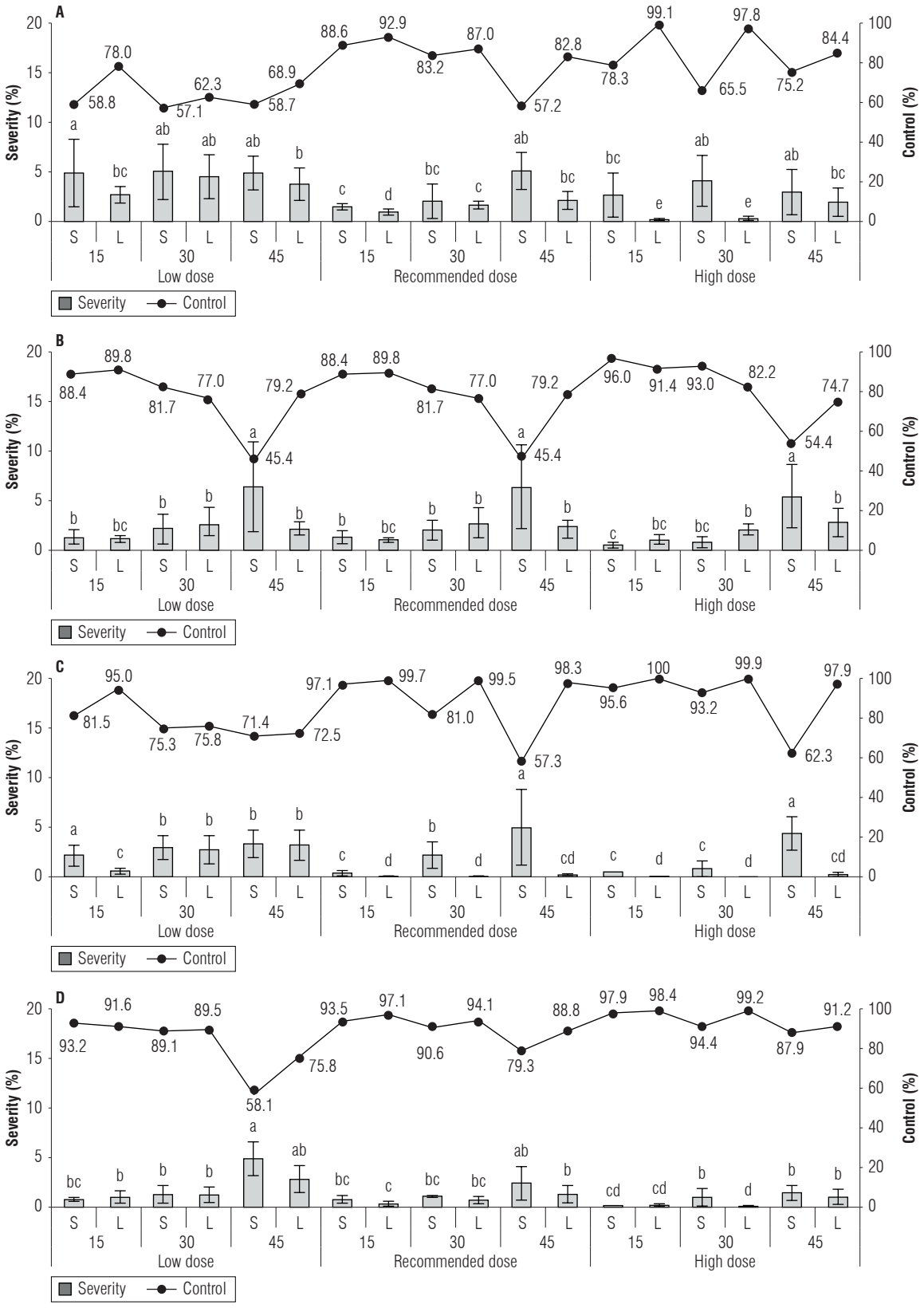

FIGURE 5. Severity index at three different doses (low, recommended, and high), at three different time intervals (15, 30, and 45 d) between the product application and pathogen inoculation, with the response signaling pathway being (L), a local effect, and (S), a systemic effect of A) potassium phosphite, B) harpin, C) SA, and D) ASM. The vertical lines on the bars indicate 95% confidence intervals (± SE, n = 3). According to Duncan's multiple comparison test at 5%, different letters on the vertical lines indicate significant differences.

SA demonstrated optimal results at high doses (5 µl ml^{-1}), followed by the recommended (1.2 µl ml^{-1}), and then the lowest dose (0.3 µl ml^{-1}), achieving disease controls of 91.5%, 88.8%, and 78.6%, respectively (Fig. 5C). The most effective disease control occurred with application intervals of 15 d, followed by 30 and 45 d, achieving controls of 94.8%, 87.5%, and 76.6%, respectively, with a predominant local signaling effect (93.2%) over systemic (79.4%).

ASM showed optimal results at high doses (800 µg ml^{-1}), followed by the recommended dose (200 µg ml^{-1}). Then, the low dose (50 µg ml^{-1}) achieved 94.3%, 90.6%, and 82.9% control, respectively (Fig. 5D). The best disease control occurred at 15 d intervals (95.3%), followed by 30 and 45 d (92.3% and 80.2% control, respectively), predominantly through local (91.7%) rather than systemic (86.8%) signaling. Similar results were reported by Guzzo *et al.* (2001), who observes ASM's protective effects ranging from 66% to 97% locally and 83% to 94% systemically at higher concentrations (400 µg ml^{-1}). However, effectiveness significantly decreases when intervals between ASM application and pathogen inoculation exceeded 49 d, even at high doses. To study their effects on coffee rust, Sancho and Diaz (2006) conducted greenhouse experiments at Cenicafé using ASM and other resistance-inducing biological products at varying concentrations. Applying the BION product (ASM) at concentrations of 1, 10, 20, 50, and 100 µg ml^{-1} effectively reduces the number of leaf lesions, with optimal application intervals observed at 8 d to induce robust plant resistance and minimize disease development time.

According to the results, treatments with these compounds consistently reduced disease severity and improved disease control compared to untreated controls. The efficacy of disease control was mainly influenced by the concentration of the product, which dictated the optimal frequency of application to avoid dilution of the treatment effects, with biweekly application intervals (every 15 d) being the best for disease control at a recommended dose. However, it is essential to note that in many of the compounds evaluated, the increase in concentration did not necessarily translate into longer application intervals (every 30 or 45 d), which led to adjusting the application intervals according to the product selected and the dose used.

In terms of signaling, local responses prevailed over systemic ones, and this behavior was primarily governed by the intrinsic properties of the molecule rather than other factors such as dose or application intervals. These types of local responses have economic and operational implications for preventive disease management plans since, as far as possible, the application process of the product should be carried out directly on the leaves to achieve better coverage, which increases application times and, therefore, the costs associated with management.

Conclusions

The direct impact of ASM, SA, potassium phosphite, and harpin on *H. vastatrix* urediniospore germination indicated varying degrees of toxicity against the pathogen. These inhibitory effects are dose-dependent, suggesting that higher concentrations of these inducers can totally suppress urediniospore germination. These results somewhat disputed the impact of this type of inducer on disease management since their responses may be more influenced by the low germination capacity of the pathogen when it comes into contact with the molecule than by the activation of the plant defense mechanisms due to its application. An undesirable effect in preventive disease management is to reduce the selection pressure on the pathogen currently exerted by chemical molecules.

Regardless of these effects, treatments with these compounds consistently reduce disease severity and enhance disease control compared to untreated controls. In general, disease control efficacy is mainly influenced by the concentration of the product, which dictates the optimal application frequency to prevent dilution of treatment effects. In terms of signaling, local responses prevail over systemic ones, and this behavior is primarily governed by the intrinsic properties of the molecule rather than other factors such as dosage or application intervals.

The present study highlights the practicality and high potential of integrating resistance-inducing compounds like ASM, SA, harpin, and potassium phosphite into disease management strategies in commercial seedbeds, particularly for managing foliar diseases such as rust. However, using these compounds may require a higher labor cost due to the requirements regarding the quality and frequency of application. For this reason, the success of implementing this type of molecule will depend more on the efficiency in controlling the disease than on its already proven effectiveness.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

JML, ALG, and CAA formulated the research aims, and JML applied statistical and computational techniques to analyze the study data. JML conducted the research and investigation process, explicitly performing the experiments; JML and CAA designed the methodology; JML and CAA wrote the initial draft; MAC translated the initial draft; JML and MAC carried out the critical review, commentary, and revision of the whole manuscript. All authors approved the final version of the manuscript.

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Calcium and nitrogen concentrations and distribution in gerbera plants as affected by nitrogen forms

Concentraciónes y distribución de calcio y nitrógeno en plantas de gerbera afectadas por las formas de nitrógeno

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ABSTRACT

Ammonium and nitrate are the two forms of nitrogen necessary for the development, metabolism, and key processes in plants. The effects of ammonium to nitrate ratio on gerbera plants and their significance in post-harvest vase life are still poorly understood. This study aimed to investigate how different nutrient solutions affect nutrient levels in various plant organs. The experiment was conducted using a completely randomized design with three replicates at the National Research Institute of Flowers and Ornamental Plants in Mahallat City, Iran, in 2018. The factors studied were the four ratios of ammonium to nitrate: 0:100, 20:80, 40:60, and 60:40, and two gerbera varieties: Stanza and Double Dutch. The results showed that nitrogen content in different plant organs (roots, stem, and leaves) increased with higher concentrations of ammonium in the nutrient solutions. The highest nitrogen content in the roots of gerbera was observed 35 d after the first flowering stem appeared, with a concentration of 4.38 mg N g^{-1} dry weight in the 60:40 ratio of ammonium to nitrate. The lowest nitrogen content was found in the flowering stem at the time of harvest, with 2.00 mg N g^{-1} dry weight in the 0:100 ratio of ammonium to nitrate.

Key words: cut flowers, leaf nitrogen content, ratio of ammonium to nitrate.

RESUMEN

El amonio y el nitrato son las dos formas de nitrógeno necesarias para el desarrollo, el metabolismo y los procesos clave en las plantas. Los efectos de la proporción de amonio a nitrato en las plantas de gerbera y su importancia en la vida postcosecha en flores de corte aún son poco conocidos. Este estudio tuvo como objetivo investigar cómo diferentes soluciones nutritivas afectan los niveles de nutrientes en varios órganos de las plantas. El experimento se llevó a cabo utilizando un diseño completamente aleatorizado con tres repeticiones en el Instituto Nacional de Investigación de Flores y Plantas Ornamentales en la ciudad de Mahallat, Irán, en 2018. Los factores estudiados fueron las cuatro proporciónes de amonio a nitrato: 0:100, 20:80, 40:60 y 60:40, y dos variedades de flores de gerbera: Stanza y Double Dutch. Los resultados mostraron que los niveles de nitrógeno en los diferentes órganos de la planta (raíces, tallo y hojas) aumentaron con concentraciones más altas de amonio en las soluciones nutritivas. El contenido más alto de nitrógeno en las raíces de las flores de gerbera se observó 35 d después de la aparición del primer tallo floral, con una concentración de 4,38 mg N g^{-1} peso seco en la proporción de 60:40 de amonio a nitrato. El contenido más bajo de nitrógeno se encontró en el tallo floral al momento de la cosecha, con 2,00 mg N g^{-1} peso seco en la proporción de 0:100 de amonio a nitrato.

Palabras clave: flores del corte, contenido de nitrógeno en las hojas, relación de amonio a nitrato.

Introduction

In studies on crop responses to mineral nutrients, the relationships between fertilizer and water management are at the forefront. For crops to develop, grow, and produce, proper management of nitrogen fertilizer and irrigation is essential. Improving irrigation schedules and nitrogen application techniques is critical for sustainable agricultural management, with attention given to their effects on individual crops (Dai *et al*., 2019). As agriculture consumes increasingly high amounts of freshwater, the problem of scarce freshwater for irrigation worsens. High irrigation expenses, rising fertilizer costs, and environmental issues, such as pollution from the overuse of nitrogen or other chemical fertilizers in agricultural production systems, are all major concerns (Mancosu *et al*., 2015).

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Since it can precisely determine the nutrient concentrations and actual requirements for each crop, hydroponics appears to be the ideal cultivation system for both soilless crop production and the introduction of unknown plant species into intensive cultivation regimes (Varlagas *et al*., 2010). Hydroponic farming is an environmentally beneficial growing method that can be used in regions with poor soil, limited acreage, and limited water supplies. Consequently, hydroponics is promising for application in such circumstances (Marques *et al*., 2019; Pradhan *et al*., 2019). In hydroponic systems, the pH of the nutrient solution can be monitored and controlled in addition to nutrient management (Bar-Yosef *et al*., 2009; Corrêa *et al*., 2008).

Nitrogen ranks as the fourth most plentiful element in plants (Kozlowski, 1985; Hopkins, 1999). In addition to being critical for plant nutrition, nitrogen is also necessary for the synthesis of coenzymes, proteins, and nucleic acids. It is essential in photosynthesis because it is the primary constituent of the chlorophyll molecule (Barker *et al*., 2015). Nitrate (NO_3^-) and ammonium (NH_4^+) are the two main types of nitrogen that higher plants absorb (Tschoep *et al*., 2009). While plant roots can also absorb ammonium in nitrate-deficient environments, most plants prefer nitrate as a nitrogen source. According to Zhonghua *et al*. (2011) and Guo *et al*. (2012), the ideal ratio of nitrate to ammonium for plant growth and development varies depending on the genotype, growth stage, environmental factors, and total nitrogen concentration supplied. Plants exhibit diverse morphological, physiological and biochemical responses that are contingent upon the kind of nitrogen provided, species, and environmental factors (Helali *et al*., 2010; Liu *et al*., 2017; Na *et al*., 2014; Prinsi *et al*., 2020; Zhang *et al*., 2019; Zhu *et al*., 2014). Modern technology, such as hydroponic farming techniques, can be a reasonable step toward improving the efficiency of water and fertilizer usage in greenhouse crops, especially since drought and water scarcity are major global issues. The current study assessed the impact of various ammonium to nitrate ratios in the nutrient solution on the nutrient concentrations in the various organs of gerbera plants.

Materials and methods

This experiment was carried out in 2018 at the National Research Institute of Flowers and Ornamental Plants of Iran located in Mahallat City, Iran, with a geographic location of 50°30' E and 33°53' N, and an altitude of 1747 m a.s.l. The trial was conducted using a randomized complete block design with three replicates. This research was conducted under greenhouse conditions with minimum and maximum temperatures of 25-30°C (day) and 15-20°C (night) and relative humidity of 50 to 70%. Fans and pads, along with a shading system, were also used to cool the greenhouse.

Factors included four nutrient solutions containing 11.25 mM nitrogen at four different ammonium to nitrate ratios (0:100, 20:80, 40:60, and 60:40) in 300 L nutrient solution tanks and two varieties of gerbera: Stanza with red flowers and Double Dutch with yellow flowers. For each experimental unit, 10 pots were used, with 3 L nutrient solution and one Gerbera seedling planted in each pot. The nutrient solution used in this experiment was prepared according to the Netherlands Greenhouse Horticulture and Vegetables Research Center (de Kreij *et al*., 2003) (Tab. 1).

One molar sulfuric acid was used to adjust the pH of the nutrient solution, considering that the optimal pH for Gerbera plants is 5.5±0.1 (de Kreij *et al*., 2003). The electrical conductivity (EC) of nutrient solutions with ammonium-to-nitrate ratios of 0:100, 20:80, 40 :60, and 60:40 was 1.94, 2.28, 2.34, and 2.6 dS m^{-1}, respectively. The substrate was perlite with a particle diameter of 0.5-5 mm. After preparing the pots and substrate, gerbera seedlings (plants with 4 to 6 leaves) were planted in 3 L pots. For the first 10 d,

TABLE 1. Concentrations of mineral nutrients in nutrient solutions.

$NH_4^+ : NO_3^-$	Total nitrogen (mM L^{-1})	NO_3	NH_4^+	P*	K (mM L^{-1})	Ca	Mg	SO_4^{2-}
0-100	12.75	12.75	0	1.25	5.5	3	1	0.25
20-80	12.75	10.2	2.55	1.25	5.5	3	1	1.78
40-60	12.75	7.65	5.1	1.25	5.5	3	1	4.33
60-40	12.75	5.1	7.65	1.25	5.5	3	1	6.85
-	Fe	Mn	Zn	Cu (µM L^{-1})	Mo	B	-	-
	35	5	4	0.75	0.5	30	-	-

* Phosphorus is in the form of $H_2PO_4^-$.

plants were supplied with compound fertilizer 18-18-18 (N-P_2O_5-K_2O) with a concentration of 1 g L^{-1}. Then, until the beginning of flowering (one month after planting of the seedlings), they were supplied with the nutrient solution listed in Table 1. Fertilizations were done 4 to 6 times daily, based on the greenhouse temperature. After that, all the plants received the same routine care during the growth period.

Concentrations of nitrate, calcium, ammonium, and total nitrogen in plant organs

The concentrations of nitrogen and calcium in the roots, stems, and leaves was measured at vegetative and reproductive stages. For this purpose, after drying the samples at 70°C for 24 h, the samples were crushed using an electric grinder. The Kjeldahl method was used to determine the total nitrogen concentration in plant organs (Bremner & Mulvaney, 1982). The calcium in the plant samples was measured using a UnicamSolaar atomic absorption spectrophotometer (Emami, 1996). The methods of Nelson (1983) and Cataldo *et al*. (1975) were used to measure ammonium concentration in plant organs (roots, leaves, and stems). Finally, the concentrations of ammonium and nitrate were reported in a mg g^{-1} dry weight.

Data analysis

SAS software version 9.4 was used for data analysis (ANOVA), and a Duncan test at the 5% level was applied to compare means. To understand the relationships between the studied traits, simple correlation coefficients among the traits were calculated.

Results

Concentrations of calcium and nitrogen in the roots 35 d after seedling planting

The results of the variance analysis demonstrated that, 35 d after seedling planting, the concentrations of nitrogen, nitrate, and calcium in the roots of the gerbera plants were significantly impacted by the ammonium to nitrate ratio at the 1% level. Additionally, the variety had a significant impact on calcium concentration at 5% and on nitrogen and nitrate concentrations at 1% (Tab. 2). Applying a portion of the total nitrogen in the form of ammonium (60%) caused a significant increase in the concentration of nitrogen in the roots of gerbera. The comparison of the means revealed that the ratio of ammonium to nitrate of 60:40 produced the highest amounts of calcium and nitrate at 0.77 and 2.26 mg g^{-1}, respectively. The lowest

TABLE 2. Mean squares from analysis of variance (ANOVA) for nutrient contents in roots of two gerbera cultivars grown under varying ratios of ammonium to nitrate.

SOV	df	Total nitrogen	Calcium	NO_3^-	NH_4^+
Rep	2	0.091	0.006	0.243	0.005
Cultivar	1	1.050**	0.0600*	2.31**	0.016ns
$NH_4^+ : NO_3^-$	3	0.496**	0.098**	2.17**	0.172**
Cultivar × NH_4^+: NO_3^-	3	0.130ns	0.020ns	0.409ns	0.003ns
Error	14	0.043	0.007	0.133	0.0030
CV(%)	-	6.16	14.37	21.99	10.27

ns, * and **: non-significant difference, significant difference at 5% and 1% of probability level. SOV – sources of variations.

TABLE 3. Comparison of the mean effects of ammonium to nitrate ratio and cultivar type on the concentrations of nutrients in gerbera roots at 35 d after seedling planting.

Treatment	Total nitrogen	Calcium	NO_3^-	NH_4^+
NH_4^+: NO_3^-		(mg g^{-1})		
0-100	3.00c	0.77a	2.26a	0.36d
20-80	3.34b	0.66a	1.91ab	0.44c
40-60	3.43b	0.51b	1.62b	0.59b
60-40	3.70a	0.50b	0.84c	0.74a
Cultivar				
Stanza	3.58a	0.56b	1.97a	0.56a
Double Dutch	3.16b	0.66a	1.35b	0.51b

Means in each column followed by the same letters are not significantly different at the 5% probability level according to the Duncan test.

amount of calcium and nitrate was obtained from the 40:60 ammonium to nitrate ratio. The application of a nutrient solution with a ratio of 60:40 ammonium to nitrate, compared to nutrient solutions with ratios of 0:100 and 20:80 ammonium to nitrate led to a significant increase in the ammonium concentration of 105.6% and 68.2%, respectively, in the roots (Tab. 3).

Concentrations of nutrients in the roots 35 d after the appearance of the first flowering stem

The variance analysis of the data (Tab. 4) revealed that, at the 1% level, there was a statistically significant effect of the ammonium to nitrate ratio on the concentration of nitrogen, nitrate, ammonium, and calcium in the gerbera roots at 35 d after the first flowering stem appeared. Also, the effect of the gerbera cultivar on the concentrations of nitrate and calcium at the 1% level and on the concentration of nitrogen at the 5% level was significant. A comparison of mean data showed that by increasing the concentration of ammonium in the nutrient solution to 60% of the applied nitrogen, the concentrations of nitrogen and ammonium in the roots of gerbera increased at the stage of 35 d after the appearance of the first flowering stem. The concentrations of nitrogen and ammonium in the roots of gerbera increased significantly by 47.9% and 70.7%, respectively, when a nutrient solution with a ratio of 60:40 ammonium to nitrate was used instead of one with a ratio of 0:100 ammonium to nitrate (Tab. 5). Additionally, the ratio of 0:100 ammonium to nitrate produced the highest concentrations of calcium and nitrate, while the ratio of 60:40 ammonium to nitrate produced the lowest concentrations of calcium and nitrate. 35 d after the emergence of the first flowering stem, the concentration of calcium in the roots of gerbera decreased when the amount of ammonium in the nutrient solution was increased to 60% of total nitrogen (Tab. 5). The calcium concentration in gerbera roots was significantly reduced by 52.1% and 37.4%, respectively, when a nutrient solution with an ammonium to nitrate ratio of 60:40 was used instead of one with an ammonium to nitrate ratio of 0:100 and 20:80.

Concentrations of mineral nutrients in leaves 35 d after seedling planting

The ammonium to nitrate ratio had a significant effect at the 1% level on the concentrations of nitrogen, nitrate, ammonium, and calcium in gerbera leaves 35 d after seedling planting according to data variance analysis results (Tab. 6). Also, the effect of the gerbera cultivar on ammonium and nitrogen concentrations was significant at the 1% level. At 35 d after seedling planting, an increase in ammonium

TABLE 4. Mean squares from analysis of variance (ANOVA) for nutrient concentrations in the roots of gerbera at the stage of 35 d after the appearance of the first flowering stem.

SOV	df	Total nitrogen	Calcium	NO_3^-	NH_4^+
Rep	2	0.063	0.006	0.383	0.047
Cultivar	1	1.58^{*}	1.12^{**}	17.25^{**}	0.036^{ns}
$NH_4^+ : NO_3^-$	3	2.38^{**}	0.66^{**}	18.11^{**}	0.79^{**}
Cultivar × $NH_4^+ : NO_3^-$	3	0.020^{ns}	0.86^{ns}	0.40^{ns}	0.002^{ns}
Error	14	0.26	0.052	1.21	0.009
CV(%)	-	13.79	23.49	15.63	9.41

ns, * and **: non-significant difference, significant difference at 5% and 1% of probability level. SOV – sources of variations.

TABLE 5. Comparison of the mean effects of ammonium to nitrate ratio and cultivar type on the concentrations of nutrients in the roots of gerbera at 35 d after the appearance of the first flowering stem.

Treatment	Total nitrogen	Calcium	NO_3	NH_4^+
$NH_4^+ : NO_3^-$		(mg g^{-1})		
0-100	2.96c	1.40a	8.95a	0.89c
20-80	4.12ab	1.07b	7.95a	0.66d
40-60	3.57bc	0.75c	6.28b	1.07b
60-40	4.38a	0.67c	5.05b	1.52a
		Cultivar		
Stanza	4.02a	0.76b	7.90a	-
Double Dutch	3.50b	1.19a	6.21b	-

Means in each column followed by similar letters are not significantly different at the 5% probability level according to the Duncan test.

content in the nutrient solution to 60% of applied nitrogen resulted in an increase in nitrogen and ammonium concentration in the gerbera leaves. The concentration of nitrogen in gerbera leaves significantly increased when a nutrient solution with a ratio of 60:40 ammonium to nitrate was employed instead of other nutrient solutions. This nutrient solution, compared with the nutrient solution with a ratio of 0:100 ammonium to nitrate, caused a significant increase in nitrogen concentration in gerbera leaves by 27.9% (Tab. 7). The average data comparison demonstrated that, when compared with other ammonium to nitrate ratios, raising the concentration of ammonium in the nutrient solution to 60% of the total nitrogen resulted in a decrease in nitrate concentration in the gerbera leaves 35 d after seedling planting (Tab. 7). Application of nutrient solutions with ratios of 60:40 ammonium to nitrate contrasted in its effect to the solutions with 20:80, 40:60, and 0:100 ammonium to nitrate. The nitrate concentration in gerbera leaves significantly decreased by 55.1%, 44.2%, and 43.3%, respectively (Tab. 7). The comparison of mean data demonstrated that, 35 d after seedling planting, when ammonium in the nutrient solution was increased to 20% of the total nitrogen applied, the concentration of calcium in gerbera leaves increased significantly relative to other levels of ammonium to nitrate. Providing gerbera plants with a nutrient solution containing an ammonium-to-nitrate ratio of 20:80 caused the calcium concentration in the leaves to increase by 14.2%. The concentration of calcium in the leaves, however, significantly decreased as the amount of ammonium in the nutritional solution increased. The nitrogen (8.8%) and ammonium (12.9%) concentrations in the leaves of Stanza plants were significantly greater than those of Double Dutch plants (Tab. 7).

Concentrations of mineral nutrients in leaves 35 d after the appearance of the first flowering stem

The effect of biostimulant substances to nitrate ratio on nitrogen, nitrate, ammonium, and calcium content in gerbera leaves was significant at the 1% level according to the results of analysis of variance (Tab. 8). In comparison to other ratios of ammonium to nitrate, raising the concentration of ammonium in the nutrient solution to up to 60% of applied nitrogen resulted in a statistically significant increase in the nitrogen concentration in the leaves of gerbera. The nitrogen concentration in the leaves of gerbera increased significantly by 32.6% when they were supplied with a nutrient solution containing 60:40 ammonium to nitrate, as opposed to a nutritional solution without ammonium. 35 d after the first flowering stem appeared, the nitrate concentration in the leaves of gerbera was much lower

TABLE 6. Mean squares from analysis of variance (ANOVA) for nutrient concentrations in gerbera leaves at 35 d after seedling planting.

SOV	df	Total nitrogen	Calcium	NO_3^-	NH_4^+
Rep	2	0.025	0.001	0.115	0.0022
Cultivar	1	0.055^{**}	0.113^{ns}	0.24^{ns}	0.0096^{**}
$NH_4^+ : NO_3^-$	3	0.645^{**}	0.416^{**}	1.923^{**}	0.038^{**}
Cultivar × $NH_4^+ : NO_3^-$	3	0.008^{ns}	0.005^{ns}	0.067^{ns}	0.0011^{ns}
Error	14	0.034	0.025	0.059	0.001
CV(%)	-	5.67	13.01	12.96	11.12

ns, * and **: non-significant difference, significant difference at 5% and 1% of probability level. SOV – sources of variations.

TABLE 7. Comparison of the mean effects of ammonium to nitrate ratio and cultivar type on the concentrations of essential nutrients in gerbera leaves at 35 d after seedling planting.

Treatment	Total nitrogen	Calcium	NO_3^-	NH_4^+
$NH_4^+ : NO_3^-$		(mg g^{-1})		
0-100	2.83c	1.34a	2.47a	0.39a
20-80	3.21b	1.53a	1.99b	0.25c
40-60	3.34b	1.08b	1.96b	0.30b
60-40	3.62a	0.94b	1.11c	0.21d
Cultivar				
Stanza	3.40a	-	-	0.31a
Double Dutch	3.10b	-	-	0.27b

Means in each column followed by similar letters are not significantly different at the 5% probability level according to the Duncan test.

when ammonium, up to 60% of the total nitrogen in the nutritional solution, was applied. Using nutrient solutions with an ammonium to nitrate ratio of 60:40 resulted in a significant decrease in the nitrate concentration of gerbera leaves by 34.9%, 27.3%, and 10.1%, respectively, when compared to nutrient solutions with ammonium to nitrate ratios of 0:100, 20:80, and 40:60. Compared to nutritional solutions with an ammonium to nitrate ratio of 40:60 or 60:40, the use of ammonium (20% of the total nitrogen) resulted in a considerable increase in calcium concentration of 25.4% and 52.6%, respectively. Furthermore, compared to the Stanza cultivar, 52.6% more calcium was found in the Double Dutch cultivar (Tab. 9).

Concentrations of nutrients in the stem 35 d after the appearance of the first flowering stem

The analysis of variance showed that the ammonium-to-nitrate ratio effect on nitrogen, nitrate, ammonium, and calcium concentrations was significant at the 1% level. Also, the effect of the gerbera cultivar on nitrogen and calcium concentration was significant at the 1% level (Tab. 10). The concentration of nitrogen in the stem increased significantly by 26.1% and 19.2%, and 10.1%, respectively, when nutrient solutions containing a ratio of 60:40 ammonium to nitrate were used instead of those containing ratios of 0:100, 20:80, and 40:60 ammonium to nitrate. The concentration of ammonium in the stem increased by 33.3%, 104.9%, and 15.1%, respectively. The highest amount of calcium was obtained from the ratio of 20:80 ammonium to nitrate at the rate of 0.5%. With the increase in the ammonium application, the amount of calcium in the stem of the gerbera plant decreased; this decrease was 52.9% and 67.7%, respectively, in the ratios of 40:60 and 60:40. The highest amount of nitrate was obtained from the 0:100 ammonium to nitrate nutrient solution at the rate of 2.5%, and with the increase in the amount of ammonium, the amount of nitrate decreased. The lowest amount was obtained from the ratio of 40:60 ammonium to nitrate at 1.96%. Based on the average comparison results, the Stanza cultivar had the highest concentration of stem nitrogen (2.4%), and the Double Dutch cultivar had the highest concentration of calcium (0.45%) (Tab. 11).

After 35 d of seedling planting, there was a considerable increase in the concentration of nitrogen and ammonium in the roots and leaves of gerbera in both phases when the concentration of ammonium in the nutrient solution was increased to 60% of the applied nitrogen. Moreover, 35 d after the appearance of the first flowering stem, the concentration of nitrogen and ammonium in the flowering

TABLE 8. Mean squares from analysis of variance (ANOVA) for nutrient concentrations in gerbera leaves at 35 d after the appearance of the first flowering stem.

SOV	df	Total nitrogen	Calcium	NO_3^-	NH_4^+
Rep	2	0.177	0.04	0.005	0.001
Cultivar	1	0.173^{ns}	2.27^{**}	0.044^{ns}	0.008^{ns}
$NH_4^+ : NO_3^-$	3	0.75^{**}	0.43^{**}	1.23^{**}	0.16^{**}
Cultivar × $NH_4^+ : NO_3^-$	3	0.01^{ns}	0.047^{ns}	0.004^{ns}	0.002^{ns}
Error	14	0.040	0.049	0.006	0.00009
CV(%)	-	7.02	15.02	3.44	8.48

ns, * and **: non-significant difference, significant difference at 5% and 1% of probability level. SOV – sources of variations.

TABLE 9. Comparison of the mean effects of ammonium to nitrate ratio and cultivar type on concentrations of nutrients in gerbera leaves at 35 d after the appearance of the first flowering stem.

Treatment	Total nitrogen	Calcium	NO_3^-	NH_4^+
$NH_4^+ : NO_3^-$		(mg g^{-1})		
0-100	2.51c	1.64ab	2.86a	0.58a
20-80	2.70bc	1.74a	2.56b	0.41b
40-60	2.93b	1.39bc	2.07c	0.29c
60-40	3.33a	1.14c	1.86d	0.19d
Cultivar				
Stanza	-	1.17b	-	-
Double Dutch	-	1.78a	-	-

Means in each column followed by similar letters are not significantly different at the 5% probability level according to the Duncan test.

stem of gerbera at the stage of flower harvest was compared with the absence of ammonium in the nutrient solution (Tabs. 3, 5, 7, 9, and 11). There were positive and significant correlations between percentage of ammonium in the nutrient solution and leaf nitrogen concentration (P<0.01, r=0.86) as well as root ammonium concentration (P<0.01, r=0.97) at 35 d after seedling planting. Similarly, at 35 d after the emergence of the first flowering stem, significant positive correlations were observed between the percentage of ammonium in the nutrient solution and root nitrogen concentration (P<0.05, r=0.74), leaf nitrogen concentration (P<0.01, r= 0.94), leaf ammonium concentration (P<0.05, r=0.82), and nitrogen concentration in the flowering stem at harvest (P<0.01, r=0.86).

Discussion

The increase in nitrogen concentration in different organs of gerbera plants as a result of ammonium nutrition can be attributed to several reasons. Firstly, the assimilation of nitrate requires more energy compared to ammonium for its uptake and conversion (assimilation) into amino acids (Rosta, 2014). Therefore, supplying plants with nitrate alone will not result in achieving maximum growth. Assimilation of 1 mole of nitrate requires 15 moles of ATP, while one mole of ammonium requires 5 moles of ATP for assimilation (Marschner, 2012). Also, about 23% of the energy from respiration is used for nitrate assimilation in the roots, while this amount is 14% for ammonium. Therefore, ammonium nutrition saves plant energy (Marschner, 2012). In a study on how nitrogen form affects nutrient absorption by *Canna indica*, Konnerup and Brix (2010) found that the plants supplied with ammonium had higher nitrogen concentrations than the plants supplied with nitrate. According to Tabatabaei *et al.* (2008), in strawberries and rudbeckia, raising the ratio of ammonium to nitrate in a nutrient solution increased the concentration of total nitrogen in the plant organs, which is consistent with the findings of the current research. The effect of the ammonium-to-nitrate ratio of nutrient solution on the nitrate concentration of different organs of the gerbera plants varied, so that the concentration of nitrate in the root and leaves of gerbera in both stages (35 d after planting seedlings and 35 d after the appearance of the first flowering stem), as well as the concentration of nitrate in the flowering stem of gerbera in the flower harvesting stage with the application of 60% nitrogen in the form of ammonium decreased significantly in comparison with the non-use of ammonium and the

TABLE 10. Mean squares from analysis of variance (ANOVA) for nutrient concentrations in gerbera stem at 35 d after the appearance of the first flowering stem.

SOV	df	Nitrogen	Calcium	NO_3^-	NH_4^+
Rep	2	6.0098	0.0082	0.082	0.0004
Cultivar	1	0.283**	0.043**	0.011^{ns}	0.012^{ns}
$NH_4^+ : NO_3^-$	3	0.326**	0.060**	0.564**	0.200**
Cultivar × $NH_4^+ : NO_3^-$	3	0.0070^{ns}	0.0023^{ns}	0.152^{ns}	0.001^{ns}
Error	14	0.0096	0.0043	0.056	0.005
CV(%)	-	4.24	16.00	10.27	11.51

ns, * and **: non-significant difference, significant difference at 5% and 1% of probability level. SOV – sources of variations, df-degrees of freedom.

TABLE 11. Comparison of the mean effects of ammonium to nitrate ratio and cultivar type on the concentrations of nutrients in the stem at 35 d after the appearance of the first flowering stem.

Treatment	Total nitrogen	Calcium	NO_3^-	NH_4^+
$NH_4^+ : NO_3^-$		(mg g^{-1})		
0-100	2.07d	0.46a	2.47a	0.63c
20-80	2.19c	0.52a	2.64a	0.41d
40-60	2.37b	0.34b	1.96b	0.73b
60-40	2.61a	0.31b	2.14b	0.84a
Cultivar				
Stanza	2.42a	0.37b	-	-
Double Dutch	2.20b	0.45a	-	-

Means in each column followed by similar letters are not significantly different at the 5% probability level according to the Duncan test.

use of 20 to 80 ammonium to nitrate ratio. A negative and significant correlation between the ammonium percentage of the nutrient solution and the root nitrate concentration (r=-0.78, P<0.01) 35 d after seedling planting confirms this decrease. Similarly, at 35 d after the appearance of the first flowering stem, a negative and significant correlation was observed between the ammonium percentage of the nutrient solution and the nitrate concentration in leaves (P<0.01, r=-0.98) (Tab. 12). The decrease in nitrate concentration due to the increasing ratio of ammonium to nitrate in the nutrient solution can be due to decreased nitrate supply for plant absorption (Helali *et al*., 2010). Ammonium may reduce nitrate uptake by inhibiting nitrate assimilation upon entry and enhancing its efflux from the roots (Kronzucker *et al*., 1999). The type of plant cultivar was found to have an impact on the concentration of high-use and low-use nutrients in various regions of the gerbera plants. Therefore, the Stanza cultivar had much higher concentrations of most studied nutrients than the Double Dutch. Similarly, the concentrations of nitrogen and ammonium in the leaves at 35 d after the seedling planting and the concentrations of nitrate and ammonium at 35 d after the appearance of the first flowering stem was significantly higher in Stanza than in Double Dutch (Tab. 9). Additionally, during the flower harvest stage, the nitrogen concentration in Stanza stem was substantially higher than that in the Double Dutch stem (Tab. 11). Genetic differences cause the difference between plant cultivars in the concentration of nutrients, and this issue has been observed in other research as well. One of the key mineral ingredients for decorative plants and cut flower pots is calcium, which helps them to develop and last longer. The form and quantity of nitrogen included in the nutrient solution has a significant impact on its absorption. According to reports, there is a decrease in calcium absorption at high ammonium ratios in the nutrient solution (Hosseini Farahi *et al*., 2014).

Conclusions

The ammonium-to-nitrate ratio in the nutrient solution significantly influenced the concentrations of nitrogen, nitrate, ammonium, and calcium in the roots, leaves, and flower stems of gerbera. Increasing the ammonium ratio to 60% of the total nitrogen supply led to a significant rise in nitrogen and ammonium concentrations in the roots, leaves, and flower stems of gerbera at both growth stages (35 d after planting and 35 d after the emergence of the first flower stem). This increase may be attributed to the lower energy requirement for ammonium uptake and metabolism compared to nitrate, as nitrate requires more energy for absorption and conversion into amino acids.

On the other hand, increasing the ammonium ratio in the nutrient solution resulted in a reduction in nitrate concentrations in the roots, leaves, and flower stems of gerbera. This decrease may be due to the inhibition of nitrate uptake in the presence of ammonium, as ammonium can competitively suppress nitrate absorption. Additionally, an increase in the ammonium ratio in the nutrient solution led to a decline in calcium concentrations in the roots and leaves of gerbera, likely due to competition between ammonium and calcium ions for uptake.

Genetic differences between gerbera cultivars also affected nutrient concentrations, with the Stanza cultivar exhibiting higher concentrations of nitrogen, nitrate, and ammonium in the roots, leaves, and flower stems compared to the Double Dutch cultivar. These variations may be attributed to the specific genetic traits of each cultivar.

Overall, this study highlights that optimizing the ammonium-to-nitrate ratio in the nutrient solution can enhance nutrient uptake and improve gerbera performance.

TABLE 12. Regression coefficients for the studied traits at different growth stages of gerbera.

Plant organ		Total nitrogen	NO_3^-	NH_4^+	Calcium
Stage 35 d after seedling planting					
Root	NH_4^+	0.69	*0.78-	**0.97	*0.81-
Leaf	NH_4^+	0.86**	0.40	0.56-	**0.95-
Stage 35 d after the appearance of the first flowering stem					
Root	NH_4^+	0.74*	0.64-	0.57	0.59-
Leaf	NH_4^+	0.94**	**0.98-	*0.82	0.53-
Flower harvest stage					
Flowering stem	NH_4^+	**0.86	0.62-	0.65	0.74*-

* and **: significant differences at 5% and 1% of probability level.

However, excessive ammonium levels may negatively impact calcium uptake, which is crucial for the quality and longevity of cut flowers. Therefore, maintaining an appropriate balance between ammonium and nitrate in the nutrient solution is essential for achieving optimal results in gerbera cultivation.

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Conflict of interest statement

The authors declare that there are no conflicts of interests regarding the publication of this article.

Author´s contributions

MAK and EB designed the experiments, MK carried out field and laboratory experiments, MAK and EB contributed to the data analysis, MAK, EB, and MK wrote the article. All authors approved the final version of the manuscript.

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Effect of phosphine dose and exposure time on postharvest quality of Hass avocado (*Persea americana* Mill.)

Efecto de dosis y tiempos de exposición a fosfina en la calidad poscosecha de aguacate Hass (*Persea americana* Mill.)

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ABSTRACT

The Hass avocado in Colombia has great export potential, although its commercialization faces restrictions due to quarantine pests. Fumigation with magnesium phosphide has become a key postharvest strategy for pest control in refrigerated fruits. However, there is limited information regarding its impact on Hass avocados. This study evaluated the effect of treatments with phosphine at different concentrations (0, 200, 400, and 800 ppm) and exposure times (36 and 72 h) on postharvest quality in avocados refrigerated at 7°C. Fruit firmness, color of the exocarp and mesocarp, weight loss, and ethylene production were analyzed using a longitudinal multivariate analysis of variance. No direct damage to fruit quality was detected related to phosphine concentration or exposure time. Although significant differences in firmness and color were observed, these effects were attributed to variations in gas concentrations, such as CO_2, inside the barrels, and the fruit maturation process. Ethylene production increased with higher doses and longer exposure times, reaching a significant peak 72 h after harvest, coinciding with the climacteric point. These differences were related to the physiological maturation process of the avocados. Magnesium phosphide did not directly affect the quality of Hass avocados under the evaluated conditions. Magnesium phosphide is considered a viable option for phytosanitary pest control, although further studies are needed to assess its effectiveness against specific avocado pests.

Key words: phytosanitary management, postharvest treatment, magnesium phosphide, longitudinal analysis, avocado export.

RESUMEN

El aguacate Hass en Colombia tiene un gran potencial de exportación, aunque enfrenta restricciones comerciales debido a las plagas cuarentenarias. La fumigación con fosfuro de magnesio se ha considerado como una estrategia clave de poscosecha para el control de plagas en frutas refrigeradas. Sin embargo, hay poca información respecto a su impacto en la calidad de frutos de aguacate Hass. Este estudio evaluó el efecto de tratamientos con fosfina a diferentes concentraciones (0, 200, 400 y 800 ppm) y tiempos de exposición (36 y 72 h) sobre la calidad postcosecha en aguacates refrigerados a 7°C. Se analizaron la firmeza de fruto, color del exocarpo y mesocarpo, pérdida de peso y producción de etileno mediante un análisis de varianza multivariado longitudinal. No se detectaron daños directos en la calidad de la fruta relacionados con la concentración de fosfina ni el tiempo de exposición al gas. Aunque se observaron diferencias significativas en la firmeza y el color de los frutos, estos efectos fueron atribuibles a variaciones en la concentración de gases, como el CO_2, dentro de los barriles, y al proceso de maduración de los frutos. La producción de etileno aumentó con dosis más altas y tiempos de exposición más prolongados, alcanzando un pico significativo 72 h después de la cosecha, coincidiendo con el punto climatérico. Estas diferencias se vincularon con el proceso fisiológico de maduración de los aguacates. El fosfuro de magnesio no afectó directamente la calidad del aguacate Hass bajo las condiciones evaluadas. El fosfuro de magnesio es una opción viable para el control fitosanitario de plagas, aunque se requieren más estudios para evaluar su efectividad frente a algunas plagas específicas del aguacate.

Palabras clave: manejo fitosanitario, tratamiento poscosecha, fosfuro de magnesio, análisis longitudinal, exportación de aguacate.

Introduction

Avocado (*Persea americana* Mill.) is a tropical crop that grows across a wide range of thermal floors, with centers of origin for different races found both in the highlands of eastern Mexico and warm areas of southeastern Mexico and Guatemala (Guzmán *et al*., 2017; Williams, 1977). The fruits of Hass avocado have high nutritional value, with monounsaturated fatty acids making up 63.5% of total fats and containing 463 mg of potassium per 100 g of pulp (Bernal & Cartagena, 2017; Ferreyra *et al*., 2016). Therefore, in tropical countries, avocado has become an important

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crop due to its high international demand. By 2022, the total production was 9,114,135 t, with Mexico as the leading producer and exporter with 27.8%, followed by Colombia with 12% (FAO, 2022). In Colombia, avocado cultivation has become an important export product, with a growth in export volume from 44,570 t in 2019 to 124,930 t in 2024. This growth continues today with the expansion of avocado markets in the USA, Japan, and China (DIAN, 2024).

The fruit export market is characterized by high demand for quality, safety and phytosanitary standards; however, tropical crops have diverse organisms that generate phytosanitary problems, such as pests and diseases (ICA & ANDI, 2016). Quarantine pests (QP) are particularly limiting in export crops because QP are potentially dangerous species that are not present or are under strict control in the importing country (Heather & Hallman, 2008; IPPC, 2024). Consequently, importing countries enforce stringent detection policies that can lead to the interception, return, or destruction of shipments, thereby imposing additional costs on producers. In Colombia, significant quarantine pests affecting avocado cultivation include *Heilipus lauri* (Coleoptera: Curculionidae) and *Stenoma catenifer* (Lepidoptera: Elachistidae) (Carabalí Muñoz *et al*., 2021).

Various alternatives exist for postharvest pest management. Fumigants like methyl bromide have been commonly employed due to their efficacy and versatility; however, their use has been restricted due to adverse effects on the ozone layer (UNEP, 2020). As a result, alternative strategies were studied for postharvest pest management, including ionizing radiation, thermal treatments, and the application of other fumigants such as phosphine (Ma *et al.,* 2024). Phosphine gas, or hydrogen phosphide (PH_3), serves as the principal alternative to methyl bromide. This gaseous compound is commercially produced from metal phosphides, usually magnesium or aluminum, in conjunction with substances that control gas release (Kim *et al*., 2016; Zou *et al.,* 2025).

Aluminum phosphide has been used for approximately 80 years as a pest control for stored grains (Arora *et al*., 2021). In recent years, magnesium phosphide has been implemented as a phytosanitary treatment for agricultural products because, unlike aluminum phosphide, it does not contain ammonium carbamate. This means that the reaction product is phosphine gas without phytotoxic residues or ethylene stimulants that affect food quality (Agrafioti *et al.,* 2019; Restrepo Giraldo, 2019). Phosphine has rapid diffusion in the treated material, so it easily reaches pest insects, entering through the spiracles and the open circulatory system (Alzahrani & Ebert, 2019; Wang *et al*., 2006). Phosphine acts at the mitochondrial level, interrupting cellular respiration, acting on Complex IV of the electron transport chain of the mitochondria, preventing normal electron transport, generating energy insufficiency, and promoting the formation of reactive oxygen species that destroy the proteins through a redox effect (Nath *et al*., 2011).

Treatments involving magnesium phosphide for fresh agricultural products are conducted in sealed environments, typically under refrigeration, where the concentration of phosphine and exposure time play critical roles (Ahmed *et al*., 2018). The volatilization rate of magnesium phosphide diminishes at lower temperatures, which may affect treatment efficacy; therefore, increasing the dosage or extending the exposure time can be necessary (Zhang *et al*., 2013). An appropriate exposure duration is essential, as sufficient time can achieve effective control even at lower concentrations (Zhang *et al*., 2015). Wason and Selladurai (2023) found that phosphine was effective in mitigating fruit fly eggs in fruits of *Syzygium samarangense*, and the color and texture of the fruits were not significantly affected at concentrations up to 0.69 mg L^{-1} of PH_3 for 24 h. However, prolonged exposure may disrupt export logistics. Conversely, utilizing high concentrations of phosphine can negatively impact control efficacy, as it may induce narcosis in certain insects, thereby limiting respiration and reducing mortality (Lampiri *et al*., 2021). Likewise, its low concentrations or insufficient exposure times increase the selection pressure on pest insects, inducing the development of resistance (Kyung *et al*., 2018; Liu & Liu, 2014). On the other hand, high concentrations of fumigants can cause phytotoxic damage in agricultural products, affecting quality (Cato *et al*., 2019). It is important to establish concentration ranges and exposure times that can control pests without affecting the quality of the agricultural products.

The objective of this research was to assess the impact of different doses and exposure times of phosphine on the postharvest quality of Hass avocados, thereby supporting future studies of insect pest management.

Materials and methods

Location and plant material

This experiment was conducted in the first semester of 2019 at the postharvest laboratory of the Faculty of Agricultural Sciences at the Universidad Nacional de Colombia, Bogotá campus. The avocados were sourced from an orchard located in the municipality of Granada, Cundinamarca

(Vereda Santa Helena) (4°30'10.9" N, 74°20'45.3" W, altitude 2500 m a.s.l.). Harvest was carried out based on visual criteria, which were subsequently validated through the measurement of the fruit dry mass percentage, reported as optimal at around 23%, serving as an indicator of harvest maturity (Carvalho *et al*., 2014; Cerdas Araya *et al*., 2014). From the total harvested fruits, those exhibiting uniform size and free from damage or abnormalities were selected for the study.

Experiment design and treatments

The experiment was designed as a bifactorial arrangement, with the first factor representing five levels of phosphine concentration (D) (0, 200, 400, 600, and 800 ppm) and the second factor comprising two exposure times (E) (36 and 72 h). Treatments were conducted under refrigerated conditions in cold rooms maintained at 7±1°C, using ten 200 L metal barrels that were placed and used as treatment chambers. Each treatment contained six avocados as the experimental unit, with six replicates. Magnesium phosphide (Fumicel placa®, ANASAC, Chile) was added to each barrel based on a commercial dosage of 3.4 g m^{-3}, which approximates a concentration of 200 ± 20 ppm. Additionally, according to the Andean standard for pesticide registration, two control treatments were included: the first without phosphine (0 ppm), and the second with a double dose (400 ppm) (ICA & ANDI, 2016; Lizarazo-Peña *et al*., 2024). The barrels were hermetically sealed, and the presence of phosphine leaks was monitored with a portable meter (PAC-7000, Drager, Lübeck, Germany). Treatments were applied starting with 72 h of exposure, followed by 36 h. Then the fruits were kept refrigerated for 30 d to simulate the export transport process, which served as the postharvest evaluation period.

Measurement of fruit variables

During the application of treatments in each barrel, the phosphine concentration was measured using colorimetric tubes based on silver salts (Detia®, Degesch, Laudenbach, Germany) with a detection range of 50 to 2000 ppm. The first measurement was taken two h after the application of each treatment and, from then on, every 12 h until the end of the exposure periods. During this period, and with the same frequency, the CO_2 concentration was monitored in the cold room and inside the barrels with treatments 0 ppm – 36 h (phosphine concentration – exposure time) and 0 ppm – 72 h using an electronic atmosphere meter (Oxybaby, Witt-Gasetechnik GmbH & Co. KG, Witten, Germany).

Destructive-type variables of fruit firmness and color (exocarp and mesocarp) were evaluated on days 0, 7, 14, 21, and 28 after the application of the treatments (DAT). In each of the samples, the following variables were evaluated in six fruits per experiment unit:

Firmness (F) measurement was made using a Universal Test Machine penetrometer with a cylindrical Magness-Taylor probe of 8 mm diameter. Following the methodology of Castellanos *et al*. (2016), the firmness was determined as the force (Newtons) necessary to penetrate the fruit; a single measurement was taken per fruit at a random point on its equatorial contour, using a speed of 10 mm s^{-1} and a maximum depth of penetration of 12 mm from the surface of the fruit.

Color was measured on the exocarp and mesocarp of the fruits with a Minolta CR-400 colorimeter (Minolta Camera Co., Osaka, Japan) using the L*, a*, and b* coordinates of the CIELAB color space. For each fruit, three points were taken in the exocarp, then the fruit was immediately cut transversely and three points were taken again in the mesocarp.

For the exocarp, a color index (ICe) was determined with a principal component analysis (PCA), integrating the standardized values of L, a and b that explained 88% of the variation and were defined by:

$$ICe = 0.546*a - 0.600*b - 0.584*L \quad (1)$$

The resulting value was used as a variable to identify differences between treatments.

For the mesocarp, the PCA generated a color index that explained 64% of the variation; therefore, it was not considered, and the values of L*, a*, and b* were analyzed independently.

Weight loss and ethylene production were estimated with non-destructive sampling, for which samples of two fruits were placed in a plastic mesh with three repetitions and evaluated on days 0, 3, 6, 10, 14, 19, 23, and 26 after the application of treatments.

For ethylene production (ET) analysis, ethylene samples were taken by drawing 1 ml of gas with a syringe through the rubber seal of the container where the avocados were stored for 1 h, and the samples were injected into a gas chromatograph (Agilent 7890A, Agilent Technologies Inc., Santa Clara, CA, USA) following the methodology proposed by Castellanos *et al*. (2017).

Weight loss (WL) was measured with the fresh mass of the fruit samples described above, using a balance (Scout

Pro® RS232, OHAUS, Mexico) with a precision of 0.01 g, calculating the percentage of weight lost between the initial weight (0 DAT) and the weight of the subsequent samples.

Statistical analysis

The experiment was designed with a bifactorial structure in complete and generalized blocks, where the blocking factor (B) represented fruit maturity at harvest. For the variables with longitudinal structure (evaluated over time) including F, WL, ET, and color L*, a*, b* of the exocarp and mesocarp, the days after treatment (DAT) was considered as a factor in repeated measurements to account for the correlational structure in temporal measurements. The effect of the factors and the differences between treatments in variables with longitudinal structure were estimated from a longitudinal multivariate analysis of variance with a permutational (non-parametric) approach as proposed by Friedrich *et al*. (2018), using the MANOVA.RM library (2019) of the R statistical software (Version 1.2.1335), along with the library's confidence intervals, to assess differences between the factor levels. The graphics were created with the package "ggplot2" (Wickham, 2016). For interpreting and discussing the interval, we refer to the confidence interval criteria outlined by Cumming *et al*. (2007).

Results and discussion

Table 1 summarizes the MANOVA results for the evaluated postharvest variables. The analysis revealed no significant effects from the blocking factor or the dose factor on any variables. However, exposure time significantly influenced all variables, except for the L* color coordinate in the exocarp, WL, and ET. The duration of the test in days after treatment (DAT) also showed significant differences across all parameters. Notable interactions were observed, including the effect of phosphine dose and exposure time (D x E) on weight loss and ethylene production, as well as the impact of exposure time and days after treatment (E x DAT) on firmness and the b* component of color in both exocarp and endocarp. Additionally, the triple interaction involving dose, exposure, and DAT demonstrated differences in the L* and a* components of mesocarp color. Given the interactions observed, the highest degree of interaction was analyzed according to Montgomery (2017), with differences interpreted through graphical representations.

The evaluated variables showed no adverse effects from the application of phosphine at different doses (D) compared to the control group (Tab. 1), indicating that magnesium phosphide concentration does not significantly impact fruit quality (Güncan *et al*., 2023; Liu *et al*., 2018). However, firmness (F), exocarp color (ICe), and mesocarp color (values a* and b*) were influenced by the duration of exposure to phosphine (E), regardless of the concentration used. Notably, these affected variables may be directly related to the CO_2 content inside the barrels at the time of applying the treatments, which reached values of 2.8% in the barrels with an exposure time of 36 h and 4.4% in barrels with 72 h exposure compared to a value of 0.8% outside the barrels in the cold storage room (Espinosa-Cruz *et al*., 2014). Avocado fruits, when interacting in confined spaces (such as hermetic barrels), experience a sharp reduction in O_2 and an increase in CO_2 (Rojas-Graü *et al*., 2009). The rate of O_2 consumption decreases with increasing CO_2 levels; thus, high CO_2 concentrations may activate both the alternative respiratory pathway and anaerobic pathways simultaneously. Considering that Hass avocado fruits cannot tolerate environments with O_2 concentrations lower than 2% without undergoing fermentation (El-Shafei, 2020; Hertog *et al*., 2003), the reduction in O_2 availability induces fermentation, resulting in the production of ethanol and acetaldehyde, which can negatively affect fruit quality, reflected in the increased production of volatile compounds in the fruits (Perotti *et al*., 2014).

TABLE 1. Summary of the *P*-values from the MANOVA.RM analysis for the main factors and interactions between factors for each of the longitudinal response variables evaluated in Hass avocado fruits treated with phosphine at different exposure times.

Variation factor	F	Exocarp color			Mesocarp color			WL	ET
		L*	a*	b*	L*	a*	b*		
B	ns	ns	ns	ns	ns	ns	ns	ns	ns
D	ns	ns	ns	ns	ns	ns	ns	ns	ns
E	**	***	***	**	ns	***	**	ns	ns
DxE	ns	ns	ns	ns	ns	ns	ns	***	***
DAT	***	***	***	***	***	***	***	***	*
DxDAT	ns	ns	ns	ns	ns	ns	ns	ns	ns
ExDAT	*	ns	ns	*	ns	ns	*	ns	ns
DxExDAT	ns	ns	ns	ns	*	*	ns	ns	ns

Abbreviations: ns: not significant, B: blocking factor (point of maturity at harvest), D: dose (effect of the phosphine used), E: exposure time to phosphine, DAT: days after treatment (effect over time), D×E: interaction between the dose and the exposure time to phosphine, F: firmness, WL: weight loss, ET: ethylene production. MANOVA. *, **, *** represents the significant differences at probability levels 0.05, 0.01, 0.001, respectively.

Fruit firmness was influenced by both exposure duration and evaluation time, with reductions observed in firmness for all durations. However, the decrease was more significant in fruits exposed for 72 h. At 21 DAT, similar reductions in firmness were noted, but by 28 DAT fruits exposed for 72 h demonstrated a significantly greater loss of firmness (Fig. 1). The avocado is classified as a climacteric fruit, and the production of ethylene is intricately linked

to the ripening process during storage (Pedreschi *et al.*, 2019). Thus, firmness, color, and weight loss are affected by chlorophyll degradation and increased enzymatic activity in pectin degradation and hemicellulose hydrolysis induced by maturation (Liu *et al.*, 2018). Firmness was affected mainly at 72 h exposure, possibly because the fruits were mostly at consumption maturity (Fig. 1), and a few were at physiological maturity, which led to greater enzymatic degradation of polysaccharides (Goulao & Oliveira, 2008; Walse & Jimenez, 2021). Similar to Garcia *et al.* (2021), a shelf-life study on avocados was conducted to assess the effects of biopolymer coatings on post-harvest parameters. Hass avocados were refrigerated at 4°C and 79% relative air humidity. Initially, the avocados had a firmness greater than 70 Newtons. By 24 d, the firmness of fruits in the control group had decreased to below 10 Newtons, demonstrating a similar trend in firmness reduction.

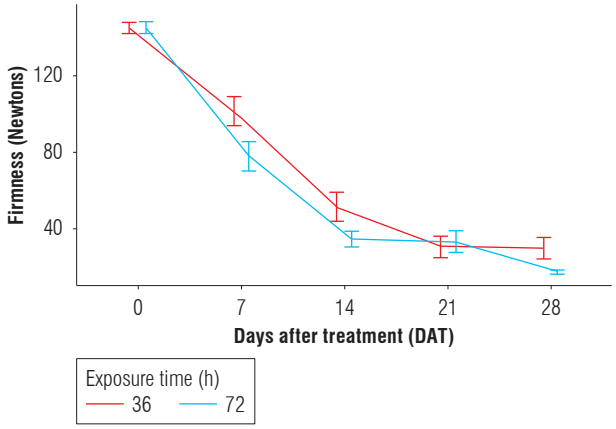

FIGURE 1. Effect of phosphine exposure time on the firmness of Hass avocado fruits. Vertical bars represent confidence intervals for the MANOVA.RM model ($P<0.05$), n = 180.

The exocarp color was significantly affected by exposure duration and days post-treatment, as shown in the L*, a*, and b* components. The L* value darkened over time, particularly with the 72-h exposure. Meanwhile, the a* component indicated a reduction in the fruit's green hue, with higher values observed from 7 d post-treatment for the 72 h exposure. Additionally, the b* component showed a decrease, reflecting a loss of yellow color, especially after the 72 h exposure (Fig. 2).

The mesocarp color was significantly influenced by the triple interaction between dose (D), exposure time (E), and days after treatment (DAT), particularly in the L* and a* components. The L* component showed a darker tone over time, which was more noticeable with 72-h exposures. The changes were subtle, making it difficult to identify clear trends. The a* component increased over time, indicating

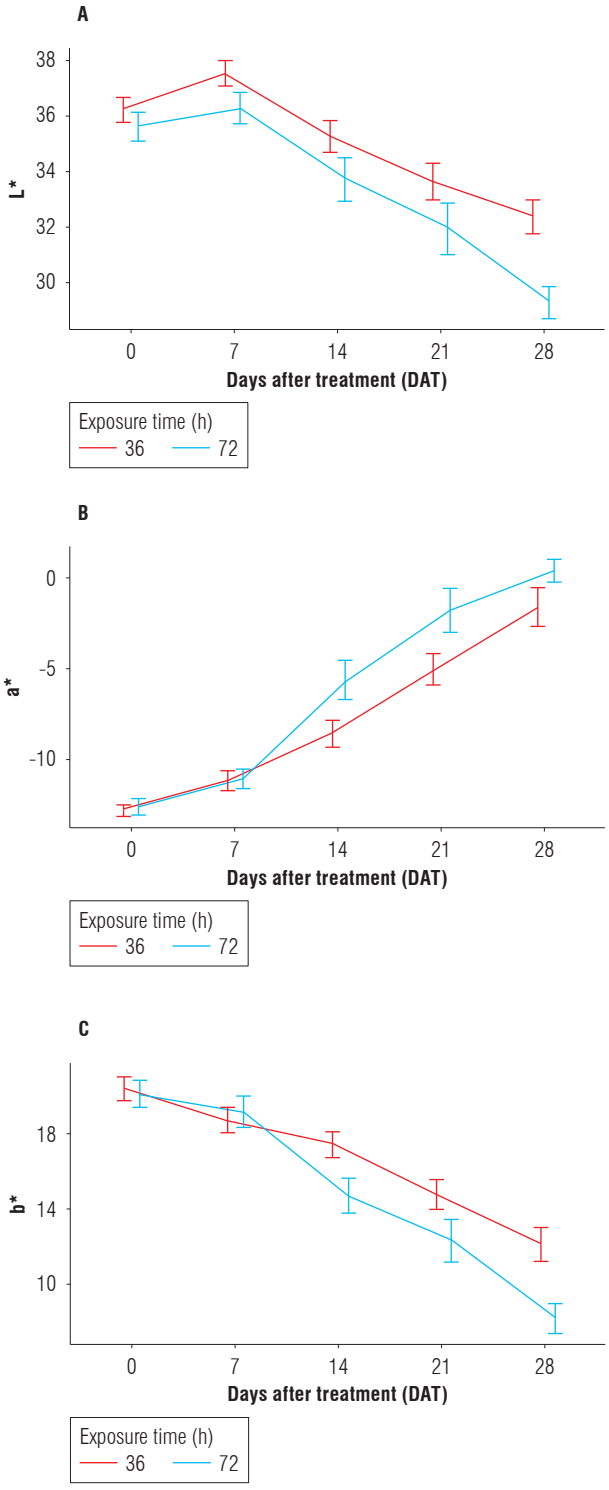

FIGURE 2. Effect of phosphine exposure time on color components (CIELAB) of the exocarp in Hass avocado fruits. The blue line represents the exposure time of 72 h, and the red line shows the 36 h. (A) component L*, (B) component a*, (C) component b*. Vertical bars represent confidence intervals for the MANOVA.RM model ($P<0.05$), n = 180.

a transition from dark green to light green, with significant differences at 21 d for 36 h exposures and at 14 d for 72 h exposures; the treatment with the least effect was 800 ppm.

The b* component was significantly influenced by the interaction between exposure time (E) and days after treatment (DAT). It decreased, reflecting a loss of yellow color, especially in the 72 h exposures. The differences between exposure times were more pronounced at the start and diminished as the experiment progressed.

The previously mentioned changes, such as the alteration in mesocarp color, are associated with changes in the exocarp. These alterations may be closely related to water loss and the subsequent dehydration of the fruit during the ripening process in storage (Pidakala *et al*., 2024; Shikwambana *et al*., 2021). Furthermore, exocarp luminosity

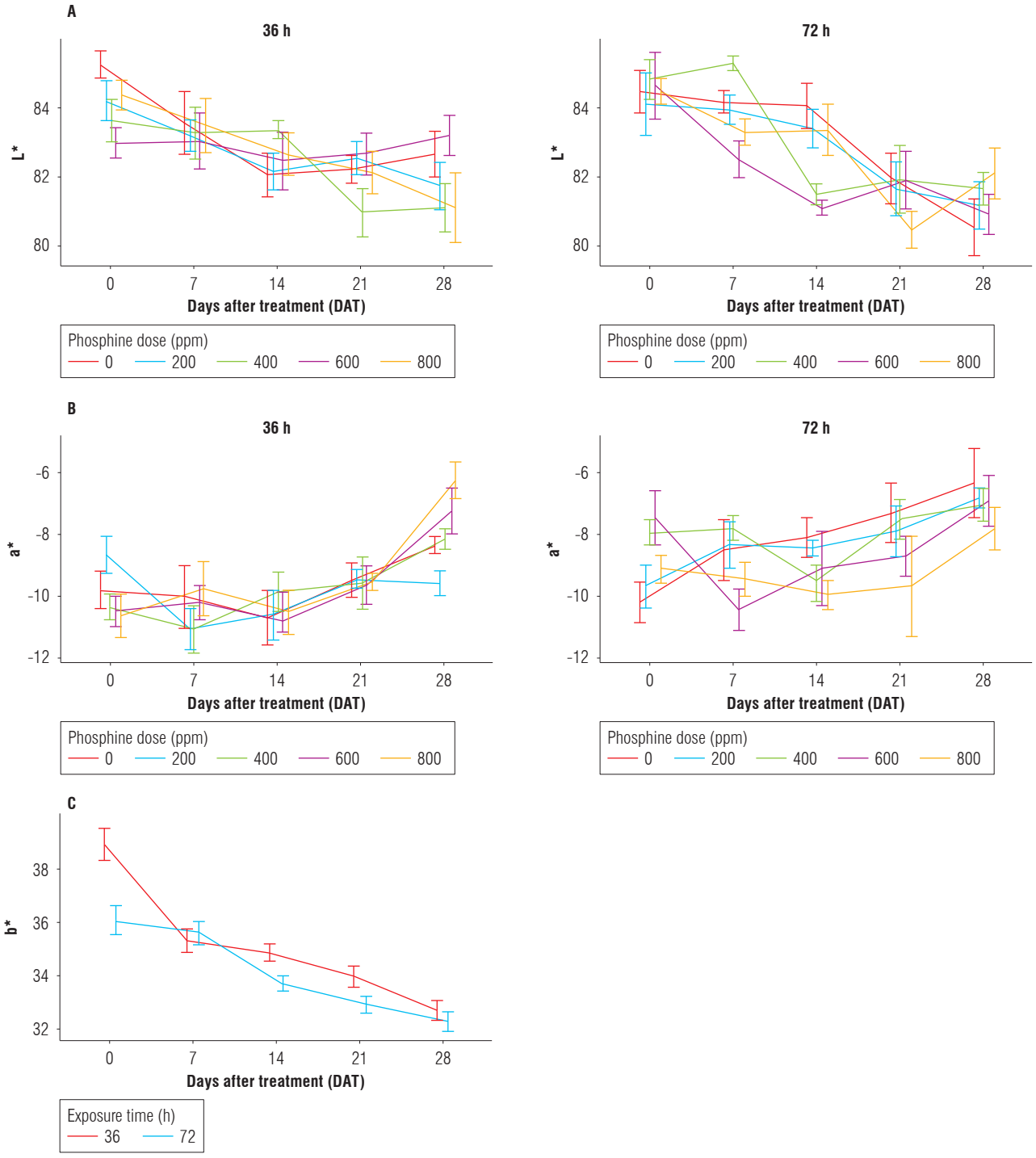

FIGURE 3. Effect on the color components (CIELAB) of the mesocarp of Hass avocado fruits from the interactions between dose, exposure time, and test time for (A) component L* and (B) component a* and (C) the interaction between exposure time and test time (DAT) for component b*. Vertical bars represent confidence intervals for the MANOVA.RM model ($P<0.05$), n = 180.

(L*) decreased in the days following treatment, indicating a reduction in brightness. Meanwhile, the exocarp color value a*, which describes the shift in color from red (positive) to green (negative), increased during the same period (Fig. 2). Since no significant differences were observed between the treatments applied at different doses (Tab. 1), color changes in the a* and L* coordinates could be associated with the fruit ripening process, which induces the degradation of chlorophyll through the enzymatic action of chlorophyllases, red chlorophyll catabolite reductases (RCCR), and pheophorbide oxygenases (PAOy). This process promotes the synthesis of cyanidin-3-O-glucoside during the ripening of Hass avocados, resulting in lower L* values and higher a* values in both the exocarp and mesocarp (Castellanos *et al*., 2016; Pathare *et al.,* 2013; Wason & Selladurai, 2023). Regarding the b* coordinate (Fig. 3), which describes changes between yellow (positive) and blue (negative), a decrease in the yellow hue of the avocado pulp (mesocarp) was observed. Although an increase in b* was expected, indicating a shift towards more yellow tones due to the synthesis of carotenoids during ripening, this observed behavior in b* is consistent with findings reported by Sierra *et al.* (2019) when modeling the ripening effect in Hass avocados. Lu *et al.* (2009) reported variations in carotenoid levels in the pulp of avocado cv. Hass at its consumption ripeness stage, with a range from 5 μg g^{-1} to 40 μg g^{-1} total carotenoids. Similarly, Rosas Flores *et al.* (2021) found carotenoid content in Hass avocado to be 17.60±1.61 μg g^{-1}, compared to a chlorophyll content of 56.85±19.41 μg g^{-1}. This suggests that, when evaluating color, the b* coordinate could be influenced by the content of these pigments, masking the effect of secondary metabolites such as carotenoids (Li *et al*., 2020).

Ethylene production was affected by the interaction of phosphine doses and exposure time. Ethylene is a signaling hormone in the ripening processes, which leads to the degradation of complex sugars that serve as a source of carbon and energy for enzymatic processes and changes in fruit color, facilitating the ripening process (Astudillo-Ordóñez & Rodríguez, 2018). Figure 4A shows that the interaction of phosphine doses and exposure times affected ethylene production; however, the interaction with the highest ethylene production was at 800 ppm at 36 h exposure, and the lowest production of ethylene occurred at 400 ppm at 36 h exposure and not at 0 ppm. Fluctuations in ethylene levels were observed, but they were not associated with the concentration of phosphine (Obenland *et al*., 2021). Instead, it is suggested that these fluctuations may have been generated by the fruit's production of carbon dioxide, a phenomenon akin to findings reported by Liu (2012), where doses of up to 2,200 ppm phosphine were evaluated for 3 d and there was no evidence of damage to fresh products, but there was damage due to accumulated CO_2. Subsequently, Liu and Liu (2014) evaluated the use of CO_2 and ethylene absorbents to reduce damage to lettuce.

For the production of ethylene over time, a decrease was observed; however, an abrupt increase was observed at 3 DAT as a result of the climacteric peak, reflecting the overproduction of ethylene in the maturation process (Fig. 4B). This behavior coincides with the report by Gwanpua *et al.* (2018) and Rosas Flores *et al.* (2016), who stated that Hass avocado fruits are sensitive to ethylene after the first 72 h after harvest, where there is an increase in the rate of ethylene production that subsequently stabilizes.

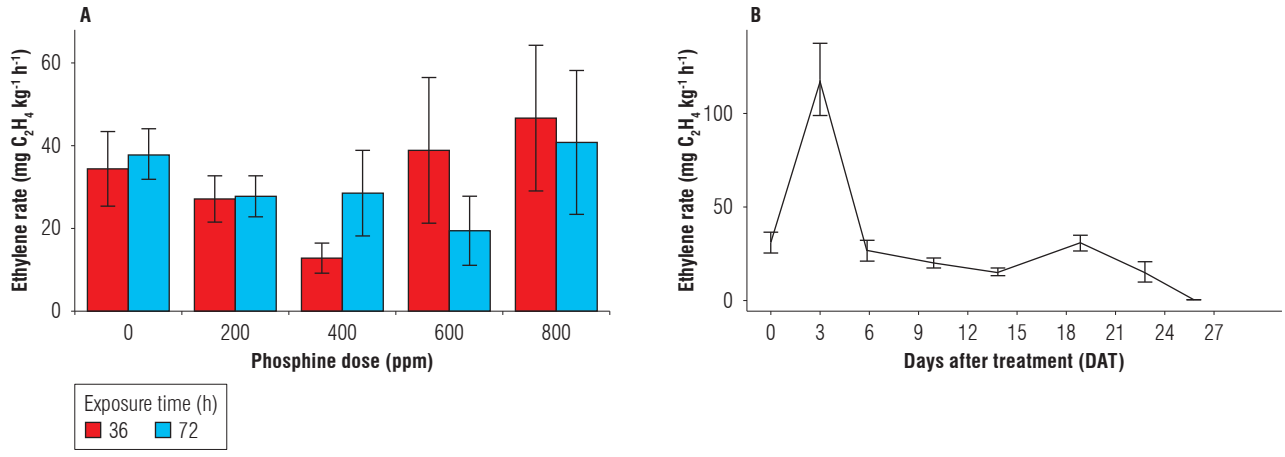

FIGURE 4. Effect of phosphine on the rate of ethylene produced by Hass avocado fruits. (A) Interaction effect of phosphine dose and exposure time, (B) effect of days after treatment (storage period). Vertical bars represent confidence intervals for the MANOVA.RM model ($P<0.05$), n = 180.

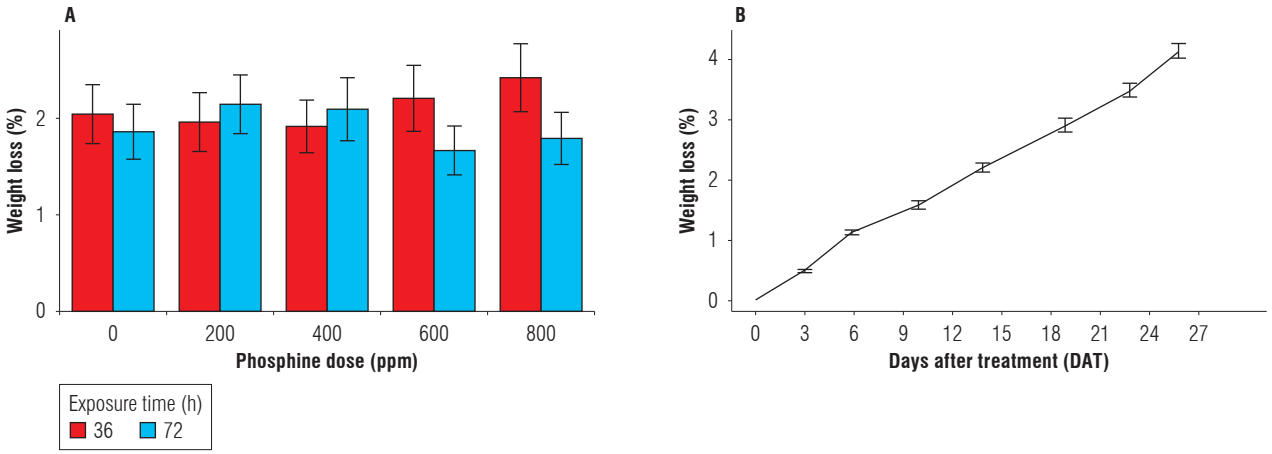

FIGURE 5. Effect of phosphine on weight loss in Hass avocado fruits. (A) Interaction effect of doses and exposure length to phosphine, (B) effect of trial evaluation days. Vertical bars represent confidence intervals for the MANOVA.RM model ($P<0.05$), n = 180.

Weight loss exhibited a linear trend throughout the trial, increasing with the number of days post-treatment, ultimately resulting in a 4% loss of initial weight by 27 DAT (Fig. 5B). The interaction between phosphine dose and exposure time significantly influenced weight loss. The highest weight loss was observed at an 800 ppm phosphine dose with 36 h exposure, which differed significantly from the combination of 600 ppm and 72 h. However, none of these interactions showed significant differences compared to the control (Fig. 5A). The greater weight loss at 800 ppm and 36 h of exposure was possibly attributed to increased respiration in the avocados subjected to high concentrations of phosphine (Fig. 5A), which led to a greater vapor pressure deficit of the avocado compared to the reduced and saturated environment of phosphine molecules, resulting in increased water loss (Espinosa-Cruz *et al.*, 2014; Kim *et al.*, 2022). However, the high concentrations of phosphine exposed for 72 h did not show marked differences with respect to the weight loss of the control (0 ppm). Similarly, the weight loss overtime was less than 5% (Fig. 5B) attributed to the ripening process. This is consistent with reports by Escobar *et al.* (2019), who noted a weight loss due to maturation of 3.84% at 5°C. Likewise, Aguirre-Joya *et al.* (2017) reported a weight loss of up to 5.56% at 7°C in fruits of 190±20 g stored for 28 d.

Conclusions

Considering the postharvest variables evaluated in this study, the applied treatments indicate that the phosphine concentrations tested did not produce adverse effects on the postharvest quality of Hass avocado fruits. This finding suggests that concentrations up to 800 ppm could be viable for future studies on the efficacy of pest management, without compromising fruit quality. Notably, exposure duration did not show a direct impact on fruit quality, nor were there significant interactions between exposure time and phosphine concentration affecting fruit quality. However, it is important to note that prolonged exposure times, such as 72 h, may lead to the accumulation of respiratory by-products, which could inadvertently accelerate fruit ripening. Therefore, it is crucial to implement strategies within the treatment containers to mitigate this effect and ensure optimal fruit preservation.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

PALP carried out the functions of conceptualization, research, data curation, formal analysis, validation, visualization and writing the original draft. SBO contributed to research, data curation, formal analysis, visualization and writing the original draft. AOHA contributed to funding acquisition, project administration, supervision, validation, writing review & editing. All authors approved the final version of the manuscript.

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Wax coating and Ag-TiO_2 nanoparticles as alternatives to preserve postharvest quality of the purple passion fruit (*Passiflora edulis* f. *edulis*)

Recubrimiento con ceras y nanopartículas de Ag-TiO_2 como alternativas para preservar la calidad poscosecha de frutos de gulupa (*Passiflora edulis* f. *edulis*)

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ABSTRACT

The purple passion fruit (*Passiflora edulis* f. *edulis*) is a highly sought climacteric fruit on the global market, but its short postharvest life makes international commercialization difficult. The objective of this study was to evaluate the implementation of wax coatings and silver-doped titanium dioxide nanoparticles (Ag-TiO_2-NPs) to preserve the postharvest quality parameters of the purple passion fruits. After the waxes and NPs synthesized using the combustion solution method were applied, the fruits were packed in plastic bags and cardboard boxes. Treatments were evaluated under two different storage conditions: room temperature (18°C, domestic market) and refrigeration (7°C, export market) + 1 week of shelf life. Physicochemical variables were measured periodically, and at the end of each storage condition, a consumer perception analysis was performed using natural language processing. The coating treatments did not favor postharvest behavior in the two experiments, and only increased brightness of the fruits was evident. In the refrigeration experiment, the application of Ag-TiO_2-NPs alone had a positive effect on the delaying parameters like respiration rate (decrease of up to 55% compared to the control), color (up to 80% less), total soluble solids (lower by ~ 10%), and titratable acidity (increased ~ 5%), with no effect on the perception of taste and visual characteristics identified by consumers. However, this treatment did not show consistent effects at room temperature (18°C). These findings support the viability of nanoparticle application as a strategy to preserve the postharvest quality of passion fruit destined for exportation and wax coatings to improve visual fruit perception.

Key words: respiration, ethylene, shelf life, natural language processing, consumer perception.

RESUMEN

La gulupa (*Passiflora edulis* f. *edulis*) es una fruta climatérica altamente deseada en el mercado global, pero con una corta vida poscosecha, lo que dificulta su comercialización en mercados internacionales. El objetivo del presente estudio fue evaluar la implementación de recubrimientos de ceras y nanopartículas de dióxido de titanio dopadas con plata (Ag-TiO_2-NPs) para preservar los parámetros de calidad poscosecha del fruto de gulupa. Después de aplicar las ceras y las NPs sintetizadas usando el método de combustión en solución, las frutas se empacaron en bolsas plásticas y luego en cajas de cartón. Posteriormente, se evaluaron los tratamientos bajo dos condiciones de almacenamiento diferentes, a temperatura ambiente (18°C, para el mercado doméstico) y refrigeración (7°C, para el mercado de exportación) + 1 semana de vida útil. Se midieron periódicamente variables fisicoquímicas y, al final del almacenamiento, se realizó un análisis de percepción del consumidor utilizando procesamiento de lenguaje natural. Los resultados indican que los tratamientos con encerado no tuvieron un efecto favorable en el comportamiento poscosecha en los dos experimentos, sólo se evidenció un mayor brillo de los frutos. Por otro lado, en el experimento de refrigeración, la aplicación de únicamente Ag-TiO_2 NPs tuvo un efecto positivo al ralentizar parámetros como la tasa de respiración (disminución hasta en un 55% comparado con el control), el color (hasta un 80% menos), los sólidos solubles totales (menores en ~ 10%) y la acidez titulable (aumentó ~ 5%), sin efecto en la percepción del sabor y las características visuales identificadas por los consumidores. Pero este tratamiento no mostró efectos consistentes a temperatura ambiente (18°C). Estos hallazgos apoyan la viabilidad de la aplicación de NPs como una estrategia para preservar la calidad poscosecha de la gulupa destinada a la exportación, y de los recubrimientos de cera para mejorar la percepción visual del fruto.

Palabras clave: respiración, etileno, vida en anaquel, lenguaje natural de procesamiento, percepción del consumidor.

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Introduction

The purple passion fruit (*Passiflora edulis* Sims f. *edulis*) is cultivated mainly in subtropical and tropical regions with an elevation between 1600 and 2300 m a.s.l. (De Armas Costa *et al*., 2022; Fischer *et al*., 2022). This Passifloraceae species is one of the most desired exotic fruits in the world and is adequately valued for its flavor, aroma, and presence of biofunctional compounds with medicinal potential (such as its high content of vitamin C, antioxidants, and fiber) that make it a healthy option for human consumption (Lozano-Montaña *et al*., 2021; Vuolo *et al*., 2019). Passion fruit is one of the most important exotic fruits in production in the tropics. Brazil is the world's largest producer of passion fruit, mainly cultivating the yellow variety (*Passiflora edulis* f. *flavicarpa*) and principally for the domestic market. Colombia is also a significant producer of these fruits, but Ecuador and Peru are major players in the international market (Fonseca *et al*., 2022). For this reason, the Colombian agro-industry has shown considerable interest in increasing crop production in recent years (Bernal Durán, 2022; Rincón Munar, 2020). For 2022, Colombia's national output of fruits reached 32,353.78 t in an area of 2,059.47 ha, with exports totaling 14,600 t (Agronet, 2023; ANALDEX, 2023).

The purple passion fruit is a berry with a spherical or ovoid shape, measuring 5.2 to 8.0 cm in length and 4.7 to 7.2 cm in diameter. It has a hard, smooth, and waxy skin (pericarp), about 3.0 to 4.5 mm thick, with a spongy, white mesocarp (Ocampo *et al*., 2020). The immature fruit is pale green and turns dark purple when ripe. The fruit weight ranges from 46 to 76 g (Ocampo *et al*., 2020). The maturity stage ideal for harvest corresponds to a fruit color of 40-50% green and 40-50% purple; at this point, the fruits reach the maximum total pulp fresh weight and the highest content of soluble solids (15.9 °Brix) and decrease contents of titratable acidity to less than 4.65% (Pinzón *et al*., 2007). During the postharvest period, passion fruits experience loss of moisture and firmness, skin darkening, microorganism growth, nutrient degradation, cell wall rupture, wrinkling, and eventual senescence, resulting in a short shelf life (Nxumalo & Fawole, 2022; Zhou *et al*., 2022). Depending on the storage conditions, the storage time of purple passion fruit can range between 13 and 48 d (Herrera *et al*., 2024). Due to its climacteric behavior, passion fruit undergoes a physiological process characterized by a significant increase in the respiration rate during ripening accompanied by ethylene biosynthesis. While this promotes ripening of the fruits, it simultaneously accelerates its deterioration (Calderón-Martínez *et al*., 2021; De Armas Costa *et al*., 2022). Therefore, it is essential to develop appropriate preservation technologies to maintain fruit quality and improve shelf life.

It is necessary to control the rate of transpiration, respiration, ethylene production, and microbial infection, as these measures allow for prolonging the shelf life of the fruits and minimizing the losses during postharvest (Barsha *et al.,* 2021; Yahia & Carrillo-López, 2018). Among the various postharvest treatments, some techniques, such as the application of edible coatings based on oil, wax, and chemical products as microbial control agents (Gemail *et al*., 2023; Nxumalo & Fawole, 2022; Zhou *et al*., 2022), are used. In this way, the application of wax acts as a protective barrier against the entry of oxygen into the fruits and the loss of fruit moisture, thus obtaining a modified atmosphere allowing the control of the ripening process and postharvest quality of the fruits (Devi *et al*., 2022; Yahia & Carrillo-López, 2018). In purple passion fruit stored at room temperature, Zhou *et al.* (2022) found that a multifunctional coating based on chitosan and tannic acid delayed weight loss, firmness, shrinkage index, and prolonged postharvest life by 7 d.

There are other postharvest management alternatives, such as the application of nanoparticles; this technology is up-and-coming and is arousing great interest in various fields due to its antifungal activity (Nevado-Velasquez *et al*., 2023; Sadek *et al*., 2022) in kiwifruit (Li *et al*., 2022) and mangosteen (Thammachote *et al*., 2023). Nanoparticles can be doped with certain elements that allow them to absorb or eliminate ethylene and extend the shelf life of fruits (Ali *et al*., 2020; Nevado-Velasquez *et al*., 2023). Titanium dioxide nanoparticles (Ag-TiO_2-NPs) are reported to inhibit ethylene biosynthesis by suppressing genes that encode enzymes of ACC synthase (ACS) and ACC oxidase (ACO) (Elatafi & Fang, 2022; Naing & Kim, 2020). Additionally, the combined use of waxes and nanoparticles is evaluated in several fruits (Cid-López *et al*., 2021; Taha *et al*., 2022) because their properties improve postharvest benefits. In this sense, in banana fruits, the addition of chitosan coating and zinc oxide nanoparticles (ZnO-NPs) generates better postharvest performance (La *et al*., 2021).

There is a trend for healthy foods like purple passion fruits to meet a series of basic parameters known as multidimensional quality (Ramirez-Gil *et al*., 2019). This includes sensory attributes, nutritional values, chemical constituents, mechanical properties, functional properties, defects, physical and visual appearance, safety, and additional aspects of consumer perception and preferences (Abbott, 1999; Ramirez-Gil *et al*., 2019; Saba *et al*., 2018). So, it is necessary to view quality from an integral

perspective, emphasizing consumer perception and the analytical characteristics that determine it (Godrich *et al*., 2020; Nicolaï *et al*., 2014; Péneau *et al*., 2006).

Purple passion fruits show great potential for countries with emerging production systems, such as Colombia (Rincón Munar, 2020). However, the fruit's current export potential is limited by postharvest problems that significantly affect multidimensional quality criteria, especially visual parameters (color, shine, shape) easily detectable by consumers. This creates a technological gap that must be addressed with highly innovative technological tools such as nanoparticles and wax coatings. However, the role of waxes and nanoparticles in the quality of purple passion fruits has not been investigated. This research aimed to evaluate the effect of waxes and silver-doped titanium dioxide nanoparticles on postharvest quality parameters of purple passion fruits destined for two well-defined markets: national and international.

Materials and methods

The fruits and their initial characterization

High-quality purple passion fruits destined for exportation were acquired through the export company OCATI S.A. (Colombia). The chosen fruits had reached harvest maturity (50-80% of their peel with a purple color), with weight between 60-80 g. They were free of physical defects or phytosanitary problems. Before beginning the experiments, the fruits were disinfected with sodium hypochlorite (5%). The experiments were conducted at the Laboratorio de Calidad y Poscosecha de Productos Agrícolas de la Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Bogotá.

In the first part of the experiment, we characterized the fruits to establish a baseline for their physical, chemical, and biochemical qualities. We selected five random samples and measured the total titratable acidity, epidermis elasticity, firmness, color index, respiratory intensity, and contents of soluble solids. The protocols and quantification details are described in the following sections. This essential characterization at the start of the experiment is presented in Table 1.

TABLE 1. Physicochemical parameters of purple passion fruits at the start of the experiment.

Parameter	Value
Total titratable acidity (% citric acid)	3.1 ± 0.7
Epidermis elasticity (mm)	13.9 ± 0.6
Firmness (N)	23.1 ± 1.3
Color index	12.9 ± 4.4
Respiratory intensity (CO_2 cm^3 kg^{-1} h^{-1})	60.6 ± 4.3
Total soluble solids (° Brix)	14.9 ± 0.4

Experimental design and treatments

We carried out two experiments with the same treatments, one at room temperature (18°C and relative humidity (RH) of 75%) and the other in refrigerated storage (7°C and RH 90%) for 43 d + 1 week of shelf life (18°C), each with a specific market focus, with room temperature for the domestic market and low temperature for the export market. In each experiment, we used an completely randomized experimental design with 4 treatments; these were the application of wax (W), the combination of wax and Ag-TiO_2-NPs (W+N), the application of Ag-TiO_2-NPs (N), and a control treatment (C) where neither the wax nor the nanoparticles were applied. Each treatment had 4 replicates, and each experimental unit comprised 5 fruits. To store the fruits, we placed them in commercial plastic bags, packaging XtendR (coded B2) from StecPac (StePacPPC, Tefen, Israel), with dimensions of 53.6 × 40 cm^2, internal gussets of 9 cm, a thickness of 0.025 ± 0.002 mm^2, and 30 perforations of 0.62 ± 0.11 mm^2 in diameter (Herrera *et al*., 2024), which generated a modified atmosphere. After that we placed this plastic packaging in a 30x20x15 cm cardboard box, specifically for international trade.

Wax application

We conducted a preliminary test to establish the optimal dilution ratio for the wax application. This test determined that a 50% dilution in water (v/v) was most effective. The dilution allowed the wax to form a uniform layer on the surface of the fruits, significantly reducing dehydration during storage. The wax imparted a notorious shine to the fruits, enhancing their visual quality, a crucial factor for consumer appeal in domestic and international markets.

A microsprinkler was used to apply a uniform film over the entire surface of the fruits, guaranteeing the total coverage of each fruit. The wax was allowed to dry. The wax corresponded to a commercial product used after harvest, "Coatings Passiflora" (Coatings SAS®, Colombia), a natural resin solution developed especially for the coating of yellow passion fruits, granadilla fruits, and purple passion fruits, with the following characteristics: boiling point of 75 ± 5°C, pH of 7.8-8.2, with a medium viscous liquid appearance, brownish color, and compatibility to be dissolved in water.

Application of silver-doped titanium nanoparticles (Ag-TiO_2-NPs)

The Ag-TiO_2-NPs used were synthesized, characterized, and provided by the Ceramics and Glasses Group,

Facultad de Ciencias, Universidad Nacional de Colombia, Medellín campus, and the Nanostructures and Applied Physics Group (NANOUPAR), Academic Directory, Universidad Nacional de Colombia, La Paz campus. Ag–TiO_2 nanoparticles were synthesized via the combustion solution method (Deganello & Tyagi, 2018; Sane *et al*., 2018) using titanium isopropoxide and silver nitrate as precursors and glycine as a reducing agent (Nevado-Velasquez *et al*., 2023). Titanium oxynitrate was formed through reactions with nitric acid. Silver doping (0-4.5 mol%) and glycine were added, followed by heating to 300°C, yielding a pale-yellow residue (Nevado-Velasquez *et al*., 2023). For this process, the combustion solution method was used to ensure size and synthesis, following the methodology and structural and morphological characterization through various techniques: XRD analysis and scanning electron microscopy. For particle size determination, approximately 400 particles were counted in 23 SEM photomicrographs for each of the TiO_2 samples with different concentrations of Ag (Nevado-Velasquez *et al*., 2023).

Ag-TiO_2-NPs were used at a dose of 400 mg kg^{-1} TiO_2 with 0.75% Ag; this was based on previous results in fruit trees, where no damage was observed to the epidermis and physical-chemical quality parameters improved along with antimicrobial activity (Nevado-Velasquez *et al*., 2023). Given the nature of the compound, the nanoparticles were applied by dissolving them in sterile distilled water, a process performed via ultrasound to ensure the correct and uniform dilution of the particles in the water. The fruits were then immersed in the solution. For the treatment of wax and nanoparticles, the fruits were immersed in the Ag-TiO_2-NPs solution as follows. After drying, the wax was applied, as explained above, mixing the nanoparticles in the wax solution and ensuring that the fruits were wholly dried to avoid any excess that could generate a spill or any section with poor coverage.

Variables evaluated in purple passion fruits

We measured nondestructive variables every week, while we determined destructive variables at the beginning of the experiment and at the end of storage. In the case of the refrigeration experiment, they were also measured at the end of shelf life. A consumer perception analysis was performed at the end of each experiment to identify consumer perceptions of visual characteristics such as shape, color, firmness, presence of external damage, and parameters of taste and flavor.

Nondestructive variables

For respiratory intensity (RI, cm^3 CO_2 kg^{-1} h^{-1}) and ethylene production (µl C_2H_4 kg^{-1} h^{-1}), we followed the protocol step by step reported by Reyes *et al*. (2024). We placed each fruit inside an airtight glass chamber with a volume of 428.94 ml. After 1 h at the same storage temperature, we determined the CO_2 emission using a portable gas analyzer (Dansensor CheckPoint 3 Ametek-Mocon, Berwyn, PA, USA). After this, the fruits continued in the chambers for 2 h, and a 1 cm^3 sample of the gas was taken from the vessel's headspace. The gas sample was injected into an Agilent Technologies 7890A gas chromatograph (GC) (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector (FID) and an HP-PLOT column (30 m x 0.55 mm x 40 µm; Castellanos *et al*., 2017).

We calculated weight loss (%) according to González *et al.* (2021) as the difference in weight of each sampling at the beginning of the experiment to a sample of approximately 60 g of fruits. We measured the fresh mass on a precision balance of 0.001 g (Ohaus-Pioneer, Colombia). We calculated the color index (CI) of the epidermis according to Cepeda *et al.* (2021), measuring the chromatic coordinates L* (lightness), a* (green/red), and b* (blue/yellow) of the CIELab color space using a Minolta CR 400 digital colorimeter (Konica Minolta, Chiyoda, Tokyo, Japan). We took three measurements in the equatorial fruit zone.

Destructive variables

We determined fruit firmness (N), and elasticity (mm) using a Lloyd Instrument LS1 digital material tester with a 1 kN load cell, a cylindrical punch of 3 mm, and Nexygen plus software. For total titratable acidity (TTA, % of citric acid), we carried out an acid-base titration with NaOH (0.1 N). Total titratable acidity was determined by incorporating 2 ml of juice in 25 ml of distilled water and proceeding to potentiometric titration until reaching pH 8.2 with a 916 Food Ti-Touch 120 automatic titrator (Metrohm, Herisau, Switzerland) (Gutiérrez-Villamil *et al*., 2023). We obtained the contents of total soluble solids (TSS) from approximately 1 ml of juice by reading in a Hanna digital refractometer with a range of 0 to 85% (Hanna Instruments, Spain).

We determined electrolyte leakage (EL, %; Eq. 1) as the electrical conductivity ratio according to Gutiérrez-Villamil *et al*. (2023), by which 10 disks of 0.5 cm in diameter were cut from the epidermis of each fruit. The disks were inserted into a plastic tube with 10 ml of deionized water and left at room temperature for 30 min. We measured electrical conductivity (EC_1) using an EC electrode. After measurement, the sample was heated at 90°C in a water bath for 15 min, allowed to cool, and the second EC (EC_2) was estimated.

$$EL(\%) = EC1/EC2 \times 100 \quad (1)$$

Consumer perception analysis

The perception of a product has been traditionally approached in two ways. The first was through expert panels, and the second was through consumer surveys. The first method is costly and presents logistical challenges, making it less viable for widespread use in these tests (Nicolaï *et al.*, 2014). On the other hand, consumer surveys traditionally require a large sample size and include specific intrinsic characteristics of the population being estimated (age, gender, race, economic income, social strata). This makes the process time-consuming and logistically demanding (Nicolaï *et al.*, 2014).

To incorporate consumer perception into our study, we designed a survey method based on ranking the importance of various quality characteristics such as appearance (internal-external), color, shine, aroma, texture (internal-external), flavor, and purchase criteria. We used an open-ended question format to capture the sensations, perceptions, and feelings generated by the fruits subjected to different treatments. We also focused on visual and taste characteristics upon consumption. We implemented a natural language processing method to extract as much information as possible and compensate for the number of respondents (n). This method analyzes the words used in open responses to find patterns and identify the emotions evoked by the fruits (positive, negative, or neutral) (Chong *et al.*, 2014; Hamilton & Lahne, 2020; Sun *et al.*, 2017).

The evaluated population consisted of students, professors, and administrative personnel from the Facultad de Ciencias Agrarias of the Universidad Nacional de Colombia, Bogotá campus. We randomly selected participants (n=25), avoiding any type of bias in selection, to ensure a diverse and plural representation. This methodology ensured that the sample adequately reflected fruit consumption, similar to how it is observed in an everyday market. We performed a sensory profile test based on a descriptive analysis, using a survey in which we characterized the fruit aroma, flavor, texture, and visual perception. We neutralized fruit flavors with soda crackers. The survey structure was implemented via Google Forms. We conducted the survey only for academic research purposes, with a commitment to the noncommercial use of the collected data. Participants were informed of the nature of the study and provided their consent before proceeding.

Statistical analysis

We used open software R 4.3.1 in which a multivariate approach analysis was implemented, using a PERMANOVA ($P<0.05$), except for the weight loss percentage, respiratory intensity, and ethylene rate variables that were analyzed employing a repeated measures model over time, and a *post hoc* analysis using Fisher's least significant difference test (LSD, $P<0.05$). For both methods, all assumptions were corroborated. The sensory perception analysis was developed using the Wordcloud libraries, and the Sentiment Intensity Analyzer tool from the nltk library using the Python programming environments.

Results

Physical parameters

Fruit weight loss had a continuous and significant increase at room temperature. Control fruits exhibited the lowest weight loss, whereas fruits treated with nanoparticles experienced a weight loss exceeding 20% (Fig. 1A). Under refrigeration, weight loss was minimal, with similar

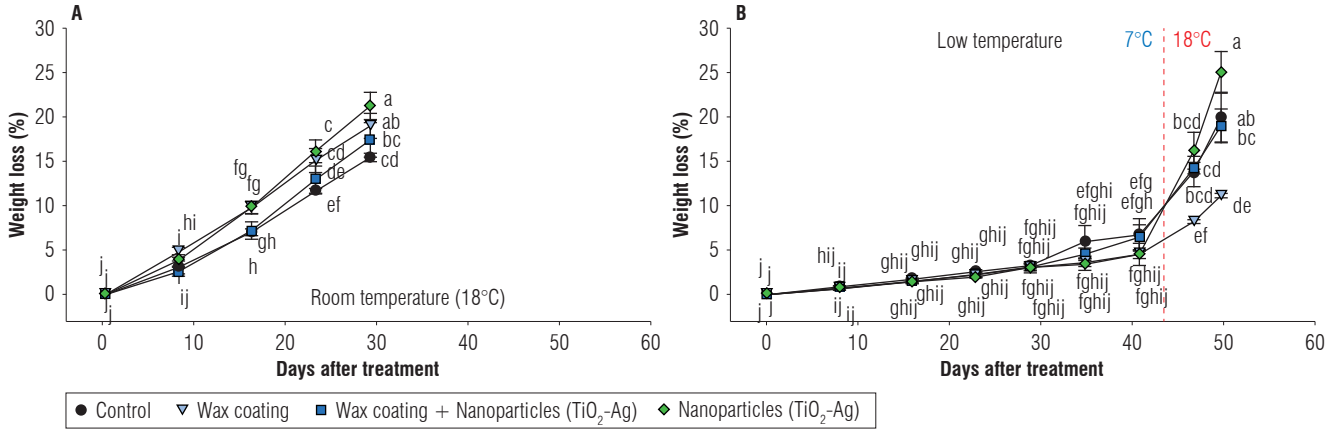

FIGURE 1. Effect of the application of wax and Ag-TiO_2-NPs on the fruit weight loss under room temperature storage (A) and refrigerated conditions of purple passion fruits stored at 7°C for 43 d and then transferred to 18°C for 7 d (B). Repetitive measures analysis was performed as a function of time. Different letters indicate significant differences according to the LSD test ($P<0.05$). The vertical bars represent the standard error (n=4). Control: fruits without wax or Ag-TiO_2-NPs application.

behavior across treatments. During shelf life, weight loss increased notably, especially in nanoparticle-treated fruits. In contrast, waxed fruits had significantly (P<0.05) lower weight loss (Fig. 1B).

Under room temperature conditions, no significant differences in the color index (CI) were observed between treatments (Fig. 2A). However, at the end of refrigeration and during the shelf life, statistical differences (P<0.05) were seen. Control fruits consistently showed the highest CI in both samples, while the lowest CI was recorded in fruits treated with nanoparticles. Additionally, the CI was higher during the shelf life than during the refrigeration stage (Fig. 2B).

There were statistical differences (P<0.05) in the elasticity of the fruits in the experiment under room temperature conditions, with increased elasticity in the control fruits

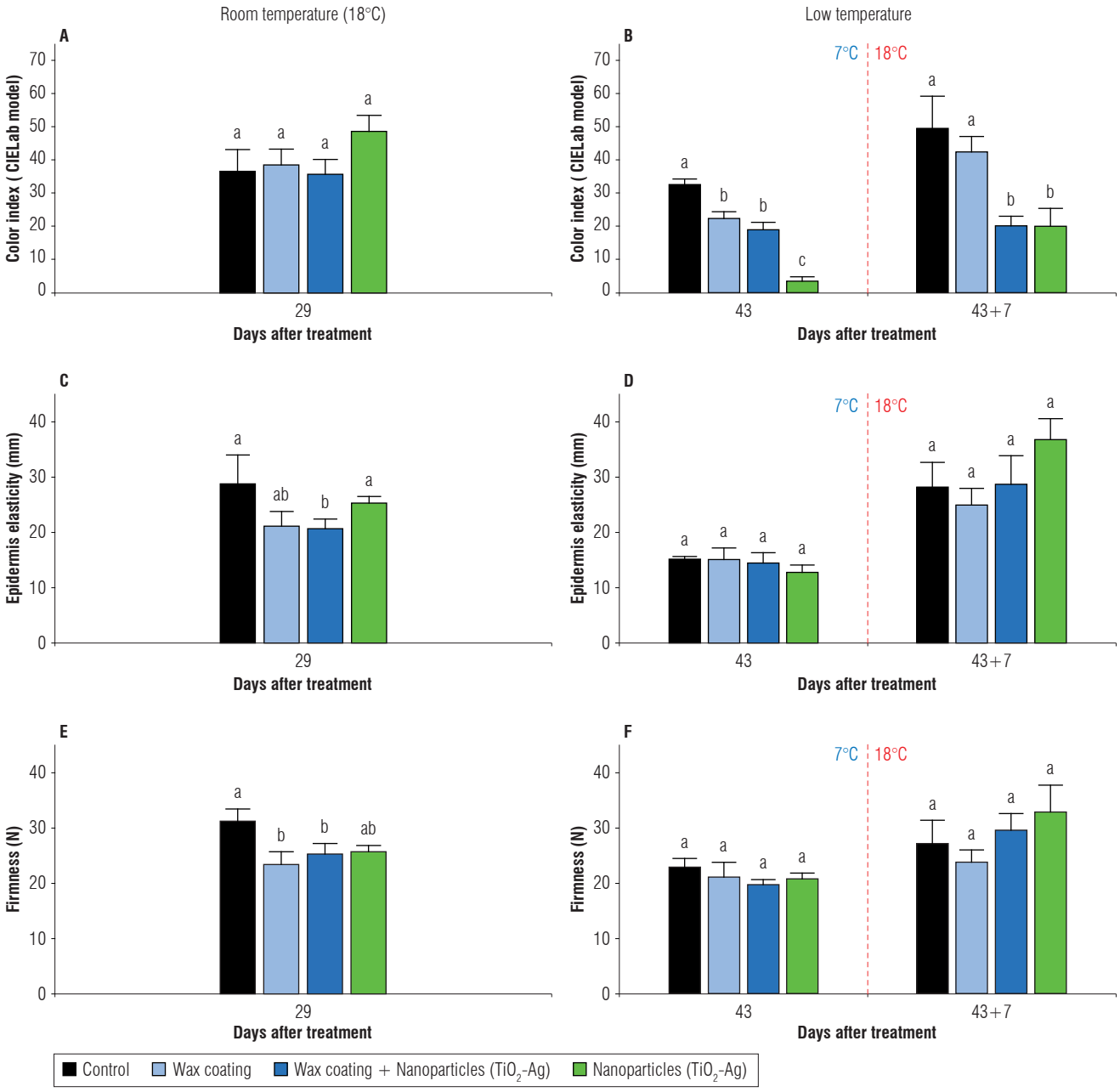

FIGURE 2. Effect of the application of wax and Ag-TiO_2-NPs on fruit color index, elasticity, and firmness during room temperature storage (A, C, and E) and refrigerated conditions of purple passion fruits stored at 7°C for 43 d and then transferred to 18°C for 7 d (B, D, and F). Repetitive measures analysis was performed as a function of time. Different letters indicate significant differences according to the LSD test (P<0.05). The vertical bars represent the standard error (n=4). Control: fruits without wax or Ag-TiO_2-NPs application.

and less in the fruits with wax + nanoparticles (Fig. 2C). Elasticity increased from the end of refrigerated storage to the end of shelf life. Still, there were no significant differences between treatments in either sampling (Fig. 2D). The control fruits had a higher ($P<0.05$) firmness at the end of room temperature storage (Fig. 2E). From the end of refrigerated storage to the end of shelf life there was an increase in firmness. Still, the treatments were statistically similar in both cases (Fig. 2F).

Biochemical parameters

In-room temperature storage, all fruits had similar acidity values (Fig. 3A). A similar situation was observed at the end of refrigerated storage. In contrast, at the end of shelf life, there were statistical differences when the highest acidity was achieved in fruits treated only with nanoparticles and the lowest value in fruits with waxing only (Fig. 3B).

There were no significant differences in treatments under room temperature conditions (Fig. 3C) for total soluble solids (TSS); however, at the end of refrigeration, there were differences ($P<0.05$): the NPs treatment showed the lowest TSS value, and the opposite occurred in the control fruits. For shelf life, there was a decrease in TSS in all treatments, but there were no significant differences between them (Fig. 3D). Under room temperature conditions, the fruit ripening process was rapid, so much so that the evaluated technologies apparently did not affect carbohydrate metabolism and, consequently, TSS were not affected. This also indicates that these treatments do not negatively impact this quality parameter related to sensory perception.

In-room temperature conditions, statistical differences were observed ($P<0.05$). The most significant loss of electrolytes occurred in the control fruits. In contrast, waxed fruits were characterized by the lowest loss of electrolytes (Fig. 4A). In refrigeration, there were no statistical differences. A marked increase in shelf life was observed, with significant differences ($P<0.05$). The fruits with only nanoparticles had the most crucial loss of electrolytes (Fig. 4B).

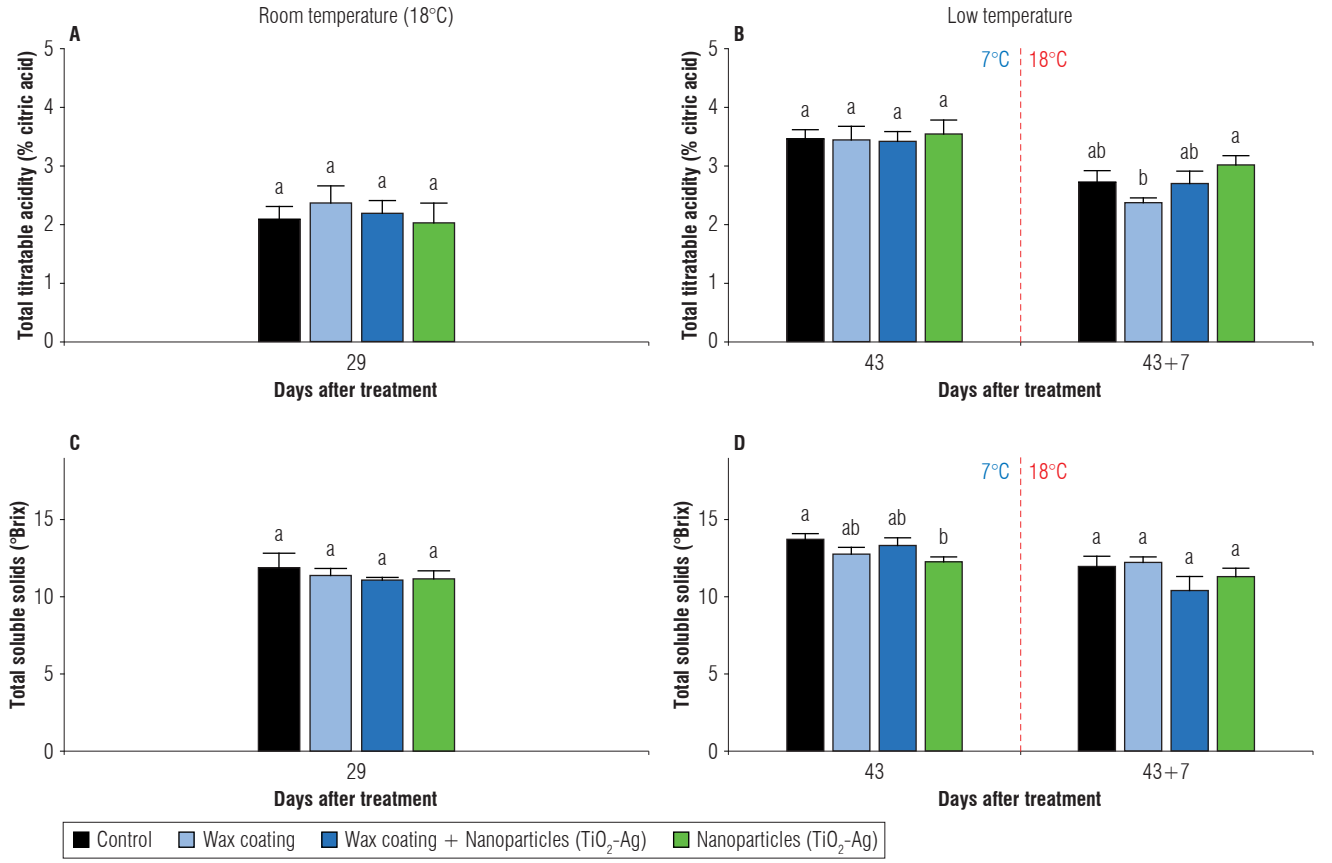

FIGURE 3. Effect of the application of wax and Ag-TiO_2-NPs on the titratable acidity and total soluble solids of purple passion fruits under room-temperature storage conditions (A and C) and refrigerated conditions of fruits stored at 7°C for 43 d and then transferred to 18°C for 7 d (B and D). Repeated measure analysis was performed as a function of time. Different letters indicate significant differences according to the LSD test ($P<0.05$). The vertical bars represent the standard error (n=4). Control: fruits without wax or Ag-TiO_2-NPs application.

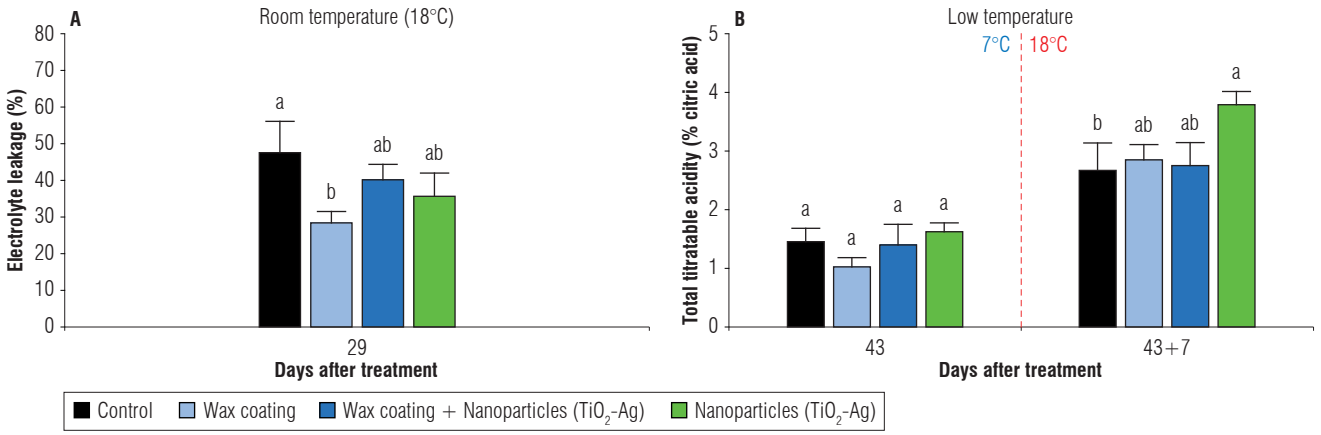

FIGURE 4. Effect of the application of wax and Ag-TiO_2-NPs on electrolyte loss from the epidermis of purple passion fruits under room temperature storage (A) and refrigerated conditions of fruits stored at 7°C for 43 d and then transferred to 18°C for 7 d (B). Repeated measure analysis was performed as a function of time. Different letters indicate significant differences according to the LSD test ($P<0.05$). The vertical bars represent the standard error (n=4). Control: fruits without wax or Ag-TiO_2-NPs application.

Physiological parameters

Under room temperature storage conditions, fruits showed increased respiration at 14 d, at which point climacteric occurred; there was a marked decrease until 21 d, and the fruits remained almost stable until the end of the experiment. The lowest respiration was obtained in fruits with nanoparticle application, and, curiously, the highest respiration was obtained with waxing (Fig. 5A). In refrigerated storage, respiration decreased progressively until 21 d, then began to increase until the end of storage and also

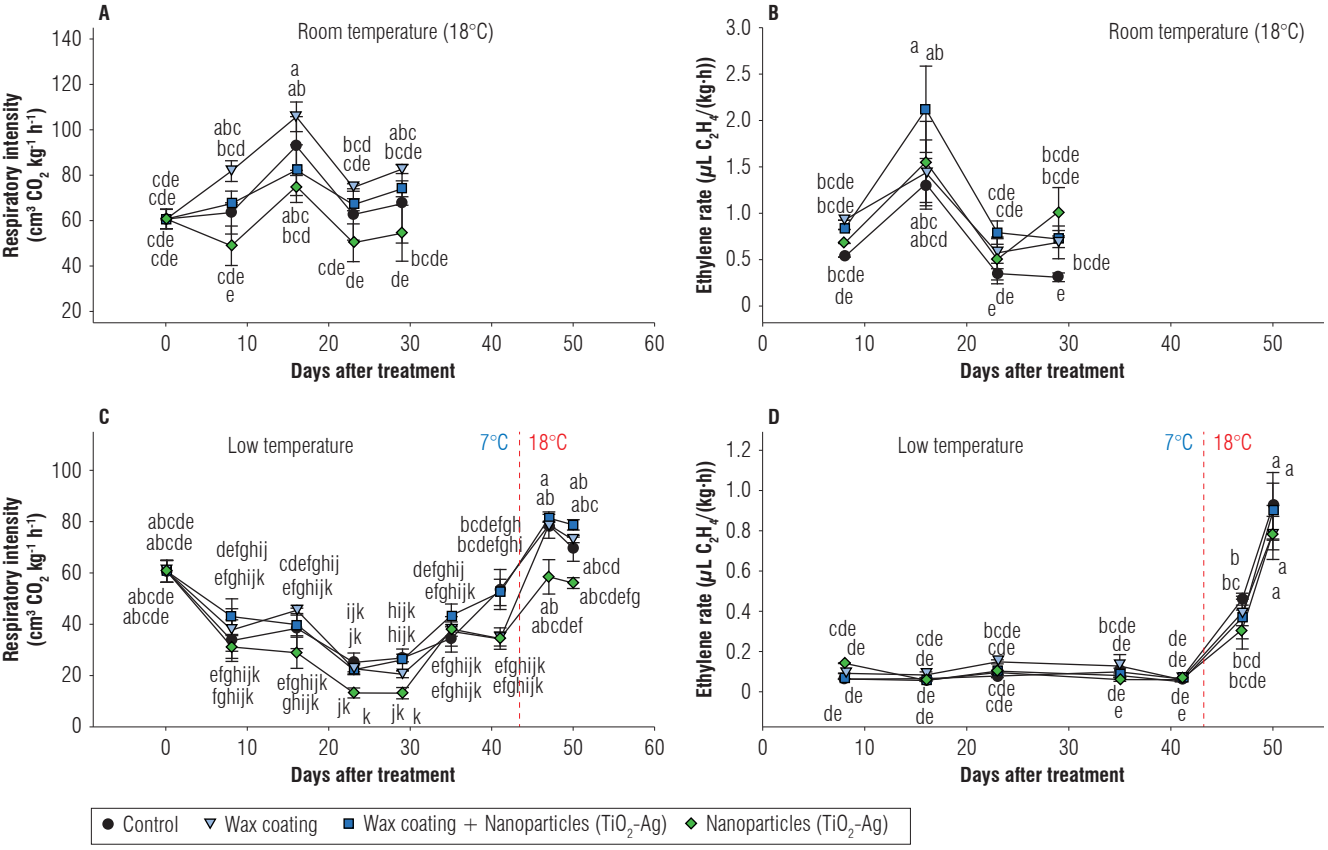

FIGURE 5. Effects of the application of wax and Ag-TiO2-NPs on the respiratory intensity and ethylene production rate of purple passion fruits during room temperature storage (A, B) and refrigerated conditions of fruits stored at 7°C for 43 d and then transferred to 18°C for 7 d (C, D). Repetitive measures analysis was performed as a function of time. Different letters indicate significant differences according to the LSD test ($P<0.05$). The vertical bars represent the standard error (n=4). Control: fruits without wax or Ag-TiO_2-NPs application.

increased during the shelf-life period. Here, it was also observed that the fruits with the lowest respiration were those treated with only nanoparticles. Respiration in the other fruits behaved similarly (Fig. 5C).

At room temperature, ethylene production increased markedly up to 18 d and then decreased. Fruits with nanoparticles and waxes had higher ethylene production during most of the experiment, while control fruits were characterized by the lowest ethylene values (Fig. 5B). Under refrigeration, ethylene remained stable as well as between treatments, but in the shelf-life period, there was a drastic increase but no differences between treatments (Fig. 5D).

Responses to sensorial analysis

The perception of purple passion fruit storaged at room temperature was mostly positive in all treatments (Fig. 6). However, there is a general lack of knowledge or low consumption of these fruits among the participants in the survey. Among the external physical characteristics of the control treatment, texture, coloration, and integrity stand out, especially of the epidermis, which is positively appreciated when smooth, shiny, and dark purple. The fruit flavor is described between acid and sweet notes with a gelatinous, soft, and smooth pulp texture similar to the same sensation that the consumption of sweet granadilla fruit gives. Another appreciated characteristic consists of the aroma of the fruits, which is considered fresh and pleasant with a citric touch (Fig. 6).

The appearance of the wax-coated fruits was more attractive as they were shiny, mostly smooth, with striking colors and aromas, and with an epidermis preserved in better conditions (Fig. 7), unlike treatments with nanoparticles, which showed a dull epidermis, mostly dehydrated, with a rough or powdery texture, and more significant visible damage, but without significantly affecting their flavor, internal texture or aroma (Fig. 7). The rough or uneven appearance of the fruit epidermis, the lightweight or low quantity of pulp, and the size of the fruit are the main undesirable characteristics when purchasing or consuming this product.

Discussion

Physical parameters of the fruits

During room temperature storage, purple passion fruits exhibited considerable weight loss with values exceeding 15% at the end of storage (Fig. 1A). This was likely due to the climacteric nature of the fruits and their high transpiration rate, given that water constitutes 90% of the fruit weight (Gemail *et al.*, 2023). Surprisingly, the use of nanoparticles led to more significant weight loss (>20%), possibly due to increased ethylene production, as observed in other fruits such as mangosteen (Thammachote *et al.*, 2023). This finding contrasts with results for loquats, where nanoparticle coatings reduced weight loss (Ali *et al.*, 2020). In contrast, wax coatings consistently minimized water loss by reducing cuticular transpiration, particularly during shelf life (Fig. 1B) (Devi *et al.*, 2022; Zhou *et al.*, 2022). Gemail *et al.* (2023) also report the effectiveness of combining wax and Ag-TiO_2-NPs in reducing weight loss in refrigerated mandarins. These conflicting results highlight the need for further research on the role of nanoparticles in managing postharvest water loss, as their effects may vary depending on the fruit species and storage conditions.

The color of the fruit epidermis, a critical indicator of ripening and quality, was also affected by nanoparticle and wax treatments. Nanoparticles, especially during low-temperature storage, delayed color changes, and the CI was approximately 4 (Fig. 2B). This result was probably because of inhibiting flavonoid biosynthesis, which influences the color of the purple fruit epidermis (Xin *et al.*, 2021). This finding is consistent with the results for mangosteen, where nanoparticles delayed anthocyanin synthesis and color changes (Thammachote *et al.*, 2023). However, the rapid ripening process at room temperature may have obscured these effects, preventing noticeable color changes. Peel shrinkage, an essential quality indicator for purple passion fruit, was significantly reduced in wax-treated fruits in correlation with increased epidermis elasticity and improved resistance to penetration (Zhou *et al.*, 2022). This observation explains the differences in the firmness of the fruits under ambient conditions, where wax treatments offered superior physical protection.

In contrast, nanoparticles had limited impacts on these physical attributes under refrigeration, suggesting that their primary action could be biochemical. This contrasts with studies on mandarins and mangoes, where nanoparticle treatments improved fruit firmness under refrigerated and ambient conditions (Chi *et al.*, 2019; Gemail *et al.*, 2023). This discrepancy underscores the need for refinement of nanoparticle and wax applications to optimize postharvest physical properties in purple passion fruit with particular attention to species-specific and environmental factors.

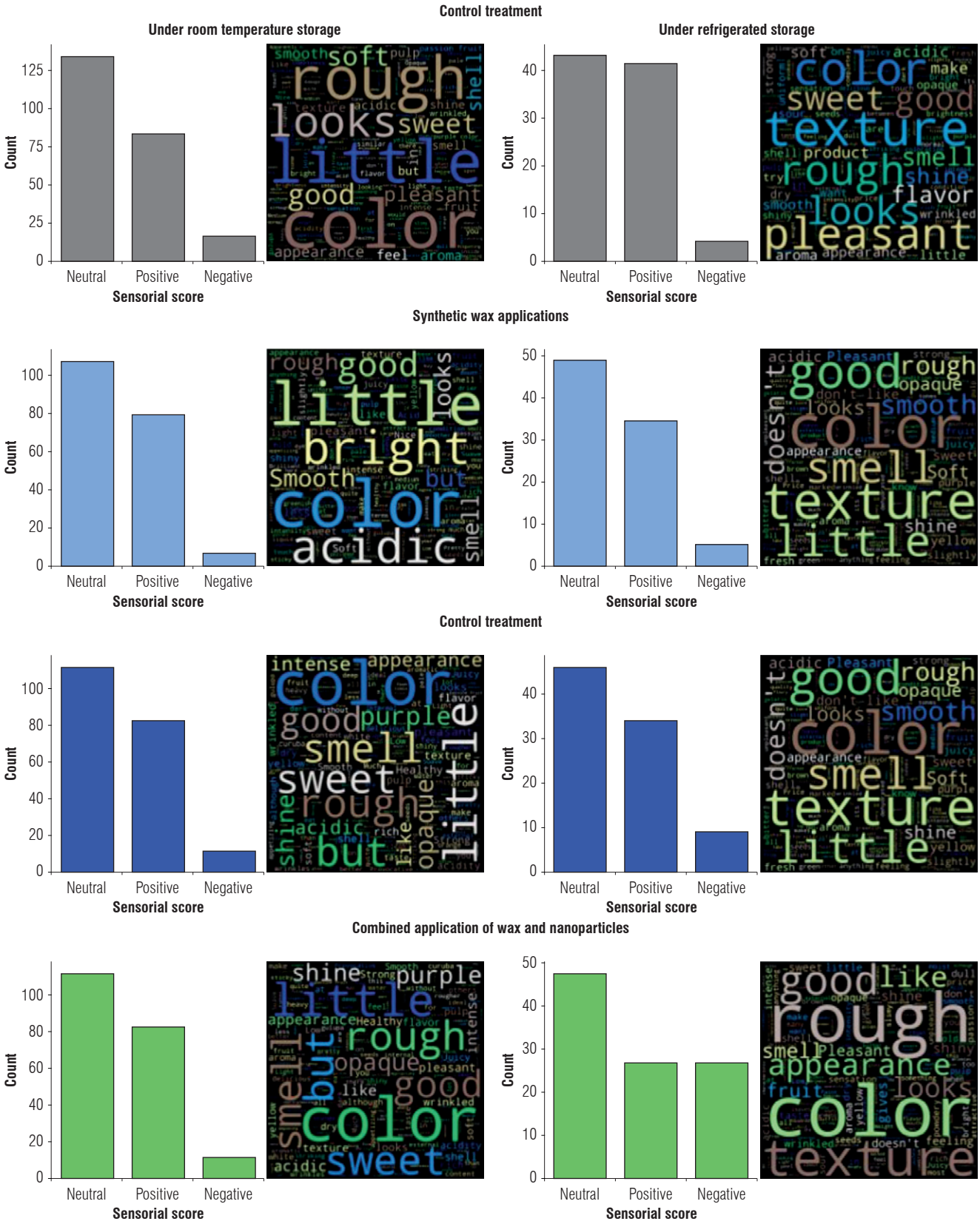

FIGURE 6. Sensory analysis corresponding to purple passion fruits with an application of wax and Ag-TiO_2-NPs and under room-temperature storage (left) and refrigerated storage (right) at the end of the shelf-life period, with their respective text analysis (word cloud). Control: fruits without wax or Ag-TiO_2-NPs application.

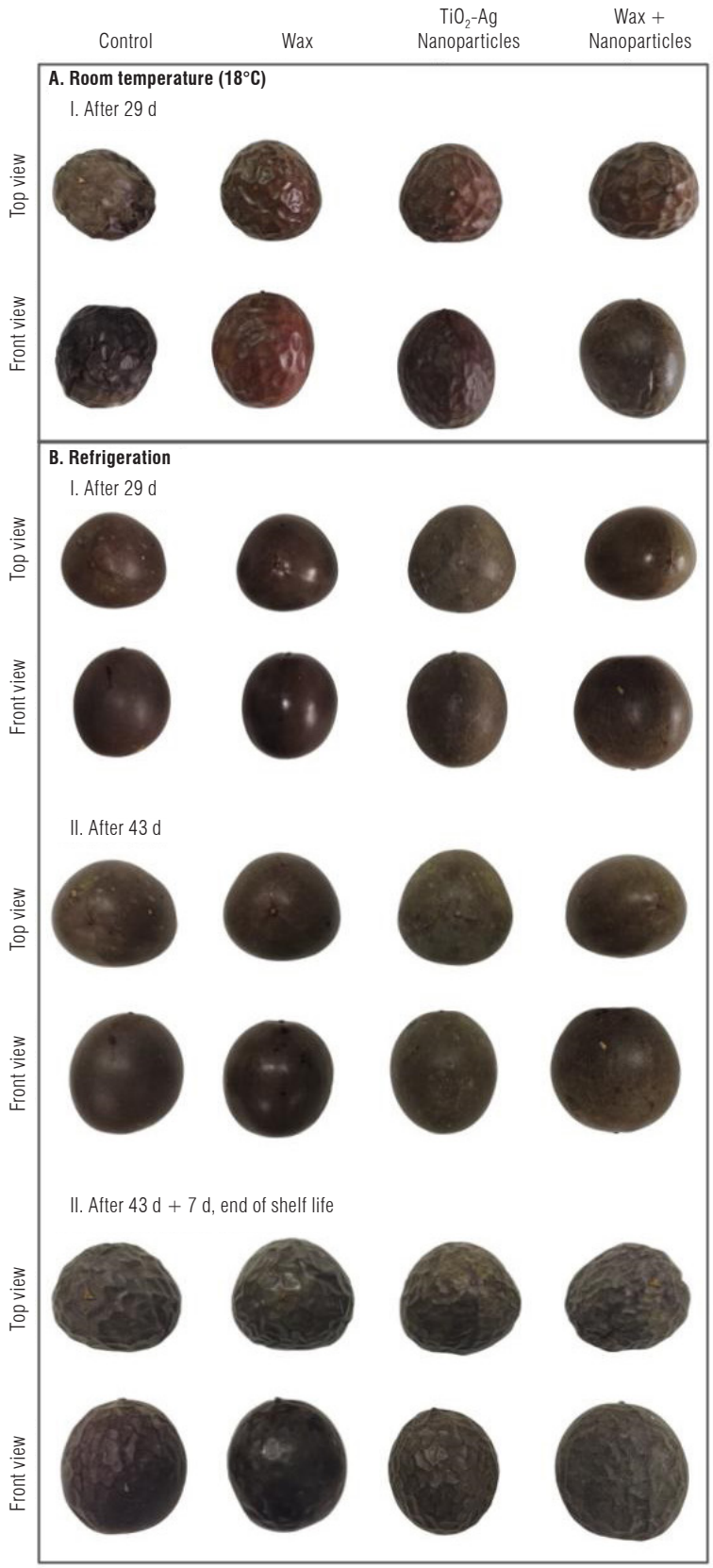

FIGURE 7. Epidermis of purple passion fruits: A) at room temperature at 18°C and B) refrigeration at 7°C with wax and Ag-TiO_2-NPs. Control: fruits without wax or Ag-TiO_2-NPs application.

Fruit biochemical parameters

The total titratable acidity (TTA) in the shelf life decreased by about 5% compared to the control (Fig. 3B). Our findings were consistent with previous studies on purple passion fruit, where the application of chitosan coatings fused with medicinal plant extracts led to a reduction in acidity (Nxumalo & Fawole, 2022). This observation aligns with similar results in loquat fruits, where coatings and Ag-NPs treatments also reduced TTA, potentially contributing to lower weight loss during shelf life (Ali *et al*., 2020). The lack of statistical differences in other conditions may be attributed to the effects of the modified atmospheric packaging on the ripening process, which could diminish the impact of different treatments.

The beneficial effects of NPs observed at the end of refrigeration were likely due to their role in reducing the respiration rate (Fig. 5). This decrease in respiration resulted in a slower increase in TSS (lower by approximately 10% for control) as a result of reduced hydrolysis of structural and reserve carbohydrates. For example, in mangoes, wax coatings, and Ag-NPs led to lower TSS than controls (Hmmam *et al*., 2021). Similarly, Ali *et al.* (2020) report a reduction in TSS in loquat fruits treated with NPs and coatings, which is attributed to the slower metabolic processes induced by these treatments.

Nxumalo and Fawole (2022) find that purple passion fruit coatings effectively prevent cell degradation and control electrolyte leaching by providing an additional protective layer. This finding was supported by our results with wax treatments, which maintained fruit quality under room-temperature storage conditions, with a value <30% of electrolyte leakage (Fig. 4). In contrast, Tavakoli *et al.* (2019) report that the application of Ag-NPs in lemon fruits control the leakage of electrolytes. This result differs from our findings in purple passion fruit, where NPs alone increased the leakage of electrolytes. Although NPs were associated with more significant weight loss, indicating possible membrane damage, they did not adversely affect quality parameters such as firmness, color, TSS, or acidity.

These results suggested that coatings and NPs can influence various postharvest attributes, but their effects are complex and vary depending on the specific fruit and storage conditions. More research is needed to refine these treatments and understand their mechanisms in different fruit species.

Fruit physiological parameters

The observed reduction in fruit respiration rate (a decrease of up to 55%, compared to the control) with the application of nanoparticles in both experiments may be attributed to the release of silver ions from Ag-NPs, which probably obstructed the Krebs cycle by disrupting intracellular ion flux and altering the respiratory electron transport system (Elatafi & Fang, 2022). In support of this, Ortiz-Duarte *et al.* (2019) reported that nanoparticles can inhibit enzyme activity in the respiratory chain. This finding is corroborated by studies of mangoes, where nanoparticles were shown to inhibit respiration during refrigerated storage and throughout shelf life (Kassem *et al*., 2022). Similarly, the application of Ag-NPs in mangosteen also significantly reduces respiration rates (Thammachote *et al*., 2023).

Our results indicate that neither nanoparticles nor wax coatings effectively inhibit ethylene production in purple passion fruit. In some cases, these treatments even led to a slight increase in ethylene levels (Fig. 5). This finding contradicts expectations based on their known roles in ethylene inhibition reported in mangoes and avocados (Kassem *et al*., 2022; Nevado-Velasquez *et al*., 2023). In the refrigerated storage experiment, the ineffectiveness of these treatments ($P>0.05$) may be attributed to the combined effects of low temperature and modified atmosphere packaging, which mimic the inhibitory effects on enzymatic activity related to ethylene synthesis.

These results suggest that while nanoparticles and wax coatings can affect respiration rates, their influence on ethylene production may be more complex and depend on specific storage conditions. More research is needed to elucidate these interactions and optimize the use of these technologies for the postharvest management of purple passion fruit.

Responses to sensorial analysis

The limited consumption of purple passion fruit, compared to more widely consumed varieties such as yellow passion fruit and sweet granadilla can be attributed to several factors. The lower commercial trajectory and limited market presence of purple passion fruit primarily contribute to its reduced availability and consumer familiarity (De Armas Costa *et al*., 2022; Franco *et al*., 2014; Gomes *et al*., 2021). Additionally, its distinctive physicochemical properties, acidity, and unique flavor profile further restrict its appeal. Many consumers in Colombia are accustomed to the milder flavors of yellow passion fruit or the sweeter taste of sweet granadilla, positioning purple passion fruit as a niche product.

Consumer perception is critical in the potential expansion of the purple passion fruit market domestically and internationally. Sensory attributes such as taste, aroma, and

appearance influence consumer preference. In this study, the application of wax coatings (application of 50% diluted wax coating) and nanoparticles (400 mg kg^{-1} TiO_2 with 0.75% Ag) positively impacted the visual appeal of the fruits by providing a bright and glossy finish. However, these treatments did not significantly affect the taste or aroma of the fruits, which are key factors in consumer acceptance.

Applying coatings and nanoparticles significantly improves the fruit external appearance and shelf life, including attributes such as color retention and reduced weight loss. However, these interventions have limited influence on enhancing the sensory qualities of taste and aroma, critical factors influencing consumer preferences and purchase decisions. To achieve successful market expansion for purple passion fruits, it is essential to focus on external quality improvements and invest in strategies to enhance its organoleptic properties. These include optimizing sweetness, acidity balance, and aromatic profile through cultivation practices, postharvest handling, and product development. Additionally, highlighting the fruit nutritional benefits and versatility in marketing campaigns can further boost its appeal and acceptance among consumers.

Our study did not evaluate the potential impact of nanoparticles on human health, as this falls beyond the scope of our research. This topic has generated extensive debate, yet current findings remain inconclusive. Future studies are expected to provide more substantial evidence to clarify the potential risks and implications (Chaud *et al*., 2021; Iavicoli *et al*., 2017).

Conclusions

These findings emphasize optimizing postharvest treatments for purple passion fruits, balancing consumer appeal with internal quality preservation. Wax coatings improve the fruit visual appearance but offer limited protection for key quality attributes, particularly under refrigeration. In contrast, Ag-TiO_2-NPs demonstrated superior effectiveness in maintaining fruit quality at 7°C by stabilizing key parameters such as respiratory rate, color, and acidity. However, both wax and nanoparticle treatments were less effective at room temperatures. Further research is needed to refine nanoparticle formulations, assess long-term safety, and explore commercial applications, with an emphasis on consumer education and a holistic approach to quality characterization.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author´s contributions

EDSS: conceptualization, research, writing - original draft, visualization, writing, and editing. HEBL: conceptualization, writing - original draft, and editing. JGRG: conceptualization, writing - original draft, and editing. All authors have read and approved the final version of the manuscript.

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The impact of vegetation cover on soil erosion in the drainage network of banana crop

El impacto de la cobertura vegetal en la erosión del suelo en la red de drenaje del cultivo de banano

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ABSTRACT

In Urabá (Colombia), precipitation generates high rates of soil erosion in banana drainage systems due to its intensity and frequency, as well as soil susceptibility resulting from exposure. One approach to mitigate this erosion is the use of a vegetation cover. The aim of this study was to determine the impact of vegetation cover on soil erosion rates in the drainage systems of a banana plantation. For this purpose, a comparison was made during the last quarter of 2022 between experimentally measured erosion rates (simulated in a greenhouse), observed erosion rates (using sedimentation boxes in field drainage channels), and potential estimation (using the USLE equation). In the greenhouse, bare soils presented higher losses at 38.16 t ha^{-1} year^{-1}, statistically differing from conventional management (CMT) and vegetation cover (VCT) treatments, which recorded values of 24.70 and 18.97 t ha^{-1} year^{-1}, respectively. A similar trend was observed in the field. Based on estimated erosion potential (USLE), no differences between treatments were identified, with CMT exhibiting the highest erosion potential at 96.47 t ha^{-1} year^{-1}. Additionally, other soil variables, such as slope and type of soil, influenced erosion susceptibility regardless of the kind of existing cover.

Key words: soil conservation, erosion estimation, *Musaceae*, soil loss.

RESUMEN

En Urabá (Colombia), la precipitación genera tasas de erosión del suelo altas en los sistemas de drenaje de banano debido a la intensidad, frecuencia y susceptibilidad del suelo resultante de la exposición. Una alternativa para mitigar esto es el uso de cobertura vegetal. El objetivo de este estudio fue determinar el impacto de dicha cobertura en las tasas de erosión del suelo en los sistemas de drenaje de una plantación de banano. Para ello, se realizó una comparación durante el último trimestre de 2022 entre las tasas de erosión medidas experimentalmente, simuladas en un invernadero, y las tasas de erosión observadas utilizando cajas de sedimentación en los canales de drenaje en campo, junto con una estimación potencial utilizando la ecuación USLE. En el invernadero, los suelos sin cobertura presentaron mayores pérdidas con 38,16 t ha^{-1} año^{-1}, diferenciándose estadísticamente de los tratamientos de manejo convencional (TMC) y cobertura vegetal (TCV), que tuvieron valores de 24,70 y 18,97 t ha^{-1} año^{-1}, respectivamente. Esta tendencia se observó de manera similar en el campo. Con el potencial erosivo estimado (USLE), no se identificaron diferencias entre los tratamientos, siendo el TMC el que mostró el mayor potencial erosivo con 96,47 t ha^{-1} año^{-1}. Notablemente, otras variables del suelo como la pendiente del terreno y el tipo de suelo influyen en la susceptibilidad a la erosión, independientemente del tipo de cobertura existente.

Palabras clave: conservación de suelo, estimativa de erosión, *Musaceae,* pérdida de suelo.

Introduction

Banana is a crop that exhibits high sensitivity to excess water (Mohd *et al*., 2021; Teoh *et al*., 2022). It is estimated that the optimal depth of the water table in soils where bananas are planted should be 1 m or deeper (Durango *et al*., 2020; Gutiérrez & Romero, 2010). The fluctuation of the water table levels near the surface or depths less than 1 m generates hypoxia or anoxia in the roots, producing root rot, which adversely affects crop yields and productivity (Moreno *et al*., 2020). In that sense, it is necessary to have efficient drainage systems which allow water tables to be maintained at appropriate depths and functioning correctly. Poorly designed and constructed drainage networks favor sedimentation or generate damages to its structure, destabilizing slopes, which causes soil movements, clogging of drainage areas, and susceptibility to erosion (Bai & Cui, 2021; Durango *et al*., 2020; Salazar, 2010).

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Water-driven erosion is one of the most critical soil degradation factors due to the displacement of particles, leaching of nutrients and organic matter, leading to a loss of soil fertility (Delgado-Bejarano *et al*., 2023; Tessema *et al*., 2020). For this reason, models have been developed to estimate water erosion, such as the universal soil loss equation (USLE) proposed by Wischmeier and Smith (1978). This equation has been used for decades and has been revised in the RUSLE version (Renard *et al*., 1991), which predicts the average annual erosivity rates of an area based on precipitation (R), soil type (K), topography (LS), vegetation cover (C), and management activities (P). Among these factors, vegetation cover shows the most variability, probably due to human activity and climate variations (Zhao *et al*., 2020).

In the case of Urabá, Antioquia department (Colombia), precipitation causes the highest erosion rates in the drainage system because of its intensity and frequency (Amellah & el Morabiti, 2021; Bai & Cui, 2021). An alternative to mitigate this adverse condition involves the use of vegetation covers on the slopes. These covers decrease the erosive process by creating a barrier effect between water droplets and the soil slope. Additionally, they promote soil particle aggregation and improve some physical properties such as porosity, structure, infiltration, and cohesion (Bai & Cui, 2021; Durango *et al*., 2020; Salazar, 2010). Cunha *et al*. (2022) mention that the implementation of conservation practices, such as well-covered grasslands, allowed for a reduction in eroded soil from 10.2 million t to 4 million t between 1986 and 2016, highlighting the importance of ground cover in reducing erosion in drainage areas such as watersheds.

This study aimed to assess the impact of vegetation covers on erosion rates in the drainage systems of banana plantation with simulations in greenhouse, field channels, and USLE.

Materials and methods

Description of study area

The research was conducted in both greenhouse and field settings. The greenhouse is located in the residential complex "Los Almendros" at km 4 on the Carepa – Apartadó road, in the municipality of Carepa, Antioquia (Colombia) (Fig. 1). The fieldwork was conducted at the Ramiro Jaramillo Sossa experimental farm located 2 km northwest of the urban center of the municipality of Carepa. Both locations belong to the Banana Research Center (Cenibanano), which is associated with the Colombian Banana Growers Association (Augura). The two experiments (field and greenhouse conditions) were carried out simultaneously and lasted three months from September to December 2022. The agroclimatic conditions of the experimental farm include average annual precipitation of 2961 mm, average temperature 27°C, altitude of 20 m a.s.l., and typical conditions of the tropical rainforest (bh-T) (Instituto de Hidrología Meteorología y Estudios Ambientales, 2022; Jaramillo, 2014).

Stage I. Greenhouse conditions

Nine sedimentation boxes were prepared using plastic containers measuring 34, 28, and 20 cm in length (l_{cs}), width (w_{cs}) and depth (h_{cs}), respectively. Each box was filled with 17 kg of soil obtained from the experimental field area, clay loam in texture, classified as Fluvaquentic Eutrudepts. The weed species *Peperomia pellucida, Selaginella* sp. and *Digitaria horizontalis*, commonly found in banana crops, were established in a mixture from rooted cuttings in three trays (three replicates). These were watered weekly until complete coverage of the trays was achieved (VCT). In three other trays, the same weed species were established, but conventional pruning was carried out (CMT). The remaining trays were left with bare soil without plants (BST).

A wooden structure was designed to support and arrange each of the trays at a 45° angle to the surface, simulating the average slope of the tertiary channels under field conditions (Fig. 2). They were randomly distributed in the section set up for the greenhouse experiment. A 3 L plastic container was attached to the underside of each box, which acted as a collector. The most frequently occurring precipitation event recorded in the banana region from 2019 to 2021 was 10 mm. This value was used in the experiment, with 10 mm of water applied to each tray weekly for eight consecutive weeks. The excess of water from each tray was recovered and stored (runoff and leachate) to determine the total volume (V_T) for the evaluation period. A subsample of 100 ml (V_s) was then taken and dried in an oven at 60°C until reaching constant weight. Once this condition was reached, the accumulated soil in the subsample (m_s) was weighed and the total soil removed from the tray during the evaluation period (m_T) was determined using Equation 1:

$$m_T = \frac{V_T * m_s}{V_s} \quad (1)$$

Stage II. Field conditions

The drainage system structure in the experimental field consisted of tertiary level drains designed to facilitate water exit from the soil profile; secondary drains removed water from the plots, while primary drains water removed from

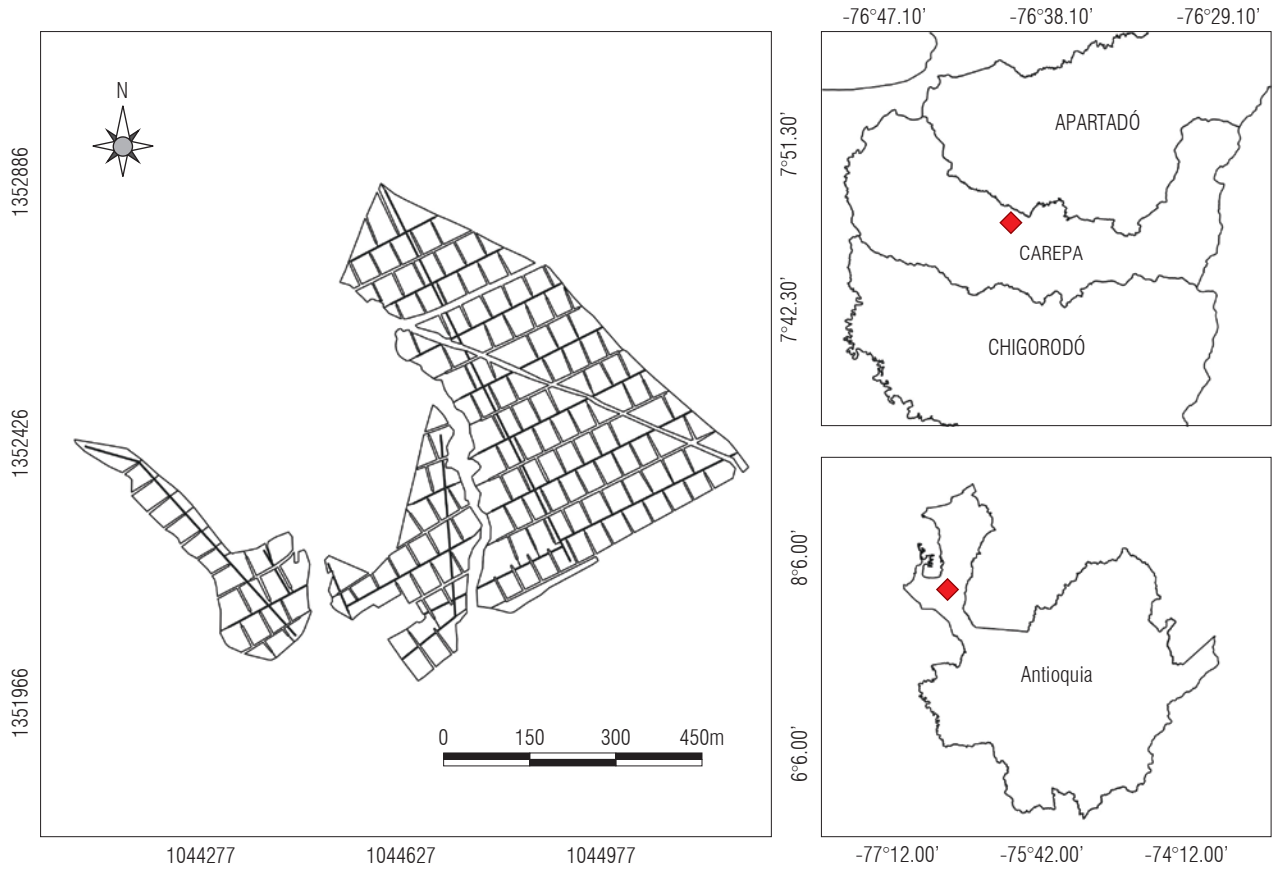

FIGURE 1. Location of the study zone in the Antioquia department (Colombia), WGS 84 Magna Sirgas Colombia West Zone (CRS:3115).

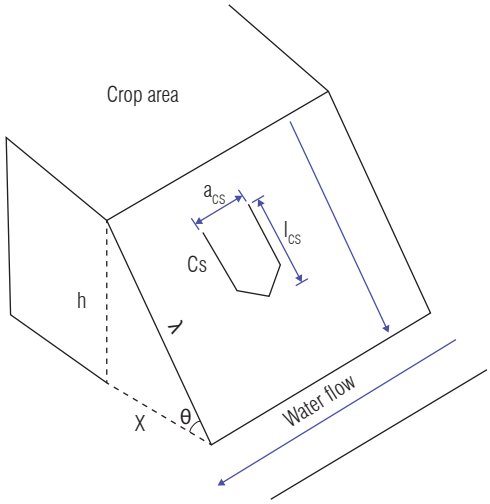

FIGURE 2. Sketch of a tertiary drainage, where y: drainage depth; x: horizontal distance relative to height; h: drainage slope; Cs: sediment collection and delimitation box; l_{cs}: box length; a_{cs}: box width; λ: slope length.

the farm. In plots 4, 9, and 10, a tertiary drain was selected and three sedimentation boxes of 50 cm by 30 cm long (l_{cs}) and width (a_{cs}) respectively were installed in the central part of these plots. The top of the box was left open to allow runoff water collected along the length of slope (λ) to enter (Fig. 2). In each box, one of the treatments described above (VCT, CMT, BST) was randomly installed.

At the beginning of the measurements, a 20 L capacity plastic container was attached to the bottom of each sedimentation box. Each week, percolated water was collected, its volume (V_T) determined, and a 50 ml subsample (V_s) extracted. Using the same methodology as in Stage I, the eroded soil was determined.

Variables

Precipitation. The precipitation data were obtained by a pluviometer located in the experimental field, which had daily records. For the study, data for the period from 2019 to 2021 were analyzed.

Modified photochemical reflectance index (MPRI). An image with a RGB (Red, Green, Blue) camera was captured over the slope of each of the treatments (Pacheco & Montilla, 2021). The sedimentation plots in the image were manually delineated, and the degree of vegetation cover was determined using the MPRI, which relates the green

(G) and red (R) bands as shown in Equation 2 (Delgado-Bejarano *et al*., 2023; Pacheco & Montilla, 2021):

$$MPRI=\frac{G-R}{G+R} \quad (2)$$

Slope (m_{se}) and *length* (λ). The inclination angle of the slope was estimated by measuring the channel depth (h) and the slope length (λ) using tape measures. The slope angle (θ) was obtained by Equation 3:

$$\theta=\sin^{-1}\left(\frac{h}{\lambda}\right) \quad (3)$$

The length (L_{se}) of the catchment area along the slope was determined as the distance between the top of the channel and the lower boundary of the sedimentation box.

Soil organic matter content. At the beginning of the experiment, soil sampling was carried out at the point adjacent to each sedimentation box, and the organic matter content of the soil was determined in the laboratory using the method of Walkley and Black (1934).

Soil permeability. The permeability was determined in each sedimentation box in the area adjacent to the soil slope by evaluating the hydraulic conductivity of the soil using Zhang's (1997) method with a mini disc infiltrometer.

Soil texture. This parameter was determined in the laboratory by the hydrometer method (Bouyoucos), finding the values of each soil fraction (clay, silt, and sand) (Jaramillo, 2014). For the greenhouse samples, soil used in the traps was analyzed, while in the field, a soil sample was taken from each trap in the area adjacent to the study channel.

Analysis by the Universal soil loss equation (A)

The Universal soil loss equation (USLE) was used to estimate soil erosion for each treatment (A, t ha^{-1} $year^{-1}$) (Eq. 4). The USLE incorporates parameters including vegetation cover (C, dimensionless), slope gradient (S, dimensionless), slope length (L, dimensionless), rainfall erosivity (R, MJ mm ha^{-1} h^{-1} $year^{-1}$), soil erodibility (K, t h MJ^{-1} mm^{-1}) and management practices (P, dimensionless):

$$A = C*LS*R*K*P \quad (4)$$

The vegetation cover factor was calculated using the vegetation index (MPRI). The parameters α and β of the equation were assigned values of 2 and 1, respectively (Eq. 5) (Almagro *et al*., 2019; Amellah & el Morabiti, 2021; Bai & Cui, 2021; Delgado- Bejarano *et al*., 2023):

$$C = e^{\left(-\alpha*\frac{MPRI}{(\beta-MPRI)}\right)} \quad (5)$$

The topographic factor was represented by the length (L) and the slope gradient (S) estimated by using equations 6, 7, and 8, which are based on the parameters m_{se} and λ, which correspond to the slope exponent and the horizontal projection of the slope, respectively. In equation 6, the parameter m_{se} takes values of 0.2, 0.3, 0.4, and 0.5 when the slope is less than 1%, between 1 and 3%, between 3 and 4.5%, or greater than 4.5%, respectively. The slope gradient (S) is a function of the slope angle in degrees (θ), which varies depending on whether this value in % (θ) is less than or greater than 9% (Wijesundara *et al*., 2018; Wischmeier & Smith, 1978).

$$L=\left(\frac{\lambda}{22.13}\right)^{m_{se}} \quad (6)$$

$$S=10.8 \sin\theta +0.03 \; ; \; \theta<9\% \quad (7)$$

$$S=16.8 \sin\theta -0.05 \; ; \; \theta\geq 9\% \quad (8)$$

The erosion caused by rain (R) was calculated as illustrated in equation 9, where P_{p_i} refers to the precipitation of the i-th month (mm), P_j to the annual precipitation of the j-th year (mm), based on the number of years in the time series n_j (Bai & Cui, 2021; Han *et al*., 2021).

$$R=\frac{\sum_1^{n_j}\left(\sum_1^{12} 1.735*10^{\left(1.5*\left(\frac{Pp_i^2}{Pj}\right)-0.8188\right)}\right)}{n_j} \quad (9)$$

The soil erodibility factor (K) refers to the effect of soil properties on its susceptibility to being eroded or transported due to the impact of raindrops. Equation 10 was used to calculate this parameter (Efthimiou *et al.,* 2020*;* Mahapatra *et al*., 2018; Wischmeier & Smith, 1978), considering soil properties of organic matter content (OM), permeability (p), structure (s), and particle size ratio (M) using Equation 11:

$$K=\left[\frac{2.1*10^{-4}(12-OM)*M^{1.14}+3.25(s-2)+2.5(p-3)}{100}\right]*0.1317 \quad (10)$$

$$M=(Silt(\%)+Sand(\%))*\left(100-Clay(\%)\right) \quad (11)$$

The p parameter was determined on a scale of 1 to 6 based on the classification of the United States Department of Agriculture (1999). For the variable s, the classification given by Wischmeier and Smith (1978) was used.

Finally, the P factor indicates the management practices that can be implemented to reduce the amount of soil transported or eroded, ranging from 0 to 1, where 0 indicates that the activity completely reduces erosion, and 1 indicates

that no conservation practice is done. Wischmeier and Smith (1978) highlighted key conservation practices, such as contour plowing, terracing, and no tillage systems. In the case of drainage networks, no conservation practices are applied, so the factor P is equal to 1.

Statistical analysis

The erosion rates directly measured through sedimentation tanks under both greenhouse and field conditions, as well as those estimated through the EEP, were considered as response variables in each case. These were analyzed based on the treatments (types of coverage) using an analysis of variance test (ANOVA). The normality of the residuals of the model and the homoscedasticity between treatments were evaluated. If the assumptions were not met, the Kruskal-Wallis non-parametric test was applied, followed by a post-hoc LSD test or Tukey test. The correlation between the parameters of the EEP with the erosive potential was determined using a Spearman correlation coefficient.

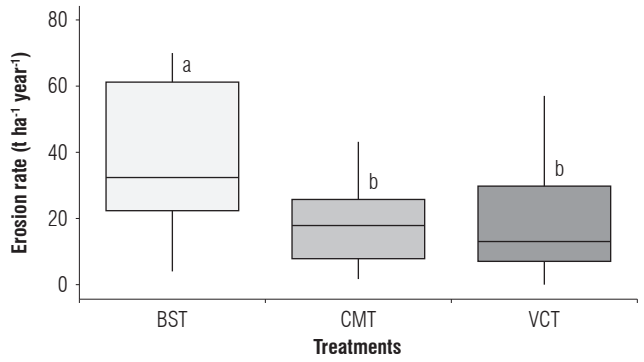

FIGURE 3. Distribution of soil erosion under greenhouse conditions. BST: Bare soil; CMT: Conventional management; VCT: vegetation cover. Different letters denote significant differences according to the LSD test ($P \leq 0.05$).

Results and discussion

Greenhouse conditions

The greenhouse evaluations revealed significant differences in soil erosion rates among the different types of vegetation cover (P=0.00533, Fig. 3).

As shown in Figure 3, BST exhibited the highest erosion rate compared to the other treatments, with a mean of 38.16 t ha^{-1} year^{-1}. This value is qualified as intermediate erosion according to the classification given by Han *et al.* (2021). This higher erosion rate is expected because the soil is completely exposed to the impact of irrigation drops and the particle transport by runoff. CMT recorded an average erosion rate of 24.70 t ha^{-1} year^{-1}. The VCT recorded the lowest average erosion values (18.97 t ha^{-1} year^{-1}). However, there were no significant differences between these two. These results show that there is a significant decrease in soil erosion with the presence of vegetation cover. However, there was no effect due to the cover management (*e.g.*, pruning). These results are consistent with those found by Bai and Cui (2021) in China, where bare soil had higher erosion rates compared to soils covered with corn and soybean crops, the latter presenting lower erosion values. These results show the importance of the barrier effect provided by soil vegetation covers and are in accordance with the coverage factor of the greenhouse experiment. In Figure 4, the BST presents a value of one (1) in the C factor, indicating that the soil is completely exposed to erosive processes. For the CMT, the C factor ranged between 0.88 and 1, showing a low level of soil protection, while the VCT presents the lowest values of C factor (0.55-0.63), indicating a greater potential of protection against erosion, although no significant differences between the latter two treatments were observed.

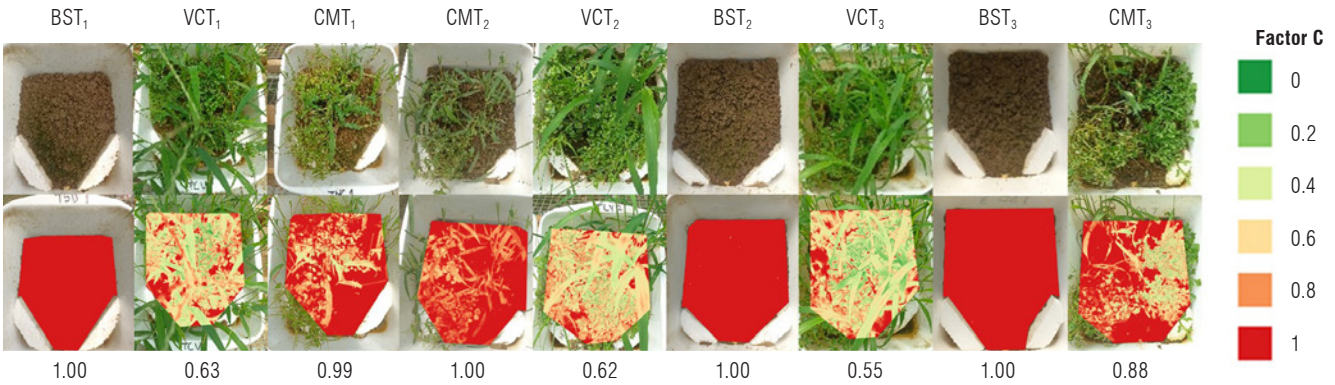

FIGURE 4. Protective effect of soil covers. BST: Bare soil; CMT: conventional management; VCT: vegetation cover. Factor C represents the effect of vegetation cover on soil erosion, where 0 indicates complete protection, and 1 indicates no protection.

Field evaluations

The accumulated weekly precipitation is shown for the evaluation period (weeks 38 to 50 of 2022). This period coincided with the rainy season that occurs in the region at the end of the year. Consequently, precipitation events were observed every week during the field experiment (Fig. 5).

Sedimentation boxes. During the field evaluations, statistical differences were found among the treatments (P=0.02126). The treatments exhibited the following order of erosion rates: BST > CMT > VCT. The mean erosion rate of BST was classified as high (50-80 t ha^{-1} year^{-1}), while those measured in VCT and CMT did not present significant differences and were classified as medium rates (25-50 t ha^{-1} year^{-1}) based on the classification by Han *et al.* (2021).

The field evaluations showed the same behavior seen in the greenhouse experiment (Stage I); there was a significant decrease in soil erosion rates with vegetation cover, but no statistical difference was found in the type of management given to the covers. These findings are in accordance with those of Chen *et al.* (2019) in China, who reported an exponential decrease in erosive rates with increasing cover crop percentages, explaining the lower average values of VCT during the evaluations. Without active management, cover crops exhibit higher leaf density which provides greater barrier effects. Huerta-Olague *et al.* (2018) also mention that the covers generally tend to decrease the runoff and erosion rates. In Mexico with four cover crops, these authors found an exponential decrease in soil erosion with all of them. Additionally, they mention that the plant structure plays a crucial role in soil protection, with denser crops demonstrating greater abilities to protect the soil against erosive agents. The effect of covers in reducing erosion rates has been reported by various authors (Beniaich *et al.*, 2023; Hou *et al.*, 2020; Koirala *et al.*, 2019; Xu *et al.*, 2019; Zhao *et al.*, 2020). Although there are no significant differences between VCT and CMT, channels with VCT management may present negative issues in operation, as these can obstruct water flow and decrease the speed at which water moves through the channel (Bond *et al.*, 2020; Salazar, 2010; Tang *et al.*, 2023; Zhao *et al.*, 2019). This situation can lead to adverse effects, especially during periods of heavy rainfall when the channels must function properly to prevent flooding and damage to plantations. Therefore, proper maintenance is recommended, including pruning and weed removal, to reduce soil erosion and preserve channel functionality.

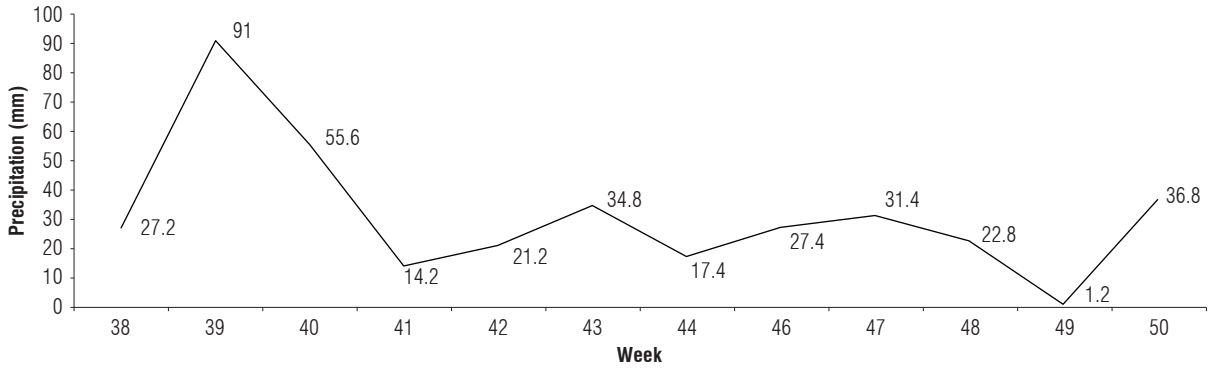

FIGURE 5. Accumulated weekly precipitation during the weeks 38 to 50 of 2022 (September-December) corresponding to the field evaluation period.

TABLE 1. Soil texture and organic matter content in the evaluated channels.

Treatments	Replicate	Sand (%)	Silt (%)	Clay (%)	Texture	OM (%)
BST	1	18.56	39.49	41.95	C	2.47
CMT	1	27.91	36.04	36.05	CL	2.24
VCT	1	23.13	38.98	37.89	CL	3.07
BST	2	20.69	36.4	42.91	C	2.60
CMT	2	24.00	39.13	36.87	CL	2.19
VCT	2	28.62	41.74	29.64	CL	2.05
BST	3	17.84	37.63	44.53	C	2.63
CMT	3	23.24	44.39	32.37	CL	2.10
VCT	3	22.49	39.71	37.8	CL	2.13

OM (%): soil organic matter content; BST: Bare soil; CMT: conventional management; VCT: vegetation cover; C: clay; CL: clay loam.

Erosive potential with USLE

The soils in the study area had an A horizon between 0 and 7 cm; based on the classification of USDA (2018), the soils were categorized as degradation class 2, which indicates a loss between 25 to 75% of the thickness of the upper horizon of 20 cm, suggesting erosive processes in the area. Additionally, the soils exhibited a structure of fine to moderate sub-angular blocky aggregates, with the characteristics of texture and organic matter presented in Table 1.

Statistical differences in potential erosion values were found between the treatments (P=0.0317, Tab. 2). Based on the classification presented by Han *et al*. (2021), all treatments fell within the very high erosion risk (80-150 t ha^{-1} year^{-1}).

TABLE 2. Erosive potential of the evaluated soil.

Treatment	Erosion (t ha^{-1} year^{-1})
BST	93.11 ab
CMT	96.47 a
VCT	87.87 b

BST: Bare soil; CMT: conventional management; VCT: vegetation cover. Different letters denote significant differences according to the Tukey HSD ($P \leq 0.05$).

These results indicated that the CMT treatment had the highest erosion potential, followed by BST and, finally, VCT. This suggests that external conditions besides the presence of covers make the soil susceptible to erosion. A Spearman correlation revealed that the LS factor had a correlation value of 0.66 and K factor of 0.46, which indicates that these two factors cause higher susceptibility to soil erosion, regardless of the coverage management. Belayneh *et al*. (2019) mentioned that the erosion rates in the Gumara watershed have a high relationship with its slopes, where steeper slopes result in higher erosion values. Additionally, Han *et al*. (2021) found that higher erosion rates are attributed to heavy precipitation (R), erodibility (K), and soil slopes (LS). Koirala *et al*. (2019) also refer to the increase in erosion with increasing land slopes.

These findings highlight that any erosion control strategies should use a comprehensive approach, considering the implementation of vegetation covers and the soil and landscape factors that contribute to the erosive potential. Therefore, a successful conservation solution must be adapted to the specific conditions of the area, incorporating practices that enhance soil properties such as soil structure and infiltration rates.

Conclusions

The presence of vegetation covers on the slopes of the channels significantly reduces the soil erosion rates compared to bare soil. This shows the protective effect of vegetation covers and emphasizes the importance of the plant cover implementation to minimize soil particle transport and control erosion in drainage systems for banana crops. However, it is crucial to properly maintain these vegetation covers to preserve the functionality of the channels without obstructing the water flow, allowing the lowering of water table levels in banana crops, especially during heavy rainfall seasons.

In addition to the influence of vegetation covers on erosive rates, other soil parameters such as texture, organic matter content, permeability, and land slopes, in this case, channel slopes, determine the erosive potential of the area. Therefore, any erosion control strategy should incorporate these factors to develop sustainable conservation solutions, including beneficial plant coverages. Such strategies should not only reduce erosion but also improve soil properties.

Conflict of interest statement

The authors declare that there are no conflicts of interests regarding the publication of this article.

Author´s contributions

LDB and LMVA designed and conducted the experiments; LMVA collected the data; LDB and DCS analyzed the data; JJPZ, MAB, SZH, and RAVV supervised the project. LDB prepared the draft of the manuscript. All authors reviewed and approved the final version of the manuscript.

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Growth, chlorophyll content, and visual symptoms of noni (*Morinda citrifolia* L.) seedlings affected by macronutrient deficiency

Crecimiento, contenido de clorofila y síntomas visuales en plántulas de noni (*Morinda citrifolia* L.) afectadas por deficiencia de macronutrientes

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ABSTRACT

Morinda citrifolia L. is a plant utilized for its food and medicinal benefits. However, a lack of information on its nutrient requirement limits the yield potential of this crop for commercial cultivation. Therefore, the response of noni seedlings to the application of complete nutrient solution with or without omission of one macronutrient (to determine the most limiting one) was studied. The treatments consisted of 7 nutrient solutions (Complete Nutrient Solution (CNS) and CNS minus (-) each of N, P, K, Mg, Ca, and S) and four application volumes (0 ml (control), 100 ml, 200 ml, and 300 ml NS) arranged in a randomized complete block design with four replicates in a greenhouse. Data on stem height, stem diameter, number of leaves, leaf area, and visual nutrient deficiency symptoms were assessed at four weeks intervals, starting from the 4th week after transplanting (WAT) in sand culture. Total dry matter yields and leaf chlorophyll content were determined at 20 WAT. The seedlings treated with CNS presented the highest growth in terms of all the variables, with no deficiency symptoms, whereas seedlings treated with CNS-N had the least growth, chlorotic leaves, and a stunted appearance throughout the period of the study. Overall, the order of growth limitation in seedlings was as follows: -N>-Ca>-Mg>-P>-K>-S>CNS. The seedlings treated with 100 ml NS had the best performance as compared to the control and other treatments. These results indicated that N followed by Ca and Mg are the most limiting macronutrients for noni seedling development and are required in relatively small quantities.

Key words: mineral nutrition, deficiency symptoms, fruit crop, nitrogen.

RESUMEN

Morinda citrifolia L. es una planta utilizada por sus beneficios alimentarios y medicinales. Sin embargo, la falta de información sobre sus requerimientos de nutrientes limita el potencial de rendimiento de este cultivo para el cultivo comercial. Por lo tanto, se investigó la respuesta de las plántulas de noni a la aplicación de una solución nutritiva completa, con o sin la omisión de un macronutriente (para determinar el más limitante). Los tratamientos consistieron en 7 soluciones nutritivas (Solución Nutritiva Completa (CNS) y CNS menos (-) cada uno de N, P, K, Mg, Ca y S) y cuatro volúmenes de aplicación (0 (control), 100, 200 y 300 ml NS) dispuestos en un diseño de bloques completos al azar con cuatro repeticiones en invernadero. Los datos sobre altura y diámetro del tallo, número de hojas, área foliar y síntomas visuales de deficiencia de nutrientes se evaluaron en intervalos de cuatro semanas, a partir de la cuarta semana después del trasplante (SDT) en cultivo de arena. Los rendimientos totales de materia seca y el contenido de clorofila de las hojas se determinaron a las 20 SDT. Las plántulas tratadas con CNS tuvieron el mayor crecimiento en términos de todas las variables consideradas sin síntomas de deficiencia, mientras que las plántulas tratadas con CNS-N tuvieron el menor crecimiento, mostrando hojas cloróticas y apariencia atrofiada durante todo el período del estudio. En general, el orden de limitación del crecimiento en las plántulas fue: -N>-Ca>-Mg>-P>-K>-S>CNS. El rendimiento de las plántulas bajo la aplicación de 100 ml de NS fue el mejor en comparación con el control y otros tratamientos. Estos resultados indicaron que el N, seguido del Ca y Mg, son los macronutrientes más limitantes para el desarrollo de las plántulas de noni y se requieren en cantidades relativamente pequeñas.

Palabras clave: nutrición mineral, síntomas de deficiencia, frutal, nitrógeno.

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Introduction

Noni (*Morinda citrifolia* L.) is an evergreen fruit-producing shrub, valuable for many health benefits and derived products (Sadino *et al*., 2024). Its fruit juice is widely consumed as nutritional tonic, while noni leaves, stem, bark, roots and flowers are used for medicinal purposes owing to their therapeutic properties (Ali *et al*., 2016). The species belongs to the Rubiaceae family and is characterized by white tubular flowers, large, bright green glossy leaves and yellowish fruits (Monroy *et al*., 2021). The species tolerates different types of soil and can grow under variable climatic conditions (Natarajan *et al*., 2023; Souto *et al*., 2016; Yashaswini *et al*., 2014). However, the fruit production, fruit quality and the nutritional composition vary among the varieties or genotypes and are directly related to the environment as well as to the cultivation system (Arya *et al*., 2022; Basar & Westendorf, 2012; Deng *et al*., 2010).

In fruit species, the lack or deficiency of essential nutrients is among the factors which can limit growth during vegetative stage and affect subsequent fruit production (Iqbal *et al*., 2023). Soil supplies most of the essential elements required by plants for optimum growth (Njinga *et al*., 2013; Toor *et al*., 2021). The availability of these elements is affected by geographic location, weather conditions, terrain, and other factors (Singh & Schulze, 2015; Wang *et al*., 2002). Tropical regions are commonly characterized by abundant and frequent rainfall, intense vertical sunlight exposure, and consistent warm temperatures, which often leads to leaching of mineral nutrients from soil and high rates of organic matter decomposition (Payne & Edis, 2012; Taylor *et al*., 2017). Under such conditions, availability and supply of some nutrient elements becomes unreliable, thus, limiting the growth of plants, especially fruit crops such as noni, under intensive cultivation. Noni has been observed to display abnormal foliar symptoms, which could be due to depletion of mineral nutrients under continuous fruit production and prevailing conditions of the tropical soils (Honey *et al*., 2012; Nelson & Elevitch, 2006). Thus, improving production and crop yield requires an appropriate fertilizer application program.

Up until now, fertilizer applications in the form of organic, mineral, and biofertilizers have been reported for noni (Caione *et al*., 2018; Sahoo *et al*., 2017). However, due to lack of information on the nutritional requirements of the species, some applications were made based on the requirements of related species (*e.g.,* coffee), while others placed emphasized on K as one of the most abundant elements in noni fruit (Melo *et al*., 2021; Souto *et al*., 2018). The basic application of fertilizer to noni plants without considering the most limiting element disregards the law of the minimum, which relates the yield level to the nutrient in lowest (minimum) supply (Brown *et al*., 2022). Moreover, the rate at which crops utilize nutrients differ between inter and intra species (Adhikari *et al*., 2023; Morgan & Connolly, 2013). Hence, this creates an ample scope for the determination of the specific limiting nutrient for noni growth. Availability of such knowledge in combination with information on soil nutrient status would provide a basis for fertilizer recommendation for this plant. Therefore, the response of noni seedlings to the application of complete nutrient solution with or without omission of one macronutrient (to determine the most limiting one) was studied in the present research.

Materials and methods

Study site

This study was conducted in a glass greenhouse of the Biotechnology Department, Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo - State, Nigeria, located at longitudes 07°23'18"N to 07°23'43"N and latitudes 03°51'20"E to 03°23'43"E. The greenhouse was situated at 215 m a.s.l. The weather reports of the location were obtained from FRIN Meteorological station and indicated average maximum and minimum temperatures of 32.4°C and 23.6°C, respectively. The mean relative air humidity was 79.2%, while mean monthly rainfall was 114 mm during the period of the study from March to August, 2023.

Treatments and experimental design

This experiment consisted of 7×4 factorial treatment combinations. The first factor was the type of nutrient solution (NS): complete nutrient solution (CNS) with macro and micronutrients, CNS minus (–) each of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), and sulphur (S). The second factor was four volumes of nutrient solution: 0 (control), 100, 200, and 300 ml. These volumes per plant were applied twice a week. The control did not receive a nutrient solution, and 200 ml of distilled water per plant was applied in the control treatment. Application volumes varied in order to expose the plants to adequate nutrient concentrations, as there were no reports establishing a particular volume of Hoagland solution for the optimum growth of the noni seedlings. The resulting 28 treatments were laid out in a randomized complete block design with four replicates, where each replicate corresponded to one plant per pot.

Growth medium preparation

Sand culture and "minus one element technique" (MOET) were used for this experiment (Burns, 1992). The river sand used was collected from the Asanmagbe riverbank in FRIN. The sand was sieved to remove particles larger than 4.5 mm, washed several times under flowing tap water until the water became clear, and then washed with distilled water. The sand was then leached with 2 N HCl solution containing 1% oxalic acid for 18 h and later washed with deionised water (Taxiarchou *et al*., 1997). It was sterilized at 100°C for 1 h in a hot air oven and was filled into polythene pots of 2 kg size, perforated at the base.

Preparation of the nutrient solutions

Stock solutions of the essential macronutrients were prepared, and the treatment solutions were made by adding the correct proportions of the required stock solutions according to Hoagland and Arnon (1938), as shown in Table 1.

Initial growth and transplanting of seedlings

Mature noni (*Morinda citrifolia* var. citrifolia) fruits were collected from the FRIN arboretum. The seeds were extracted and sundried. Seeds of similar size were sorted, clipped at the tip and propagated in leached, sterilized river-sand substrate at 100 seeds per basket. Seedlings began to emerge three weeks after sowing. Seedlings of uniform height at the four-leaf stage were carefully removed, their roots rinsed and transplanted into the growth media (sand) at one seedling per pot. The seedlings were watered with distilled water for the first week, followed by the application of 50% complete Hoagland solution during the second week to stabilize the plants. Subsequently, nutrient solutions according to each treatment were applied by the slop method, twice a week (McCall & Nagakawa, 1970). A plastic plate was placed under each pot to prevent outside root extension, while leachate was not reused.

Data collection

The biometric response of the plants in terms of stem height (cm), stem diameter (mm), number of leaves, and leaf area (cm^2) was assessed at four-week intervals, starting from the 4^{th} week after transplanting (WAT). Total dry matter (shoot + roots) (g/plant) and leaf chlorophyll content were determined at 20 WAT. The seedlings were carefully uprooted, the sand rinsed off the roots and then separated into leaves, stems and roots. The plant parts were then put in separate envelopes, labelled according to treatments and oven dried to constant weight at 70°C in an electric oven. The oven-dried weights of each of the plant parts were added together to obtain the total dry biomass. Nutrient deficiency symptoms in the plants were visually assessed along with the collection of biometric data. Stem height was determined with a meter rule. Stem diameter at the base was evaluated using a digital vernier calliper. Leaf area was measured with a portable leaf area meter (YMJ-B®, Hinotek), while the number of leaves was assessed by counting. Relative contents of chlorophyll (SPAD units) were measured in a third or fourth pair leaf from the top with a hand-held chlorophyll meter (TYS-B®, Hinotek).

Statistical analysis

The data collected were subjected to analysis of variance using GenStat (version 4), while the significantly different means were separated using the Duncan Multiple Range Test at 5% probability.

TABLE 1. Composition of the "minus one element culture" solution.

Stock solutions	Molarity (M)	Complete solution (ml L^{-1})	Stocks to be added to omit an element (ml L^{-1})					
			-N	-P	-K	-Ca	-Mg	-S
KNO_3	1	5	-	6	-	5	6	6
KH_2PO_4	1	1	-	-	-	1	1	1
$Ca(NO_3)_2 \cdot 4H_2O$	1	5	-	4	5	-	4	4
$MgSO_4 \cdot 7H_2O$	1	2	2	2	2	2	-	-
$CaSO_4 \cdot 2H_2O$	0.01	-	200	-	-	-	-	-
K_2SO_4	0.5	-	5	-	-	-	3	-
$Ca(H_2PO_4)_2 \cdot H_2O$	0.05	-	10	-	10	-	-	-
$Mg(NO_3)_2 \cdot 6H_2O$	1	-	-	-	-	-	-	2
Iron solution		1	+	+	+	+	+	+
Trace element solution		1	+	+	+	+	+	+

+ indicates presence and - indicates absence of a particular mineral element.

Ferric-sodium salt of EDTA (18.4 g L^{-1}) was used to replace Fe tartrate in the Hoagland solution. Trace element solution consisted of H_3BO_3 (2.86 g), $MnCl_2 \cdot 4H_2O$ (1.81 g), $ZnSO_4 \cdot 7H_2O$ (0.22 g), $CuSO_4 \cdot 5H_2O$ (0.08 g), and $H_2MoO_4 \cdot H_2O$ (0.02 g) in 1 L of distilled water.

Results

Plant height

The growth of noni seedlings, as affected by different nutrient solutions, indicated significant differences ($P \leq 0.05$) between the treatment means for plant height at 8 to 20 weeks after transplanting (WAT) (Fig. 1A). Moreover, results at 12, 16, and 20 WAT followed a similar pattern, except for the plants treated with CNS-Ca. Seedlings treated with complete nutrient solution CNS had the highest mean heights of 20.4, 34.7, and 43.0 cm at 12, 16, and 20 WAT, respectively, which were significantly taller than the seedlings in each of the other treatments at the respective growth periods. These were followed by seedlings treated with CNS-S, which were taller than seedlings of other treatments, except the CNS treatment, at the same periods. The plant height in the CNS-Ca treatment decreased gradually towards 20 WAT. The smallest heights of 7.4, 8.7, and 9.7 cm at 12, 16, and 20 WAT, respectively, were observed in seedlings treated with CNS-N (Fig. 1A).

Stem diameter

The analysis of variance revealed significant differences ($P \leq 0.05$) between the nutrient solutions for stem diameter at 8, 12, 16, and 20 WAT. Seedlings treated with CNS had significantly higher stem diameter compared to those from other treatments throughout the period of study (Fig. 1B). At 8 WAT, the stem diameter of seedlings treated with CNS-Mg, CNS-S, and CNS-Ca were comparable, with each significantly higher than the stem diameter of seedlings treated with CNS-K and CNS-N. The smallest stem diameter was observed in seedlings treated with CNS-P at 8 WAT. At 12 and 16 WAT, the seedlings treated with CNS-S had thicker stems than seedlings treated with CNS-Ca. Similarly, the stem diameter of seedlings treated with CNS-K and CNS-P were similar and significantly higher than that of seedlings treated with CNS-N, with the smallest values at 12 and 16 WAT. At 20 WAT, the stem diameter of seedlings subjected to CNS-S was significantly higher than in the other treatments, while the seedlings treated with CNS-N had the smallest stem diameter (Fig. 1B).

Number of leaves

The number of leaves of noni seedlings treated with various NS significantly differed ($P \leq 0.05$) among the treatments from 8 to 20 WAT (Fig. 1C). The seedlings treated with CNS-Ca produced the highest number of leaves at 8 WAT, while those supplied with CNS-N had the least number of leaves. At 12 WAT, the seedlings treated with CNS had the highest number of leaves, whereas seedlings treated with CNS-Mg had the least number of leaves. Moreover, the number of leaves produced by seedlings treated with CNS-Mg decreased gradually from 8 to 20 WAT, while the number of leaves in the CNS-Ca treatment decreased from 12 WAT onward, reaching the lowest values at 16 and 20 WAT. At 16 and 20 WAT, the seedlings treated with CNS produced the highest number of leaves, closely related to those treated with CNS-K, and significantly higher than the number of leaves for other treatments at 20 WAT (Fig. 1C).

Leaf area

Significant variations ($P \leq 0.05$) in leaf area were found between the treatment means at successive weeks of growth, except at 4 WAT. At 8 WAT seedlings treated with CNS-Ca had the highest leaf area (135.9 cm^2), which was not significantly different from values observed for seedlings treated with CNS (133.1 cm^2), CNS-Mg (123 cm^2) or CNS-S (117.7 cm^2) (Fig. 1D). The smallest leaf area (52.9 cm^2) during this period was observed in seedlings treated with CNS-P, which was closely related to the value obtained for seedlings treated with CNS-N. At 12 WAT, the seedlings treated with CNS had significantly higher leaf area compared to other treatments. Seedlings treated with CNS-N had the smallest leaf area at 12 WAT. Results at 16 and 20 WAT followed a similar trend. Seedlings treated with CNS had the highest leaf area at 16 and 20 WAT (1097.7 and 1363.5 cm^2, respectively), significantly exceeding the leaf area of other treatments at both periods. The seedlings treated with CNS-N had the smallest leaf area at both periods. The leaf area of the seedlings treated with CNS-Ca declined gradually from 12 WAT to 20 WAT (Fig. 1D).

Total dry matter accumulation

The analysis of variance indicated that the effects of NS, application rates, and their interactions were highly significant ($P \leq 0.01$) on the total dry matter yields of noni seedlings at 20 WAT. Seedlings treated with CNS had the highest total dry matter (15.45 g/plant), which was significantly higher than values for each of the other treatments (Fig. 2A). This was followed by seedlings treated with CNS-S with 8.08 g/plant. The dry matter of seedlings treated with CNS-K (5.42 g/plant) was similar to that of seedlings treated with CNS-P (4.40 g/plant), which was also not different from the dry matter of seedlings treated with CNS-Mg (3.36 g/plant). Moreover, the dry weight of seedlings treated with CNS-Mg was closely related to those of the CNS-Ca treatment (2.65 g/plant). The least biomass (1.52 g/plant) was observed in seedlings treated with CNS-N at the same period (Fig. 2A). Between the NS volumes, the seedlings under the least application volume (100 ml NS) accumulated the highest dry matter (8.91 g/plant), which was significantly higher than the seedling

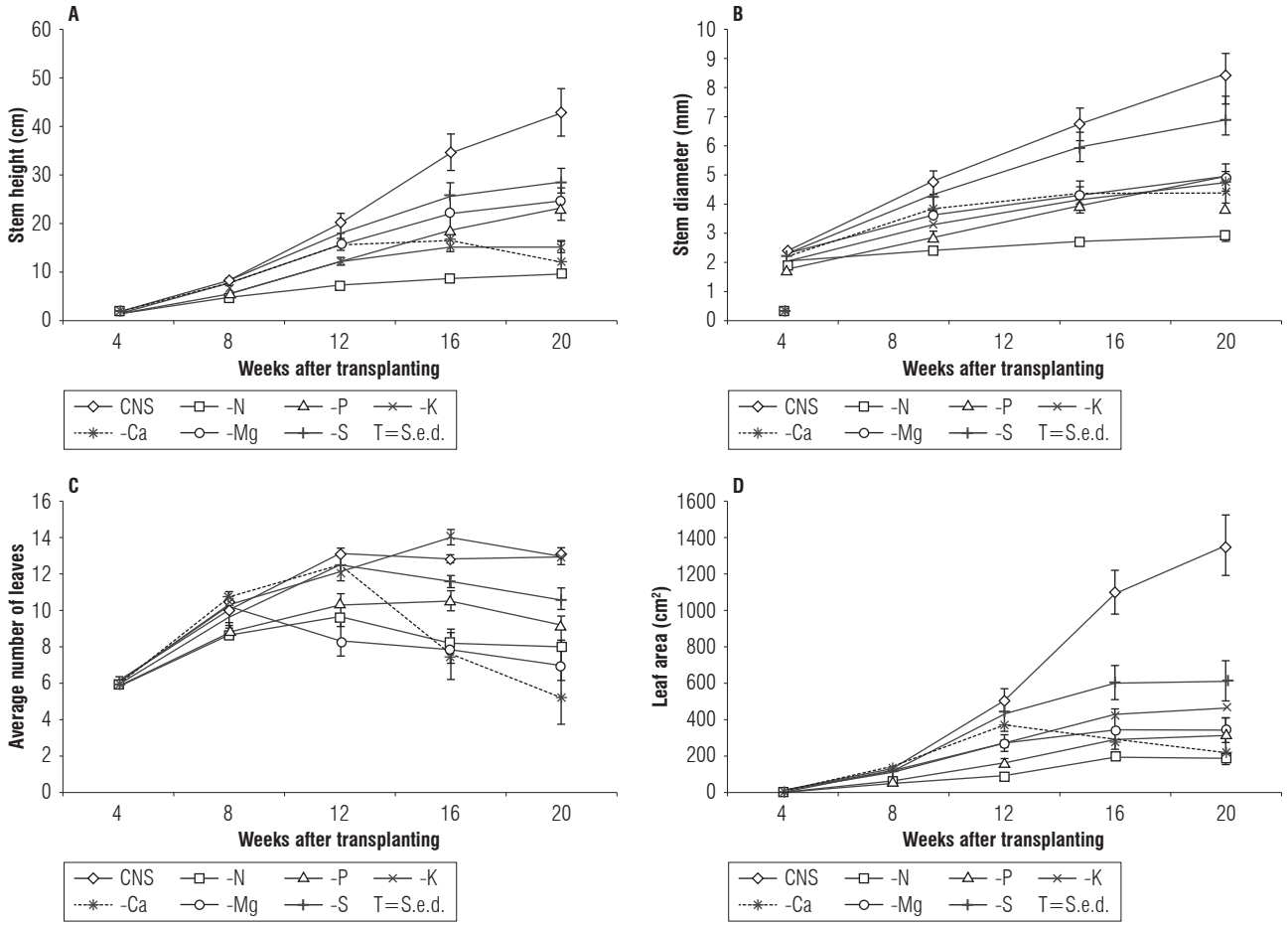

FIGURE 1. Effects of the nutrient solutions on (A) plant height, (B) stem diameter, (C) number of leaves, and (D) leaf area of noni seedlings during 20 weeks after transplanting into sand culture. Error bars correspond to standard error (n = 16). CNS - complete nutrient solution.

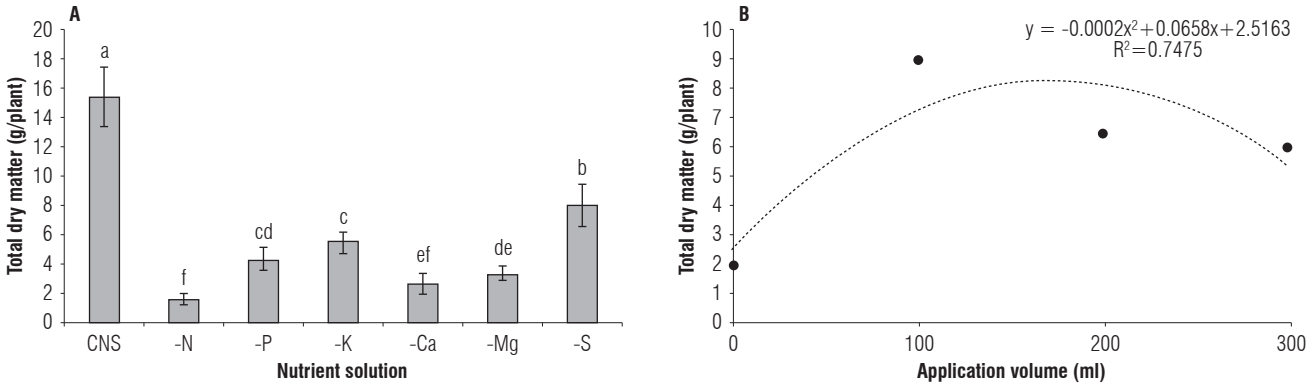

FIGURE 2. Effects of (A) nutrient solutions and (B) application volumes on total dry matter of noni seedlings at 20 weeks after transplanting into sand culture. Means with different letters indicate significant differences according to the Duncan test ($P \leq 0.05$). Error bars correspond to standard error. CNS - complete nutrient solution.

weights obtained for each of the other volumes (200 or 300 ml of NS) and control which had the least seedling weight at 20 WAT (Fig. 2B).

Furthermore, results of the effects of nutrient solution volumes on plant height, stem diameter and leaf area showed significant differences ($P \leq 0.05$) for means of the variables at successive weeks of growth (Fig. 3). Seedlings treated with 100 ml NS had the highest plant height, stem diameter and leaf area, while the control seedlings had the least values for these variables at 8 to 20 WAT. Conversely, the control seedlings had the highest number of leaves, while seedlings

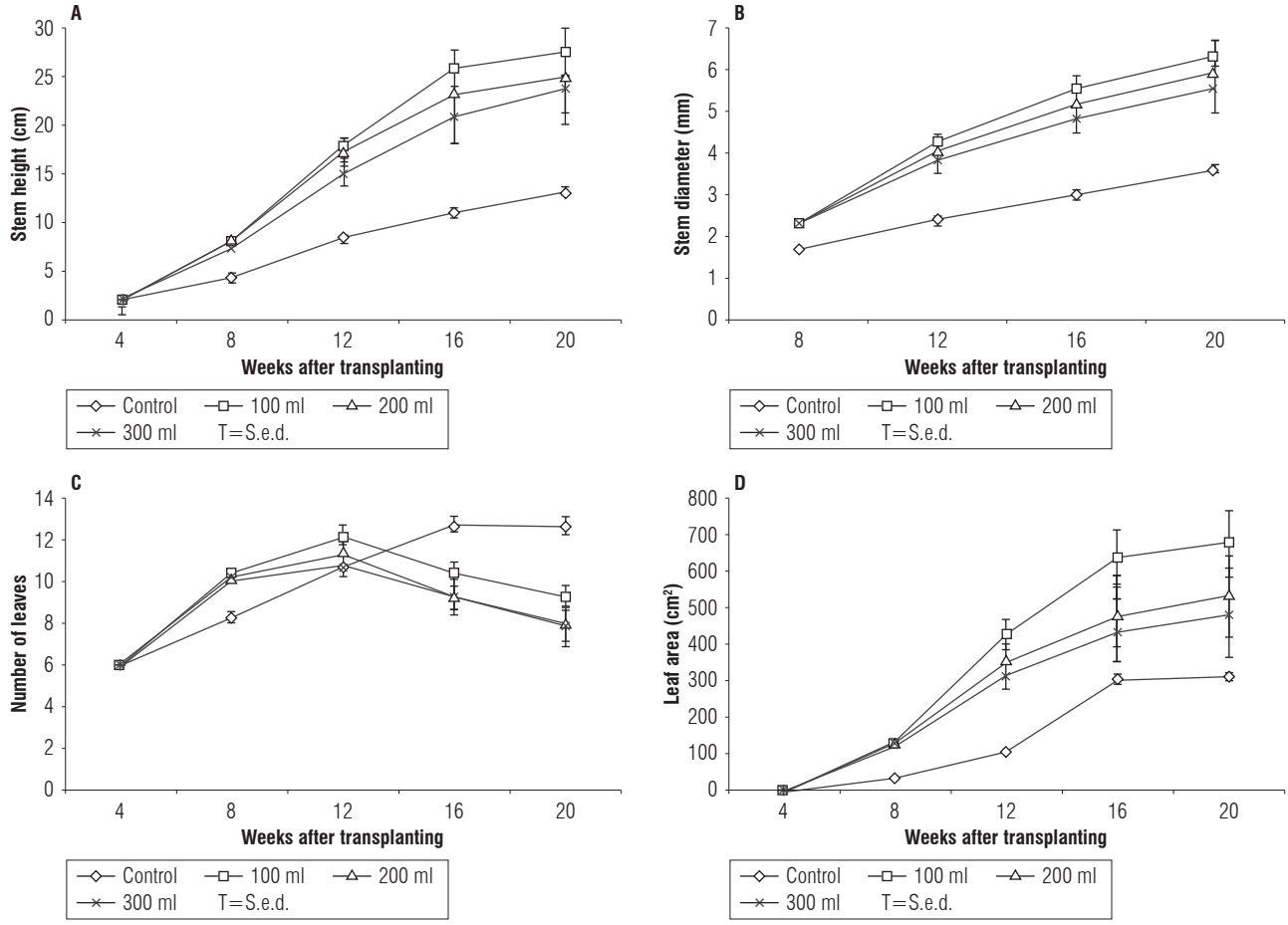

FIGURE 3. Effects of nutrient solution application volumes on (A) plant height, (B) stem diameter, (C) number of leaves, and (D) leaf area of noni seedlings during 20 weeks after transplanting into sand culture. Error bars correspond to standard error (n = 28). Control - distilled water.

treated with 100 ml, 200 ml, or 300 ml of NS decreased in number of leaves from 12 to 20 WAT (Fig. 3C). The results of the interactive effects of NS and application volumes on the seedlings also revealed significant differences for means of all variables at successive weeks of growth.

Leaf deficiency symptoms

Observations on leaf deficiency symptoms of noni seedlings indicated that plant growth was affected by the different NS treatments (Fig. 4). The growth of the seedlings was uniform with no visible deficiency symptoms at 4 WAT. However, at 8 WAT, seedlings treated with nutrient-deficient solutions began to show deficiency symptoms, which persisted and became severe at 16 and 20 WAT (Fig. 4). Seedlings treated with CNS showed no deficiency symptoms throughout the periods of observation (Fig. 4A). In contrast, stunted growth and chlorosis, which started in the older leaves and progressed to the younger leaves, were observed in seedlings treated with CNS-N (Fig. 4B). Similarly, small leaves and retarded growth were identified in seedlings treated with CNS-P (Fig. 4C), whereas brown patches in the third pair of leaves and crinkled stems were observed in seedlings treated with CNS-K (Fig. 4D). Seedlings treated with CNS-Ca produced interveinal chlorosis in young fully expanded leaves, burned leaf margins, and shoot tips die-back (Fig. 4E). Similarly, necrosis and premature leaf fall were noticed in seedlings treated with CNS-Mg (Fig. 4F), while seedlings treated with CNS-S had interveinal chlorosis on young and recently matured leaves (Fig. 4G) compared with seedlings treated with CNS at the same growth period.

Chlorophyll content

The chlorophyll (CHL) content in the leaves of noni seedlings varied significantly with respect to different nutrient solutions applied (Fig. 5). The seedlings treated with CNS had the highest CHL content (71.7 SPAD value), which was significantly higher than values for each of the other treatment solutions at 20 WAT (Fig. 5A). The lowest CHL content was recorded for seedlings treated with CNS-Ca at

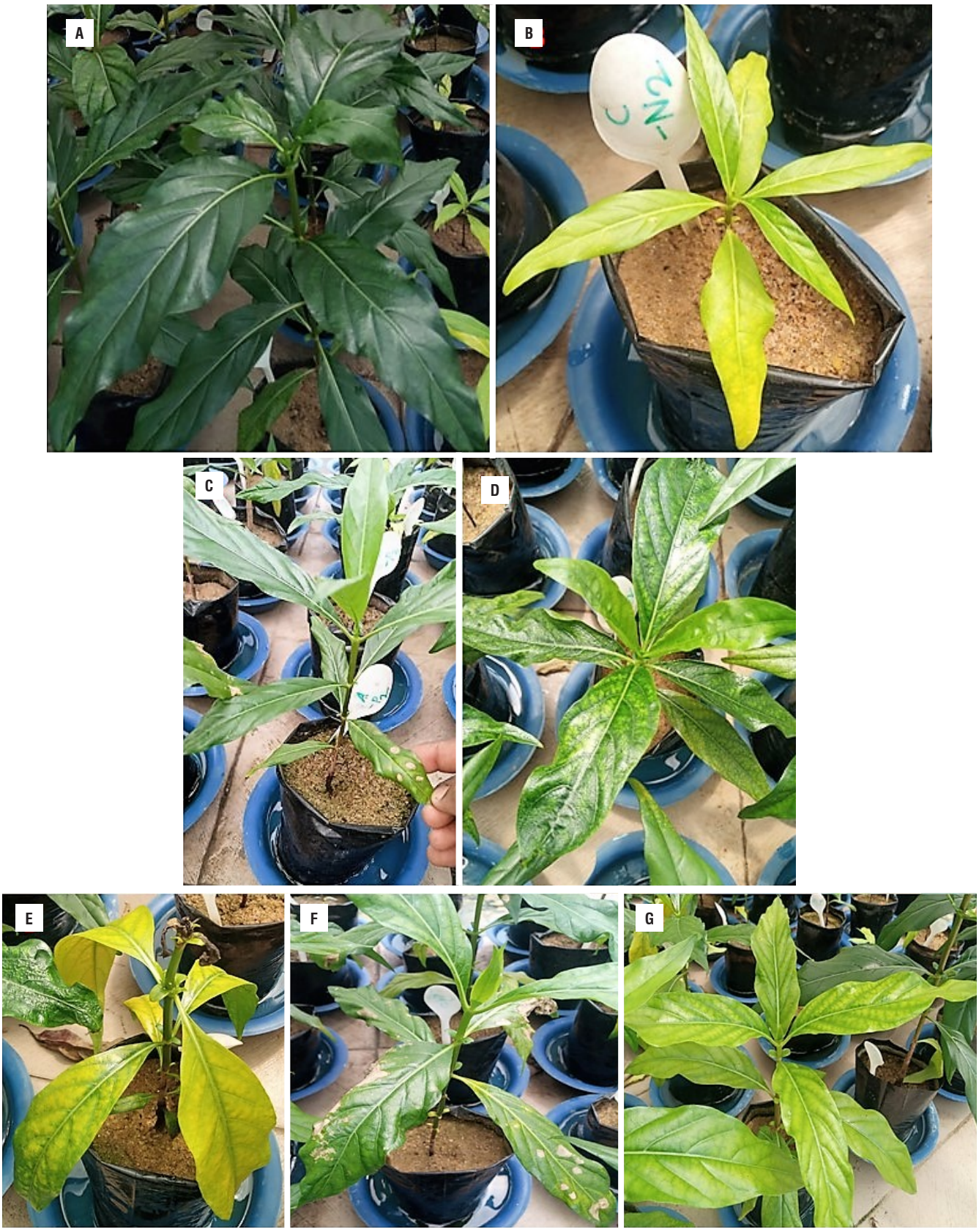

FIGURE 4. Macronutrient deficiency symptoms in noni seedlings at 16 weeks after transplanting into sand culture as affected by nutrient solutions (200 ml applied twice a week). The seedlings were treated with: A) Complete Nutrient Solution (CNS); B) CNS – N; C) CNS – P; D) CNS – K; E) CNS – Ca; F) CNS – Mg; and G) CNS – S.

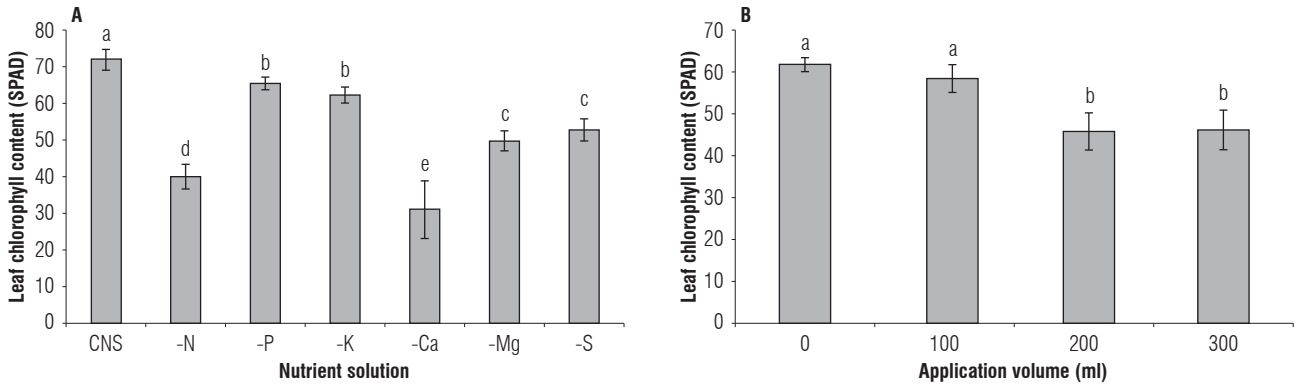

FIGURE 5. Effects of (A) nutrient solutions and (B) application levels on leaf chlorophyll content of noni seedlings at 20 weeks after transplanting into sand culture. Means with different letters indicate significant differences according to the Duncan test ($P \leq 0.05$). Error bars correspond to standard error. CNS - complete nutrient solution.

20 WAT. The CHL content in ascending order was CNS-Ca < CNS-N < CNS-Mg < CNS-S < CNS-K < CNS-P < CNS (Fig. 5). The CHL content was also affected by the levels of NS applied (Fig. 5B). The seedlings treated with only distilled water had CHL contents similar to those treated with 100 ml NS. The CHL values for these treatments were significantly higher than those recorded for seedlings treated with 200 ml or 300 ml NS at 20 WAT.

Discussion

The highest growth was observed in seedlings watered with complete nutrient solution in terms of plant height, stem diameter, number of leaves, leaf area, and total dry matter, which could be attributed to the availability of all the essential nutrients in the CNS used (Hoagland & Arnon, 1938). These results indicated that the concentration of the essential nutrients in the CNS, even at the least application volume (100 ml NS), was sufficient for normal growth in noni seedlings while providing a basis for comparison with other treatment solutions applied in the present study. Moreover, the better growth observed at the lowest application volume could be attributed to the higher efficiency of the photosynthetic apparatus used by the plants (Lambers *et al*., 2008). This was evident in the highest values of leaf area, total dry matter, and chlorophyll content observed for the least NS volume compared with control and other volumes (200 ml and 300 ml of NS). This indicated that noni seedlings might not require a high nutrient dose but the balanced nutrition for optimum growth.

Essential elements are needed for optimum growth serving as integral parts of plant metabolic pathways. Their functions cannot be substituted by another element, and without them no plant can complete its life cycle (Toor *et al*., 2021). These assertions were obvious in the results obtained for seedlings treated with CNS deficient in a macronutrient (N, P, K, Ca, Mg, and S). Generally, the pattern of growth of seedlings under each of the deficient nutrient solutions followed Liebig's "law of the minimum" which states that the nutrient in shortest supply determines the growth or yield level (Kihara *et al*., 2022). Consequently, the absence of an essential macronutrient in each of the solutions might have deprived the plant of necessary metabolic activities, resulting in retarded and suppressed growth observed in the seedlings compared with those under CNS application. Hitherto, there are reports on fertilizer application to noni plants (Caione *et al*., 2017), but studies on nutrient omission study are lacking. Hence, these results could be related to those of Costa *et al*. (2024), where complete Hoagland and Arnon nutrient solution was applied to coffee plants (a species related to noni) to obtain leaves with contrasting elemental compositions.

Nitrogen is involved in the formation of amino acids, proteins, chlorophyll, and vitamins in plants (Fathi, 2022). Lack of these functions might have caused the short, thin plants and overall chlorosis observed in noni seedlings treated with CNS-N. Moreover, the least growth observed in the same seedlings in terms of plant height, stem diameter, leaf area, and total dry matter at successive weeks after transplanting indicated that nitrogen is the most limiting macronutrient for the optimum growth of noni at seedling stage. Starting from 8 WAT, the seedlings in CNS-N were underdeveloped, and this phenomenon persisted until the end of the study. Similarly, the poor growth observed in the seedlings subjected to the CNS-Ca treatment at 20 WAT underscores the importance of Ca in formation of the cell wall middle lamella and cell membranes (Thor, 2019). Initially, rapid growth was observed in the CNS-Ca seedlings up to 12 WAT. However, the malformed new leaves, low chlorophyll content, cupped leaves, burned tips,

and stem die-back at later stages (16 to 20 WAT) could be attributed to the absence of Ca in the nutrient solution. Calcium influences N uptake, cell growth, and cell division as well as water movement in plants (Ramírez-Builes *et al*., 2020). Deficiency of these functions might have caused the reduced growth observed in the seedlings, even at higher concentrations of other nutrients, considering Ca as the second most limiting nutrient for noni growth after N. These results could be related to those of Flores *et al*. (2016), where omission of N and Ca among other deficient nutrients (P, K, Mg, and S) mostly affected the growth of coffee plants in terms of lower dry matter accumulation; therefore, nitrogen was selected as the most required mineral element in coffee at this stage of growth. In another study, N followed by Ca were also reported as the most demanded macronutrients in *Coffee canephora* (Ramírez-Builes *et al*., 2020).

Next to Ca, Mg is the limiting macronutrient for noni growth, as revealed by the results of this study. The necrosis observed in the seedlings treated with CNS-Mg was specific to the treatment throughout the periods of study and was indicative of Mg deficiency (Fig. 4F). Magnesium is a central cation in chlorophyll molecules and acts as an enzyme activator. It is required for stabilization of nucleic acids and ATP formation (Kathpalia & Bhatla, 2018). The deficiency of Mg in mature leaves of seedlings treated with Mg-deficient solution might have led to reduced photosynthetic activities and reduced influx of mineral nutrients through xylem (Tanoi & Kobayashi, 2015). This, in turn, might have caused necrosis and defoliation, culminating in the reduced total dry matter in the CNS-Mg seedlings compared with those treated with CNS at 20 WAT. On the other hand, the deficiency symptoms observed in the present study with nutrient solutions deficient in each of P, K and S proved that each of these macronutrients is essential for optimum growth of noni seedlings. However, the requirement for these nutrients by noni is not as important as that of N, Ca and Mg (Subramanian *et al*., 2023). This was evident in better growth observed for the seedlings under application of the solutions deficient in each of P, K, and S compared with seedlings to which solutions deficient in each of N, Ca, and Mg was applied.

Conclusions

Noni seedlings under various deficient macronutrient solutions exhibited different deficiency symptoms and growth limitations. Nitrogen, followed by Ca and Mg, are the most limiting macronutrients required for optimum growth of noni at vegetative stage. These nutrients are also required in relatively small amounts, considering the best performance of noni seedlings under the least level of nutrient solution applied. Consequently, the present study has provided an insight into the most required macronutrients for noni, and the results should serve as a guide for fertilizer recommendation for the species.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

JOA and EAA formulated the overarching research goals and aims. All authors designed the methodology. JOA conducted the research, data/evidence collection and statistical analysis. JOA wrote the initial draft. AOO contributed to the results presentation. AEA oversaw and led the research activity planning and execution. All authors reviewed the final version of the manuscript.

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Methodology to identify spatial patterns in coffee (*Coffea arabica* L.) production

Metodología para identificar patrones espaciales en la producción de café (*Coffea arabica* L.)

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ABSTRACT

Coffee farming, a lifeline for numerous families in the mountainous regions of Latin America, faces challenges due to climate change and production variability, which complicate the use of forecast models at the territorial level. In response to these challenges, territorial inference has gained relevance, especially with the advancement of Geographic Information Systems (GIS), which provide useful tools for territorial analysis. Although spatial models are increasingly applied in GIS, coffee farming, like many agricultural subsectors, is hindered by a lack of information and spatial methodologies. This work proposes a methodology to identify spatial patterns of homogeneous production areas. Data from 140 farms, representing 3,900 members of the coffee grower cooperative of Andes, dispersed over 200,000 ha, were analyzed between 2019 and 2021. The variables used to measure productivity included the number of fruits per tree, the average fruit weight, planting density, and the conversion rate of cherry coffee to dry parchment coffee. A simple linear regression model was employed, and spatial dependency analyses were performed using the global and local Moran's Index to identify clusters of territorial subdivisions. The data were processed in R language, and the GeoDa™ program was used to obtain the spatial weight matrix. Territorial units with similar characteristics for high-quality mountain coffee production were identified through spatial dependency indicators. The methodology can contribute to estimating coffee production in large territories, improving the reliability of information and allowing for more informed decision-making to optimize coffee farming in mountainous areas.

Key words: mountain coffee production, clustering, Moran's index, spatial dependency, territorial planification.

RESUMEN

La caficultura, una fuente de sustento para numerosas familias en las regiones montañosas de América Latina, enfrenta desafíos debido al cambio climático y la variabilidad de la producción, lo que complica el uso de modelos de pronóstico a nivel territorial. A pesar de estos desafíos, la inferencia territorial ha ganado relevancia, especialmente con el avance de los Sistemas de Información Geográfica (SIG), que ofrecen herramientas útiles para el análisis territorial. Aunque los modelos espaciales se aplican cada vez más en SIG, la caficultura, al igual que muchos subsectores agrícolas, se ve limitada por la falta de información y metodologías espaciales. Este trabajo propone una metodología para identificar patrones espaciales de áreas de productividad homogénea. Se analizaron datos de 140 fincas, representando a 3900 miembros de la cooperativa de caficultores de Andes, distribuidos en 200.000 ha, entre 2019 y 2021. Las variables utilizadas para medir la productividad incluyeron el número de frutos por árbol, el peso promedio de los frutos, la densidad de plantación y la tasa de conversión de café "cereza" a café pergamino seco. Se empleó un modelo de regresión lineal simple y se realizaron análisis de dependencia espacial utilizando el Índice de Moran global y local para identificar agrupamientos de subdivisiones territoriales. Los datos se procesaron en el lenguaje R, y se utilizó el programa GeoDa™ para obtener la matriz de pesos espaciales. Mediante indicadores de dependencia espacial, se identificaron unidades territoriales con características similares para la producción de café en zonas montañosas. La metodología puede contribuir a estimar la producción de café en grandes territorios, mejorando la confiabilidad de la información y permitiendo una toma de decisiones más informada para optimizar la caficultura en áreas montañosas.

Palabras clave: producción de café de montaña, agrupamiento, índice de Moran, dependencia espacial, planificación territorial.

Introduction

The coffee industry plays a vital role in the economies of Colombia and Latin America, supporting millions of livelihoods, particularly in mountainous regions. However, coffee farming faces increasing challenges due to climate change and production variability, which complicate the effectiveness of traditional forecast models at the territorial

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level. These challenges highlight the need for more precise and adaptable methodologies to estimate coffee production, ensuring better resource allocation and supply chain stability (Mendoza *et al.,* 2013).

Estimating coffee production is essential for strategic planning across the supply chain, particularly for cooperatives and stakeholders involved in futures contracts, who rely on accurate yield predictions to mitigate financial risks (Choperena Bedoya & Couleau, 2021). Traditionally, coffee yield estimations have depended on economic and climatic variables. However, these factors alone provide limited precision in addressing the complexities of coffee production, especially in diverse topographies and heterogeneous farming conditions (Aparecido *et al*., 2017; Gil Serna, 2012; Moraes-Oliveira *et al*., 2017). Since the 1950s, forecasting models have been developed to assist in decision-making for fertilization planning, labor scheduling, and input purchases, helping producers optimize resource allocation. Over time, more than 50 factors influencing coffee production have been identified, spanning soil properties, climate conditions, social dynamics, agronomic practices, and crop management strategies (Montoya Restrepo *et al*., 2009).

Despite these advancements, forecasting models still face significant limitations. The high variability in phenological characteristics across coffee varieties (Ramírez Builes, 2014) and the reliance on flowering stages for prediction have yielded modest results, with determination coefficients around 0.4 (Rendón-Sáenz *et al*., 2008). Additionally, many models developed from experimental farm data lack field validation, reducing their applicability to traditional farms, which often operate under different environmental and management conditions.

To address these limitations, territorial inference has emerged as a promising approach for improving yield estimation. The increasing adoption of Geographic Information Systems (GIS) has facilitated more comprehensive spatial analyses, enabling the identification of productivity patterns over large areas. However, coffee farming, like many other agricultural subsectors, still faces a lack of spatial methodologies for production estimation.

To improve the accuracy of yield estimates, recent studies have explored artificial vision techniques for fruit counting on branches, achieving high precision (R^2>0.9) (Ramos *et al*., 2017). However, this method faces practical challenges in the field, as variations in the number of axes, branches, and branch types affect implementation. Artificial vision has also been applied to satellite and drone imagery to assess factors like plant density, leaf health, and coffee fruit ("cherry") development (Abreu Júnior *et al*., 2022; Martello *et al*., 2022; Tanaka *et al*., 2015). This approach is particularly promising in the context of climate change, where unpredictable conditions limit the reliability of traditional models. Although high costs are a limitation (Benos *et al*., 2021; Chlingaryan *et al*., 2018; Kouadio *et al*., 2018), artificial vision remains valuable for tasks such as fruit counting, an essential parameter in assessing coffee productivity (Adane & Bewket, 2021; Beksisa *et al*., 2018).

Advances in artificial vision continue to support the development of forecasting models adapted to image data (Cheng *et al*., 2017; Eugenio *et al*., 2020; Khaki & Wang, 2019; Qiao *et al*., 2021; Tsai & Chen, 2017; Wang *et al*., 2017; Zhang *et al*., 2019). Furthermore, spatial analysis techniques have introduced new approaches to territorial-level modeling, enabling the identification of homogeneous areas, which can improve resource management in agricultural landscapes. In a context of resource scarcity, spatial technologies allow for optimized land, water, and input use, promoting sustainability and efficiency (Anselin *et al*., 2004; Bivand *et al*., 2013). This integration of spatial techniques enables informed decision-making, improves productivity, and mitigates environmental impacts, addressing the pressing challenges of food security and sustainable production.

This research proposes a novel methodology to identify homogeneous productivity zones by analyzing data from 140 farms representing 3,900 cooperative members, covering over 200,000 ha in the coffee-growing region of the Andes in Colombia.

The proposed approach integrates spatial dependency analyses using Moran's Index to identify clusters of productivity within territorial subdivisions. A simple linear regression model is employed to analyze key productivity indicators, including fruit count per tree, average fruit weight, planting density, and cherry-to-parchment conversion rate. The data are processed in the R language, and the spatial weight matrix is generated using GeoDa™, ensuring robust geostatistical analysis.

This study contributes to the ongoing efforts to improve coffee production estimation at the territorial level, offering a methodology that enhances information reliability, decision-making processes, and strategic planning. By leveraging spatial statistics and GIS, this approach provides a scalable framework for identifying high-quality coffee production areas and ultimately optimizing coffee farming in mountainous landscapes.

This study proposes a methodology for identifying spatial patterns in coffee production using historical data (2019–2021) from selected farms in Southwest Antioquia, Colombia. The research focuses on analyzing variables related to production, along with images of all harvested fruits per tree. The primary goal is to identify specific areas within the region where productivity can be predicted based on the number of coffee fruits per tree. Additionally, the study examines the relationship between fruit count and the average weight of a sample of 30 fruits from the same tree, establishing a foundation for refined production forecasts at the local level.

Materials and methods

This study develops a methodology to identify homogeneous areas within rural zones. In Colombia, the smallest territorial unit is called a "vereda" (small village), which this study will refer to as the Minimum territorial subdivision (MTS). The study associates certain relevant variables to this MTS to predict coffee production. A simple forecasting model was implemented to estimate the number of fruits per tree at specific times, using three years of harvest data from the Southwestern Antioquia subregion, one of Colombia's most productive coffee areas.

Localization

The study area included the municipalities of Andes, Ciudad Bolívar, Betania, Hispania, and Jardín, all located in the Southwestern Antioquia subregion, with a potential of 3,900 producers (Fig. 1).

Sampling and data collection

Farms were selected from the Cooperandes Producers Association database, with sampling conducted from January to March for secondary harvests and from August to September for main harvests in 2019, 2020, and 2021. Farms were chosen randomly with stratified sampling by agroecological zone, and data collection on each farm included:

- Producer contact: Farms with at least three productive lots of varying ages and with no ongoing fumigations were selected;
- Lot selection: Three diverse lots per farm were prioritized, excluding those near renovation areas or with harvest issues;
- Tree selection: From each lot, three trees representing different productivity levels were chosen, avoiding those near paths or water sources;
- Fruit harvesting: All fruits from selected trees were labeled with producer and farm information;
- Sample imaging: Field personnel photographed fruit samples with geolocated smartphones;
- Fruit counting: Fruit images were analyzed using the CounThings© app (Countthings, 2021) with a mung bean template for better performance. The count data were stored in a CSV file;
- Production weighing: The total harvest per tree was recorded in grams as this is how data are stored in the cooperative database;
- Individual weight measurement: Thirty fruits per tree were individually weighed with a precision balance (model LS220A) at the cooperative's lab.

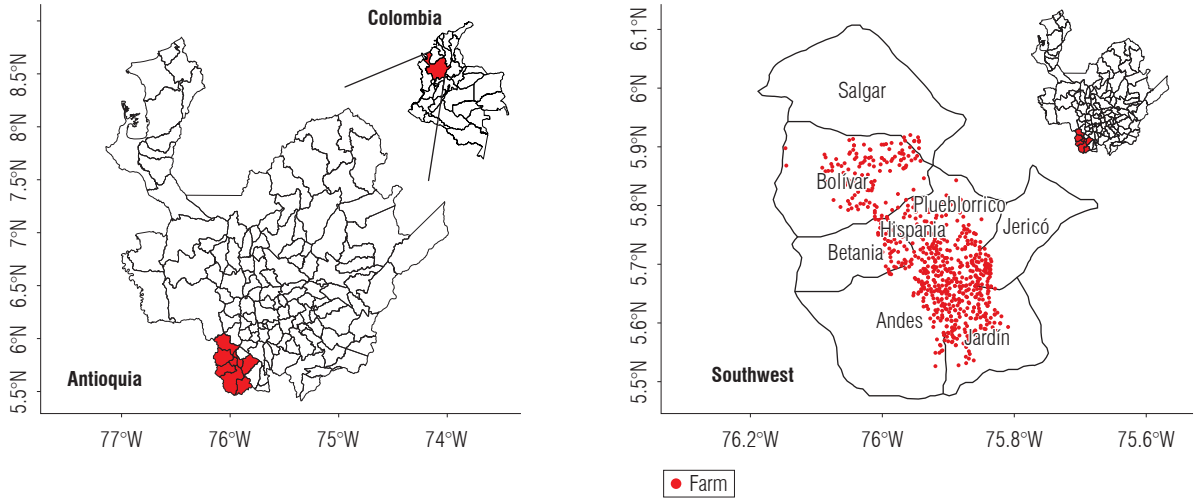

FIGURE 1. Distribution and location of the universe of producers within the territory: A) area of influence, B) territorial distribution of the farms.

Formation and selection of the dataset

Cooperandes provided 2019–2021 production data, including variables such as average fruit weight per tree, fruit count, planting density, and conversion rates for cherry coffee to dry parchment coffee, along with identifiers for lot, farm, and municipality. After data cleaning, 512 valid records from 161 farms remained. Using the National Administrative Department of Statistics (DANE) database, polygons of territorial subdivisions (MTS) were mapped for the municipalities of Andes, Ciudad Bolívar, Betania, Hispania, and Jardín. Farm coordinates were aligned with official maps from the Geographic Institute Agustín Codazzi (IGAC, 2021), resulting in 140 georeferenced farms. Each farm was assigned an MTS identifier, and the mean of each variable was calculated per MTS. Data analysis was performed in R (Bivand *et al*., 2013).

Proposed production model

A linear model (Eq. 1) obtained by regression was used to estimate the number of fruits per tree. It was determined that a sample of 30 fruits could represent the total population of fruits on each tree, regardless of the variability in axes, branches, or nodes that the trees may have.

$$Y_i = \beta_0 + \beta_1 X_i + \varepsilon_i \quad (1)$$

where Y_i is the number of fruits per tree, β_0 is the interception, β_1 is the slope, X_i is the average weight of 30 coffee fruits (g), and ε_i the error term (Montgomery & Peck, 2006).

An analysis of the proposed model was conducted in the municipalities where the samples were obtained, seeking to determine if the detected areas had better determination coefficients than the models at the municipal level. Table 1 indicates the parameters and statistical significance of the proposed model at the municipal scale, showing that only the municipalities of Andes, Betania, and Ciudad Bolívar had significant models (P<0.1). However, these models explain little variability of the phenomenon (adjusted R^2 between 0.14 and 0.3), and they do not adapt to the higher (Jardín) or lower (Hispania) zones of the territory, making their practical application challenging in conditions of high climatic variability. The normality assumptions were verified using the Shapiro-Wilk test to analyze the models.

This analysis aims to identify spatial autocorrelation in a dataset, revealing patterns of clustering or geographic dispersion among its values. Spatial autocorrelation suggests that the values of the variables have some type of spatial association, meaning that nearby observations are more likely to be associated compared to observations farther apart for the same variable. The Moran's index models this relationship, allowing the identification of the magnitude of the degree of spatial autocorrelation between the areas that define the study region. Through standard statistical tests, a statistically significant level for spatial correlation was calculated, which allows the determination of specific areas within the territory (Moran, 1950).

This is achieved by constructing a matrix of neighborhood or spatial weights matrix, for which missing data must first be imputed. For this study, the nearest neighbor method was used, which consisted of interpolation based on the arithmetic mean of the neighbors, specifically the closest or first-order neighbors. In this process, the following software programs were used for data processing, cleaning, and visualization: The R language (R Core Team, 2020) and the open-access GeoDa™ software (GeoDa Foundation, 2020).

The calculation of Moran's Index, or Moran's spatial autocorrelation coefficient (Eq. 2), was based on the correlation between a variable's values and the values of its neighboring observations (Moran, 1950).

$$MI = \frac{n}{\sum_{i=1}^{n}\sum_{j=1}^{n} w_{ij}} \frac{\sum_{i=1}^{n}\sum_{j=1}^{n} w_{ij}\,(z_i-\bar{z})(z_j-\bar{z})}{\sum_{i=1}^{n}(z_i-\bar{z})^2} \quad (2)$$

where MI is Moran's Index, wij is the Spatial Weights Matrix (Neighborhood Matrix), zi and zj are the observed values of the variable in areas i and j, $\bar{z}$ is the expected value of the variable, and n is the number of areas (MTSs).

With the Moran test, the observed Moran's Index was compared against the expected distribution under the

TABLE 1. Analysis of models at the municipal level.

Municipality	Multiple correlation coefficient	Determination coefficient R^2	R^2 adjusted	Typical error	Interception	Slope	F	Critical value of F
Andes	0.37	0.14	0.12	801.95	6,041.67	- 3,503.38	9.63	0.00
Jardín	0.14	0.02	-0.04	925.44	3,747.28	- 1,465.72	0.35	0.56
Hispania	0.53	0.28	0.20	783.76	5,118.43	- 2,041.45	3.43	0.10
Betania	0.35	0.12	0.09	1,175.8	5,762.76	- 2,713.96	3.68	0.07
Ciudad Bolívar	0.55	0.30	0.26	908.71	8,832.80	- 5,796.82	6.84	0.02

null hypothesis of no spatial autocorrelation. When the observed Moran's coefficient is significantly different from zero, the null hypothesis is rejected, indicating that spatial autocorrelation exists in the data (Moran, 1950).

LISA (Local Indicators of Spatial Association) is a dimensionless index representing the number of standard deviations by which an area deviates from the mean of its surrounding.

The calculation of LISAs, like Moran's Index, represented a weighted measure of correlations based on a neighborhood criterion (Anselin, 1995), for which the following equation was used:

$$MI = \frac{n}{\sum_{i=1}^{n}\sum_{j=1}^{n} w_{ij}} \frac{\sum_{i=1}^{n}\sum_{j=1}^{n} w_{ij}\,(z_i-\bar{z})(z_j-\bar{z})}{\sum_{i=1}^{n}(z_i-\bar{z})^2} \quad (3)$$

where IMi is the Moran Index of area i, Ji is the neighbors set of area i, Zj is the mean of observations of areas neighboring area i, n is the number of areas in area J (surrounding MTSs), and W_{ij} is the matrix of spatial weights for group J of rural MTSs (neighborhood matrix).

The result of the local spatial dependence test was depicted in a map of local spatial significance to visualize and understand the spatial structure of the data. In the graphs, the values of the variables – fruit weight, total fruits, planting density, and conversion – were placed on the x-axis for each geographical location, and the calculated values of spatial autocorrelation of the data at each location (or IMi) were positioned on the y-axis. Using these graphs, each MTS was classified according to its autocorrelation pattern, which, for this study, was conducted using LISAs. The classification included categories such as high-high (high spatial concentration of high values), low-high (low spatial concentration of high values), high-low (high spatial concentration of low values), and low-low (low spatial concentration of low values). Other authors have employed this classification for spatial analysis of climatic variables (temperature, rainfall, and humidity) in Colombia (de Corso Sicilia *et al*., 2017).

The local spatial dependence test was used to detect specific areas with clustering or association patterns among the same variable within neighboring areas. Additionally, to test the hypothesis that these spatial autocorrelation relationships may indicate other relationships among those variables, a model was proposed to explain the number of fruits per coffee tree. The coefficient of determination of this model for the identified MTS groups was proposed as the level of predictability represented by this model.

In the study, it was established that an R^2 greater than 0.7 indicated forecastable areas. Areas with R^2 values between 0.5 and 0.7 were considered modelable, while those with R^2 values below 0.5 or without local or global spatial dependence were considered undetected areas.

The research hypothesis proposed that at least one MTS group exhibits both global and local spatial dependence for the variables Fruit weight and total fruits per tree, and that these variables are significantly correlated and can form a regression model (Eq. 1).

Results

Exploratory spatial analysis of the variables

The following table shows the descriptive results of the variables by municipality.

Figure 2 illustrates the frequency distribution of the variables of fruit weight, total fruits per tree, cherry coffee to dry parchment conversion, and planting density. The Shapiro-Wilk test was applied to these variables, showing the W values and their respective "*P*" values for each. For the variable fruits per tree, the W value is relatively high, suggesting a slight deviation from normality (*P*-value = 0.06); however, upon examining the distribution graph, its tendency towards a normal distribution is evident, similar to the other variables.

TABLE 2. Overview of the variables used in the study.

Municipality	Average fruit weight (g)		Fruits per tree (quantity)		Conversion (kg cherry coffee/kg dry parchment coffee)		Planting density (trees ha^{-1})	
	Average	Deviation	Average	Deviation	Average	Deviation	Average	Deviation
Andes	1.03	0.17	2,579	1,415	5.36	0.62	6,036	1,390
Betania	1.04	0.21	2,829	1,264	5.19	0.81	6,619	1,454
Ciudad Bolívar	1.04	0.17	2,906	1,380	5.28	0.52	5,571	995
Hispania	0.98	0.18	3,131	1,093	5.41	0.75	6,838	1,808
Jardín	1.18	0.29	2,186	1,408	5.23	0.65	5,581	1,036

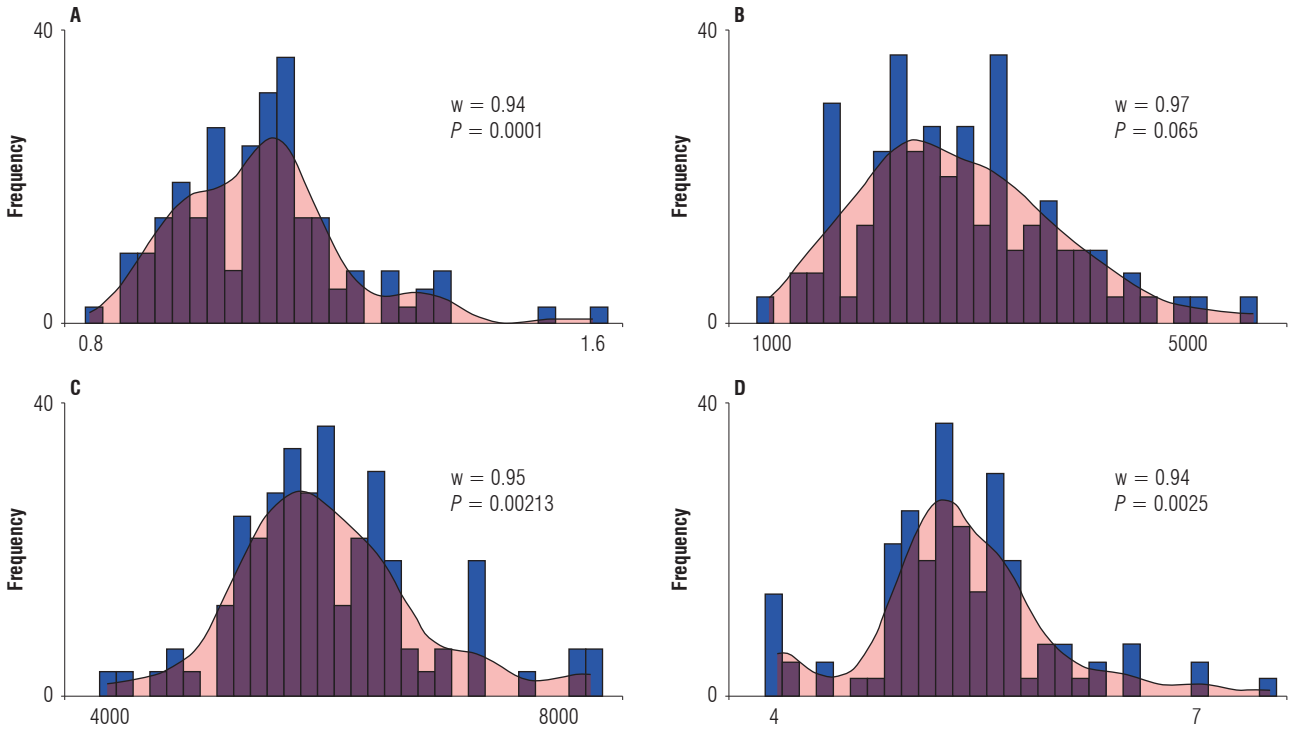

FIGURE 2. Frequency distribution of the variables used in the study: A) fruit weight, B) number of fruits per tree, C) planting density, D) conversion rate of cherry coffee to dry parchment coffee.

Some variables may cluster according to their values; however, this visual analysis is subjective and requires more robust techniques, such as LISAs.

Table 3 presents the calculated correlation between the variables. It is important to note that a negative correlation (-0.537) was detected between conversion and the number of fruits per tree. A negative correlation indicates that more efficient systems (lower conversions, *i.e.*, more technified) have a greater quantity of fruits. Despite its biological logic, this relationship also suggests the need for a different model specification. Therefore, in this study, the conversion variable was not used to refer to the harvest forecast, given the initially proposed model (Eq. 1).

TABLE 3. Correlation between variables.

Variable	Conversion	Planting density	Weight per fruit	Fruits per tree
Conversion	X	-0.085	0.277	-0.537
Planting density		X	-0.045	0.150
Weight per fruit			X	0.386
Fruits per tree				X

The neighborhood criterion used was the queen and first-order, employing the GeoDa™ program (Chasco Yrigoyen, 2006). To create the connectivity map, the average of the surrounding areas was assigned to the zones with missing data to avoid disconnected areas. The MTSs with missing data and the connectivity map are shown in Figure 4.

Spatial dependence indices

Table 4 presents the Moran's I values and their global test for each of the studied variables. The spatial dependence index is relatively low, despite all variables showing global spatial dependence. The variable with the highest spatial dependence was Fruit Weight. The global index indicated that at least one pair of MTSs exhibited spatial dependence. Subsequently, local spatial dependence was calculated to detect homogeneous areas or groups of MTSs.

TABLE 4. Global spatial dependence tests based on the Moran's Index (MI).

Item	MI	Value of *P*	Statistical significance
Planting density	0.3276	1.30E-10	***
Conversion	0.2183	9.60E-06	***
Fruits per tree	0.1760	0.0002849	***
Weight per fruit	0.3341	5.32E-11	***

*** highly significant.

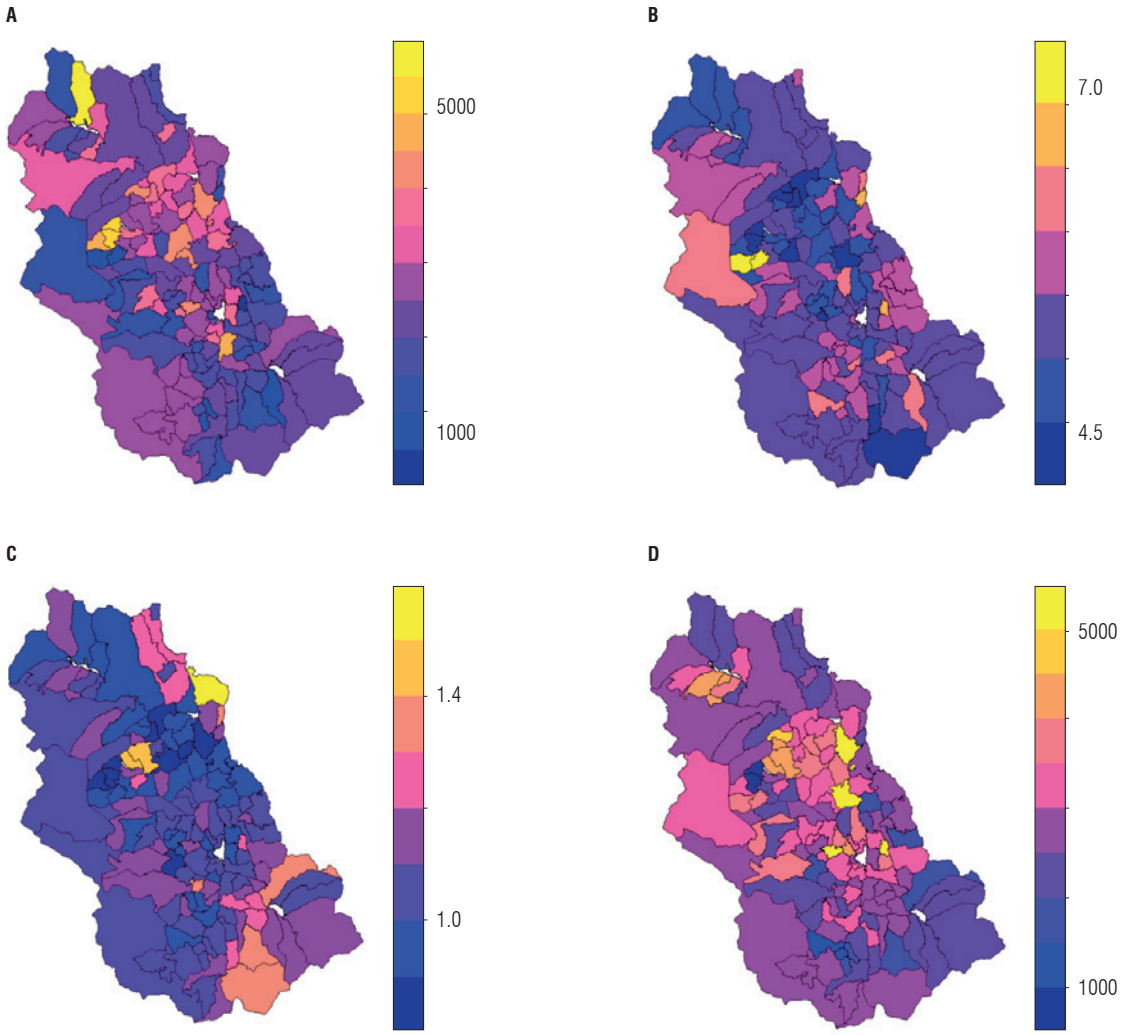

FIGURE 3. Spatial distribution of the study variables: A) number of fruits per tree, B) conversion rate of cherry coffee to dry parchment coffee, C) fruit weight, D) planting density.

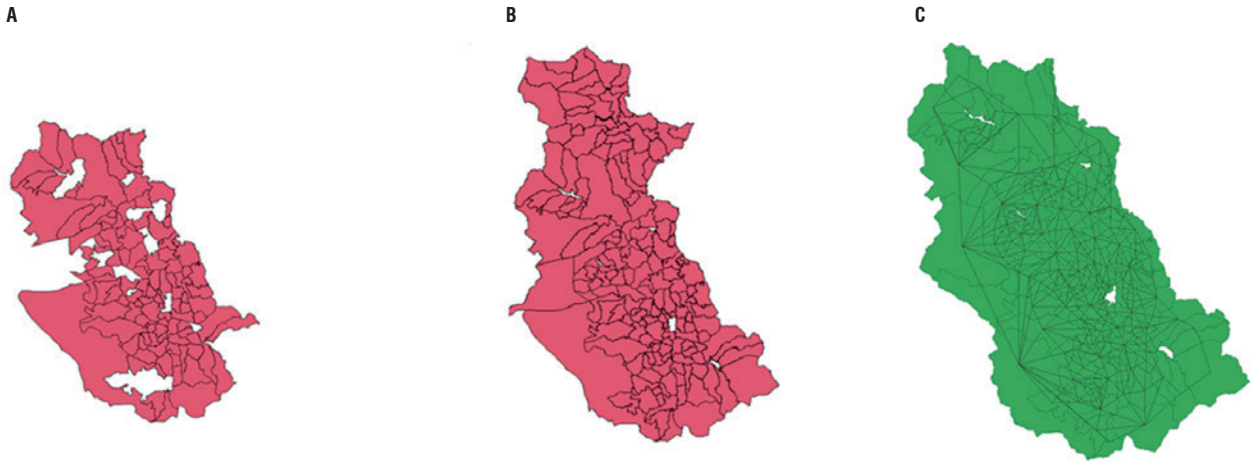

FIGURE 4. Spatial data and connectivity matrix: A) missing data, B) compete data, C) connectivity map.

Local spatial dependence index (IMi)

Figure 5 shows the estimated values of the LISAs, identifying areas with homogeneous characteristics.

Using local spatial autocorrelation analysis (LISA), MTSs presenting local dependence for the analyzed variables were identified, resulting in a total of 7 MTSs with significant spatial dependence for fruit weight and fruits per tree. Table 5 presents the LISA values for the identified MTSs. Notably, out of these 7 MTSs, two also exhibited local dependence for density and conversion variables. This suggests that in these areas, all variables considered in this study could be

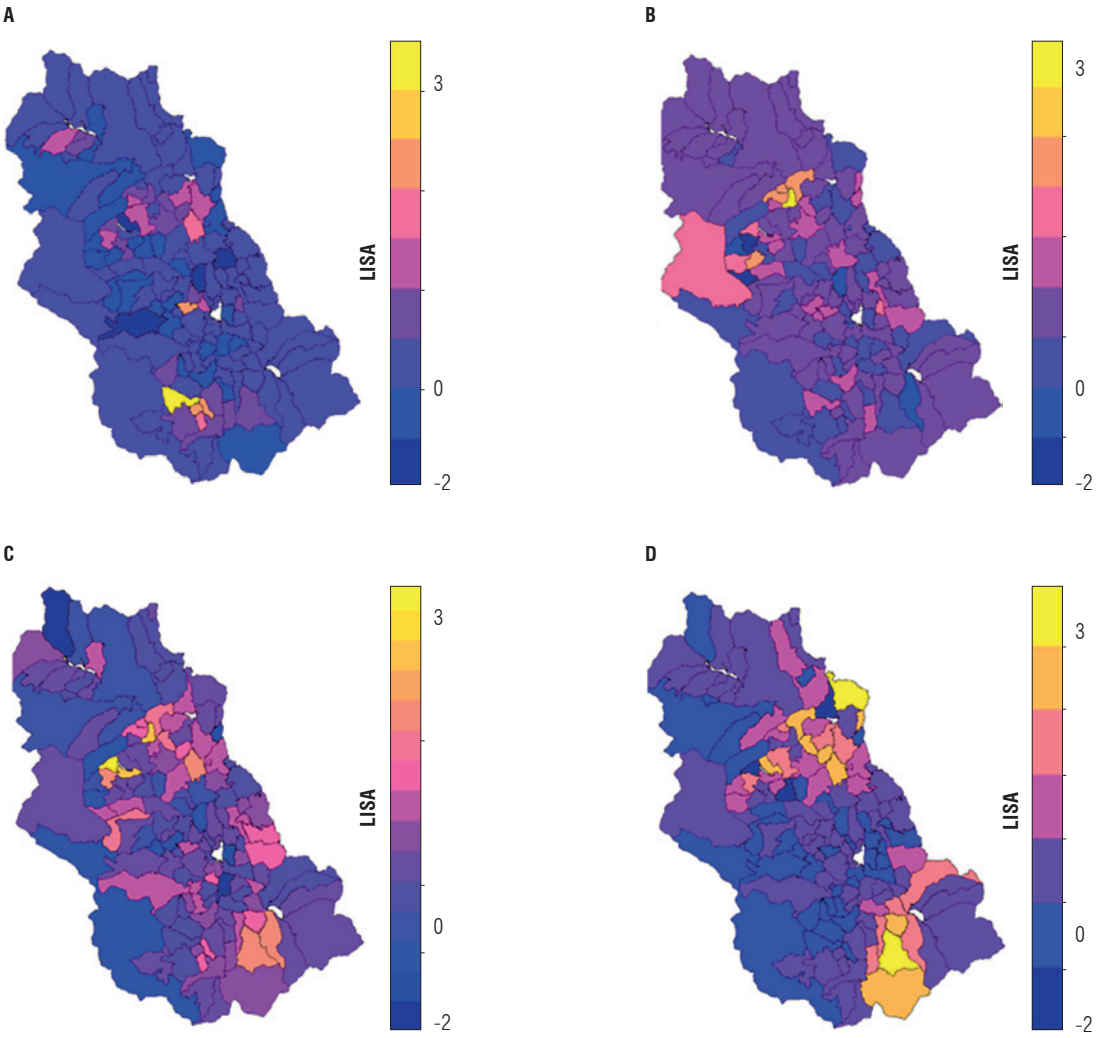

FIGURE 5. Local Moran indices: A) planting density, B) conversion rate of cherry coffee to dry parchment coffee, C) number of fruits per tree, D) fruit weight.

TABLE 5. Local evidence of spatial dependence of the selected areas.

Municipality	MTSs	*P* value Fruits per tree	*P* value Weight of fruit	*P* value Planting density	*P* value Conversion
Jardín	Macanas	0.02*	0.03*	0.02*	0.73
Jardín	Gibraltar	0.04*	0.00*	0.24	0.14
Jardín	Verdún	0.05*	0.00*	0.14	0.53
Hispania	La Armenia	0.02*	0.00*	0.05*	0.03*
Hispania	La Seca	0.03*	0.00*	0.01*	0.20
Betania	Las Animas	0.00*	0.00*	0.00*	0.04*
Betania	El Tablazo	0.03*	0.01*	0.20	0.00*

*$P<0.05$ according to IMi test. MTS – Minimum territorial subdivision.

used in a forecast model for total parchment coffee for this smaller zone. However, this study focused on the analysis of fruit-related variables and total fruits, suggesting a forecast that could extend to cherry coffee.

The degree of spatial dependence between space and the analyzed variables can be observed in the Moran plots (Fig. 6). The Moran plots show that the variables "fruit weight" and "planting density" exhibit higher spatial dependence, while " number of fruits per tree" and "conversion" show lower spatial dependence. Additionally, it is important to highlight that all variables present positive spatial dependence.

Analysis of the detected areas

The results of the linear regression models and their statistical significance for the group of detected MTSs, the group of detected MTSs plus their surroundings, and the group of all MTSs are shown in Table 6. It was demonstrated that, in the detected areas, a production model that explains 79% of the number of fruits per tree from their mean weight performs better, and that the detected area and its surroundings explain 62% of the variability of the phenomenon.

In Figure 7A, the five municipalities included in the study are shown. In Figure 7B, the non-detected areas are shown

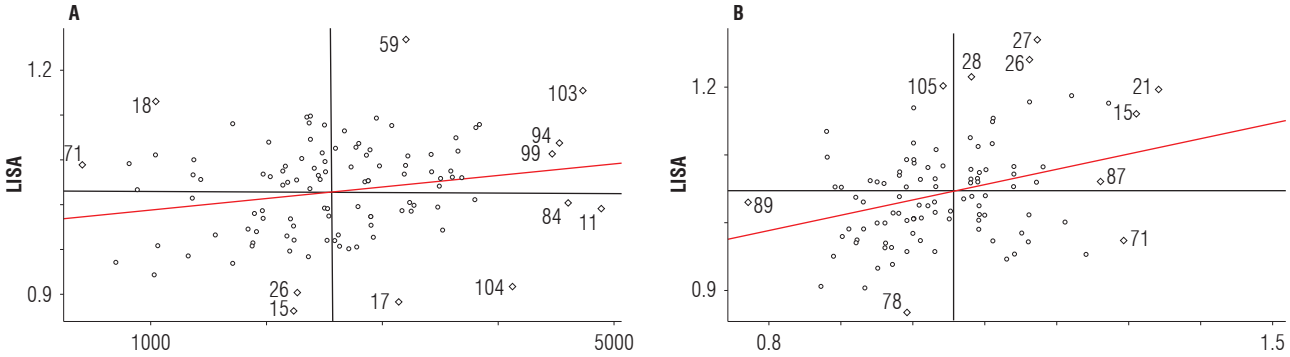

FIGURE 6. Moran graphics: A) number of fruits per tree, B) fruit weight.

TABLE 6. Parameters of the production models according to the selected MTSs.

Model	Slope	Interception	MTSs	Total area (ha)	R^2
Detected area	-4,423	7,376	7	6,482	0.79
Detected area and surrounding	-5,372	8,344	30	20,361	0.62
Study zone	-3,180	5,906	140	109,626	0.15

MTS – Minimum territorial subdivision.

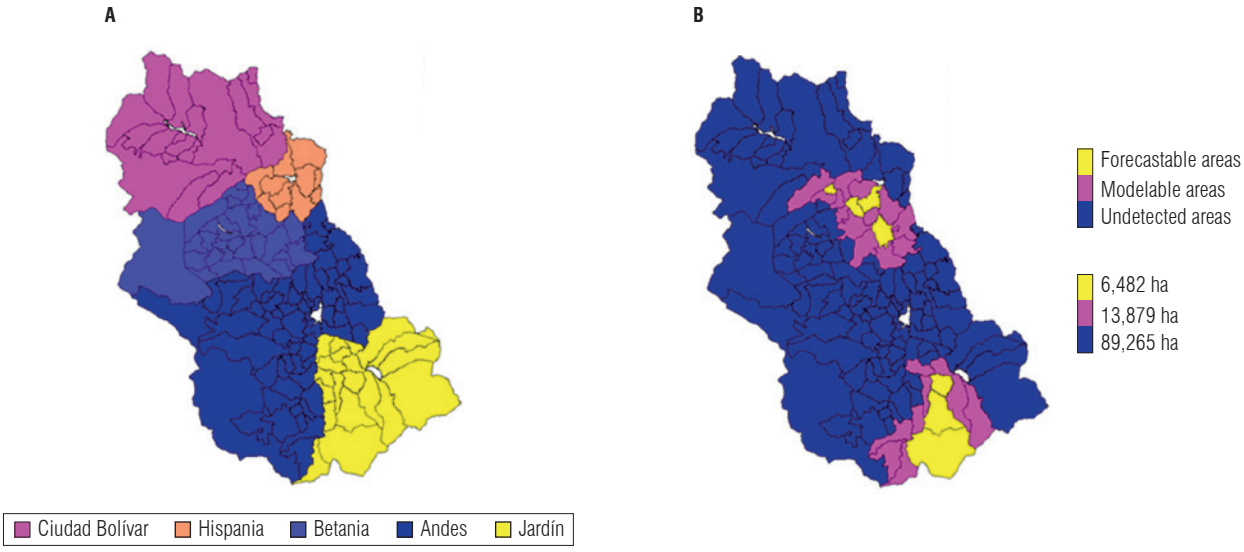

FIGURE 7. Location of the detected areas or MTSs: A) municipalities, B) detected areas.

in blue, the predictable areas in yellow (6,400 ha), and the modelable areas, which are the surrounding zones, in magenta (13,800 ha). Comparing the modelable area with a small municipality, such as Hispania, it can be observed that the area is slightly larger than the municipality. This finding implies that a space covering slightly over 20,000 ha has been identified where it is possible to apply a production model under the conditions of the present study.

Table 7 presents the most homogeneous MTSs according to spatial criteria and the relationship between the study variables.

The results suggest that a classifier of MTSs can be created according to their level of predictability. For the data in this study, the proposed model allows predicting production in an area of over 6,000 ha (R^2 = 0.79) and reducing uncertainty (R^2 = 0.61) to slightly over 20,000 ha, which constituted the most homogeneous set of MTSs and their surroundings. It should be noted that in the detected area, there was a wide variety of cultivars (Castillo, Colombia, Cenicafé 1, Caturra, Catimor, among others), in different stages of development and under different cultivation systems. Despite this, the methodology identified spatial patterns that make it possible to predict production using fruit weight as the only variable.

It is important to note that, from a territorial sampling perspective, the present approach required weighing only 30 fruits per tree, from three trees per lot, in three lots per farm, and three farms per MTS, to estimate coffee production in fruits per tree over an area of a little over 20,000 ha. This method can be easily implemented in the territory and represents a significant contribution to the coffee sector in mountainous areas, as it allows covering larger areas with minor adaptations to the parameters, resulting in practical and easy-to-apply models in rural areas.

Future projections and implications for regional coffee farming

The results of this study establish a solid foundation for improving agricultural planning in mountain coffee farming. Identifying areas with higher predictability allows for optimizing resource allocation, improving harvest planning, and reducing uncertainty in commercialization. However, to maximize impact and scalability, it is essential to incorporate advanced tools in computer vision and artificial intelligence.

Use of artificial intelligence and computer vision in yield prediction

The spatial analysis of coffee production can be enhanced with image detection and classification models, such as YOLO (You Only Look Once) (Bazame *et al*., 2021) and Mask R-CNN (Chen *et al*., 2019)which would allow them to allocate optimal labor and equipment, as well as other resources for harvesting, transportation, and marketing. Accurate estimation of the number of strawberry flowers and their distribution in a strawberry field is, therefore, imperative for predicting the coming strawberry yield. Usually, the number of flowers and their distribution are estimated manually, which is time-consuming, labor-intensive, and subjective. In this paper, we develop an automatic strawberry flower detection system for yield prediction with minimal labor and time costs. The system used a small unmanned aerial vehicle (UAV, to identify patterns in crop development. The integration of drone and satellite imagery with segmentation algorithms would enable:

- Estimating fruit load per tree through automated fruit detection in high-resolution images;
- Identifying phenotypic and developmental variability in large-scale plantations, correlating visual characteristics with productivity;

TABLE 7. Description of the detected MTSs.

Municipality	MTS	Area (ha)	Conversion (kg cherry coffee/kg dry parchment coffee)	Planting density (trees ha^{-1})	Average fruit weight (g)	Fruits per tree
Jardín	Macanas	3187	4.00	5935	1.31	2245
Jardín	Gibraltar	1310	5.40	4713	1.34	1045
Jardín	Verdún	522	5.38	5548	1.27	1552
Hispania	La Armenia	296	4.74	6573	0.93	3303
Hispania	La Seca	472	5.60	6987	0.95	2507
Betania	Las Animas	576	4.73	6497	0.93	3665
Betania	El Tablazo	119	4.36	6470	0.92	3485

MTS – Minimum territorial subdivision.

- Determining fruit maturity based on color changes, optimizing harvest timing;
- Monitoring crop health, detecting early signs of water stress, nutrient deficiencies, or pests.

Scalability and model expansion

Combining spatial data with computer vision models would extend yield prediction to larger territories, generating detailed information without the need for direct field measurements. To achieve this, key steps include:

- Expanding the collection of images and geospatial data, incorporating historical records and new sensors;
- Developing an automatic image labeling system, facilitating the creation of databases for training more accurate models;
- Integrating this methodology into agricultural monitoring platforms, allowing producers to access real-time predictions.

Conclusions

The proposed methodology allowed for identifying a regression model associated with coffee plant productivity, which estimates the number of its fruits based on a sample of 30 fruits, facilitating on-field monitoring of lot and farm productivity. The proposed methodology combines linear regression models and spatial analysis and could serve as a complementary tool for estimating coffee production in addition to traditional methods, contributing to the precision and reliability of coffee harvest forecasts in mountainous areas. The results of this study enable the identification of specific areas within the territory where coffee production can be jointly estimated. This result is valuable for farmers and cooperatives as it allows them to focus their efforts and resources on the most productive areas and better plan outreach activities led by cooperatives within the territory. The application of this methodology could facilitate more informed decision-making in mountainous coffee farming, enabling more sustainable and profitable practices in coffee production, benefiting both farmers and the sector. The incorporation of computer vision and machine learning in yield prediction would strengthen precision coffee farming, enabling more efficient and sustainable decision-making. This approach would position the region as a leader in agricultural innovation applied to mountain coffee farming.

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Conflict of interest statement

The authors declare no conflicts of interests related to the publication of this article.

Author contributions

CACV: conceptualization, methodology, software, data curation, writing – original draft preparation, visualization, research, writing – review & editing. IDAT: writing – review & editing. FJRC: methodology, writing – review & editing. ELMC: writing – original draft, writing – review & editing. All authors reviewed the final version of the manuscript.

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Native mycorrhization of onion in response to the application of bioinoculants, inorganic fertilization, and water deficit

Micorrización nativa de la cebolla en respuesta a la aplicación de bioinoculantes, fertilización inorgánica y déficit hídrico

Erika Lorena Blanco[1*], Luimar Álvarez[2], Yulimar Castro[3], María Eugenia Pulido[1], Fermín Rada[4,5], and Jorge Paolini[6]

ABSTRACT

Arbuscular mycorrhizal fungi are important components of the soil microbiota which interact with other beneficial microorganisms in the rhizosphere. The synergistic effect of this naturally occurring association between the soil and introduced microorganisms to improve growth and to cope with biotic and abiotic stresses plays a key role in crop productivity. In this research, the native mycorrhizal colonization of onion (*Allium cepa* L.) was evaluated in the presence of a plant growth promoting microbial consortium (MC), under inorganic fertilization and water deficit regimes, in a semiarid region of the Venezuelan Andes. The main objectives were to determine the colonization potential of arbuscular mycorrhizae and to quantify the presence of mycorrhizal spores in soil under field conditions. An onion crop was established with normal irrigation (NIr) (100% ETc) and water deficit (WD) (67% ETc), with different fertilization treatments (MC with 0%, 50%, and 100% NPK). Native mycorrhizal colonization was determined by counting vesicles and arbuscules in the roots of onion plants in all treatments, and mycorrhizal spores in the soil of the experimental plot were quantified before and after the trial in the rhizospheric soil for each treatment. The treatments showed no significant differences in native mycorrhizal colonization, but the soil spore count was higher in the MC 100% NPK NIr treatment. Additionally, the mechanization of the plot significantly reduced the presence of mycorrhizae in the soil, suggesting that greater implementation of non-conventional practices could improve preservation of biodiversity and increase soil health through agricultural management.

Key words: *Allium cepa*, mycorrhizae, plant-microorganism interactions, plant growth promoting microorganisms-PGPM, water stress.

RESUMEN

Los hongos micorrízicos arbusculares son componentes importantes de la microbiota del suelo e interactúan con otros microorganismos benéficos en la rizósfera. El efecto sinérgico de esta asociación que se produce de forma natural en el suelo y los microorganismos introducidos para mejorar el crecimiento y combatir el estrés biótico y abiótico, desempeña un papel clave en la productividad de los cultivos. En esta investigación se evaluó la colonización micorrízica nativa de la cebolla (*Allium cepa* L.) en presencia de un consorcio microbiano (CM) promotor del crecimiento vegetal, bajo un régimen de fertilización inorgánica y déficit hídrico, en una zona de cultivo semiárida en los Andes venezolanos. Los objetivos principales fueron determinar la colonización de micorrizas arbusculares y cuantificar la presencia de esporas micorrízicas en el suelo en condiciones de campo. Se estableció un cultivo de cebolla con riego normal (IrN) (100% ETc) y déficit hídrico (DH) (67% ETc) y diferentes tratamientos de fertilización NPK con y sin el consorcio microbiano CM (CM con 0%, 50% y 100% NPK). Se determinó la colonización de las micorrizas nativas mediante el conteo de vesículas y arbúsculos en las raíces de las plantas de cebolla en todos los tratamientos, y se cuantificaron las esporas micorrízicas en el suelo de la parcela experimental antes de establecer el ensayo, y luego del ensayo en el suelo rizosférico para cada tratamiento. Los resultados no mostraron diferencias significativas entre los tratamientos en cuanto a la colonización micorrízica nativa, pero el conteo de esporas en suelo resultó mayor en el tratamiento 100% NPK CM IrN. Se determinó que la mecanización de la parcela redujo significativamente la presencia de micorrizas en el suelo, sugiriendo que una mayor implementación de prácticas no convencionales preserva la biodiversidad e incrementa la salud de los suelos con manejo agrícola.

Palabras clave: *Allium cepa,* micorrizas, interacción planta-microorganismo, microorganismos promotores del crecimiento vegetal-MPCV, estrés hídrico.

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Introduction

Mycorrhizae are symbiotic associations generated between certain fungi and plant organs which are, in most cases, roots (Huey *et al*., 2020). This association increases the surface area of the roots, allowing the plants to absorb water and nutrients more efficiently from a large volume of soil (Sun *et al*., 2018).

Arbuscular mycorrhizal fungi (AMF) aid plants to establish and survive in unfavorable environments by different means, inducing greater tolerance of the roots to pathogens by acting directly as protectors of the root system (Liu *et al*., 2018), improving plant mineral nutrition and increasing plant biomass, and by improving plant-water relations, since mycorrhizae enhance resistance to water stress (Eroğlu *et al*., 2020). For these reasons, AMF inoculation is being incorporated as a strategy applied to agroforestry and silvicultural practices to colonize the root-soil environment, improve plant growth, yield, nutrient content, and soil fertility (Jacott *et al*., 2017).

Plant growth-promoting microorganisms (PGPM) in the rhizosphere are beneficial bacteria and fungi of great importance in Agronomy, due to their prominent role in sustainable agricultural practices (Brauer *et al*., 2019). Plant growth-promoting rhizobacteria (PGPR) help to improve plant growth and health in various ways (Dheeman & Maheshwari, 2022). The direct mechanisms involve the availability of mineral nutrients and production of phytohormones that contribute to plant growth and adaptation (Shome *et al*., 2022). PGPR can also act indirectly by eliminating or reducing the load of phytopathogens through biocontrol activity, *i.e*., secreting various growth inhibitors such as lytic enzymes, bacteriocins, and antibiotics or by inducing natural resistance of the host plants (Ehinmitan *et al*., 2024).

The coexistence of PGPM and AMF in the rhizosphere is highly beneficial for growth and development of most plants (Hashem *et al*., 2016). This synergistic effect is the result of positive interactions between these microorganisms, playing an important role in mitigating a wide range of biotic and abiotic plant stresses such as drought, salinity, heavy metal exposure and pathogens, in addition to increasing plant growth and yield (Soussani *et al*., 2023). Therefore, the evaluation of the inoculation of these beneficial microorganisms represents a favorable option for the sustainable management of ecosystems (Chamkhi *et al*., 2022). Their greatest potential lies in directly influencing the reduction of the use of pesticides and chemical fertilizers that harm the environment (Gupta, 2020).

Onion cultivation may require a great investment due to the high cost of certified seeds, industrial fertilizers containing NPK and agrochemicals and other inputs to maintain its production cycle. This means that its marketing costs could be excessive. In turn, these practices have significantly modified the ecological balance of soils and have altered soil microbial populations, acidified or increased the salt content, and decreased organic matter content in soil (Wolińska *et al*., 2017). The indiscriminate application of inorganic products to soil could be reduced by using microorganisms with biofertilizing, biocontrolling and biostimulating characteristics, which would enable sustainable crop production with the least use of chemical compounds (Blanco & Castro, 2021). In onions, inoculation with *Azospirillum* and mycorrhizae in combination with inorganic NPK fertilizer increased bulb diameter, bulb dry weight, total dry weight per plant, and bulb yield, as well as improved the cost-benefit ratio (Singh *et al*., 2017). Similarly, inoculation with two mycorrhizal species (*Funneliformis mosseae* and *Claroideoglomus etunicatum*) under drought conditions increased the content of proline, soluble proteins, and total carotenoids in plants (Muhsen *et al*., 2019). Therefore, it is important to determine how other native beneficial microorganisms (mycorrhizae) influence these processes and determine the effects they exert on the soil flora to improve their growth.

For the above reasons, our hypotheses were: 1) the growth of native mycorrhizae in soil increases in the presence of a bioinoculant and with a lower dose of industrial fertilizers, as this growth would be stimulated by the synergistic action of the beneficial microbial consortium (MC), and the colonization process would not be inhibited by the presence of agrochemicals; 2) native mycorrhization improves under conditions of water deficit, due to enhanced root growth, which enables plants to explore a greater soil volume for water and mineral nutrients. To validate these hypotheses, the aim of our study was to determine the native mycorrhizal colonization of onion (*Allium cepa* L.) in the presence of a native plant growth-promoting MC of the Venezuelan Andes, under an inorganic fertilization regime and water deficit conditions in a semiarid region. Based on previous research, we aimed to determine whether there was a synergy between the PGPMs used and the native mycorrhizae of the experimental plot and whether this influenced the favorable effects of MC on the onion crop. This will allow us to stimulate the preservation of biodiversity and increase the quality of soils through agricultural management.

Materials and methods

Study site and experiment design

The field experiment was carried out from February to mid-June 2017 at the IIAP-ULA Experimental Station in San Juan de Lagunillas, Mérida, Venezuela, at 1,100 m a.s.l. (UTM coordinates 8º30'75'' N; 71º20'28'' W). The soil of the plot corresponded to an Aridisol (USDA, 2020). The experimental site was characterized by an average rainfall of 509 mm, with two maximum peaks in April and October, average temperature of 23.7ºC, annual evaporation around 1,500 mm, vastly exceeding precipitation, so that crops require irrigation year-round (Blanco, Rada, Paolini *et al*., 2021). The soil characteristics (15-cm depth) were: sandy loam texture, water retention capacity 27%, organic matter 3.6%, pH 6.0, electrical conductivity 0.14 mS cm^{-1}, total N 0.12%, available P 30 mg kg^{-1}, K 568 mg kg^{-1}, Ca 2060 mg kg^{-1} and Mg 1872 mg kg^{-1} (Blanco, Rada, Paolini *et al*., 2021). The experimental plot was prepared by mechanization through disc plow and harrow passes, and the application of a pre-emergent and selective herbicide (ai: S-metolachlor) at a dose of 2 L ha^{-1} for weed control, one month before seedling transplant. Weeding during the trial was done manually, and irrigation was localized. An onion hybrid, F1 2000, was used, and seedbeds were prepared as described by Blanco, Rada, Paolini *et al*. (2021). These plants were transplanted 45 d after sowing (das) in six rows, for a total of 120 plants per bed and corresponding to a density of 360,000 plants ha^{-1}. Seedlings were planted in 2.4 m long x 1.4 m wide beds (3.36 m^2), spatially separated from each other by a trough to avoid contamination between treatments.

Microbial inoculum

The bioinoculant used corresponded to a microbial consortium (MC) of the strains ME01 (*Rhizobium tropici*) + Leu2A (*Bradyrhizobium japonicum*) selected for their plant growth-promoting effects on *A. cepa* seedlings (Blanco, Rada, Castro *et al*., 2021). The bioinoculated treatments received 2.5 ml of the MC ($1x10^8$ cells ml^{-1}) per seedling on day of transplant, and were reinoculated with 5 ml of the inoculum at the same concentration in the field, 15 d after transplant (dat). The applied MC belonged to the strain collection of the Laboratorio de Fitobiotecnología of the Universidad de Los Andes, Venezuela.

Experimental design

A 2×4 bifactorial design in completely randomized blocks with 3 replicates was used in the experiment. The irrigation factor consisted of two frequencies which corresponded to a given evapotranspiration value (ETc): NIr (daily irrigation; 100% ETc) and WD (irrigation every 3 d; 67% ETc) beginning 20 dat, this latter frequency corresponded to severe water stress (Blanco, Rada, Paolini *et al*., 2021). The fertilization factor consisted of four levels referenced to 100% of the nutrient requirement of NPK for onion yielding 40 t ha^{-1}. For this reference, net extraction per ha was 247 kg N, 240 kg P_2O_5 and 240 kg K_2O, which corresponded to 577 g of inorganic fertilizer with a commercial formula 15-15-15 NPK (ammoniacal N 10.3%, nitric N 4.7%; assimilable phosphorus 15%; potassium chloride 15%) plus 5.4 g of urea for each bed (3.36 m^2) (Blanco, Rada, Paolini *et al*., 2021). These fertilization rates were combined with the application of the MC biofertilizer, resulting in treatments as follows: MC+0% NPK, MC+50% NPK, MC+100% NPK, and 100% NPK (non-inoculated control). The fertilization schedule is given in Blanco, Rada, Paolini *et al*. (2021).

Native mycorrhizal colonization of onion roots

Root sampling of *A. cepa* plants was carried out during the experiment to evaluate the percentage of native colonization and the presence and number of mycorrhizal spores in the rhizospheric soil. For this, root sampling was carried out according to Sánchez de Prager (1999). Four plants were chosen at random, and root samples were collected from the first 10 cm, placed in polyethylene bags, and taken to the laboratory, where they were washed with distilled water to eliminate any rhizospheric soil residues.

Roots were stained using the procedure described by Phillips and Hayman (1970), which consisted of five steps to obtain an ideal staining and observe the mycorrhization within the roots: 1) thinning of the previously washed rootlets: very thin rootlets were placed in test tubes and 10% KOH was added to cover them and heated for 10 min in boiling water. Afterwards, the KOH was removed, and the rootlets were washed five consecutive times with distilled water; 2) bleaching of the roots: 10% H_2O_2 was added to cover the roots for 3 min, then removed, followed by seven consecutive washes with distilled water until the excess was removed; 3) acidification: the roots were covered with 10% HCl for 3 min at room temperature, and then washed five consecutive times until the acid was completely removed according to Sánchez de Prager (1999); 4) staining the roots: the roots were covered for 10 min with trypan blue (0.05%) and then heated in a boiling water bath for 15 min. The dye was then removed without rinsing; 5) decolorization of the roots: the roots were treated with lactoglycerol (lactic acid, glycerol, and water in a 1:1:1 ratio) and processed to determine the percentage of mycorrhizal colonization.

To determine the percentage of mycorrhizal colonization, stained roots were mounted on slides for evaluation under an optical microscope (McGonigle *et al*., 1990). The roots were placed in Petri dishes for better handling and eight segments approximately 1 cm long were taken using dissection needles and placed parallel to each other on a slide. Drops of clean lactoglycerol were added to keep the roots moist and preserve them for a longer time. A coverslip was placed on each slide, air bubbles were removed, and the edges were sealed with nail polish, allowing them to dry for 5 min. Finally, three parallel and equidistant lines were marked on the back of the slide, perpendicular to the rootlets to be examined under the microscope. At each intersection between the lines and the rootlets, the presence of vesicles and/or arbuscules was recorded, regardless of the intensity of mycorrhization. A value of one (1) was given for the presence and zero (0) for the absence of these structures. Colonization was determined with the following equations:

$$\text{Vesicular colonization (\%)} = \frac{\text{Nº of segments with vesicles}}{\text{Total Nº of segments}} \times 100 \quad (1)$$

$$\text{Arbuscular colonization (\%)} = \frac{\text{Nº of segments with arbuscles}}{\text{Total Nº of segments}} \times 100 \quad (2)$$

Quantification of mycorrhizal spores in the soil

For the isolation and quantification of mycorrhizal spores in the rhizosphere, soil from the chosen plants was used to determine native mycorrhizal colonization of the roots, following the method described by Sánchez de Prager (1999). The soil was obtained by inserting a gardening shovel vertically adjacent to the bulb, at a depth of approximately 4-8 cm. Using these subsamples for each treatment and replicate, a composite sample of more than 100 g was prepared and placed in previously labeled polyethylene bags, transported in ice containers to the laboratory and kept at a temperature of approximately 6ºC until processed.

For the separation of spores from the soil, a centrifugation in sucrose method was used (Sieverding, 1991). One hundred g of rhizospheric soil were introduced into a beaker with approximately 2,000 ml of distilled water. The sample was stirred on a stirring plate for 5 min and left to rest for 3 min to eliminate large particles by sedimentation. The suspension was passed through a series of sieves between 500 and 44 µm and washed with plenty of water. This process was repeated twice consecutively to collect the spores on a 44 µm sieve. The spores were transferred to a 50 ml centrifuge tube, and the volume was completed to 20 ml of distilled water. The sample was then centrifuged at 1800 rpm for 2 min to eliminate the supernatant. Twenty ml of distilled water and 20 ml of a 50% sucrose suspension were injected into the bottom of the centrifuge tube, taking care not to disturb the gradient, and again centrifuged at 3500 rpm for 2 min until a central ring formed within this gradient. Spores were extracted from this ring using 5 ml pipettes. They were placed on the 44 µm sieve, washed thoroughly with sterile distilled water and placed on a Doncaster plate to quantify them in a stereoscopic microscope (Thz75 Leica model 1446275). Additionally, spore counts were performed in the non-rhizospheric soil (before the test) and in non-rhizospheric soil without fertilizers or inoculate under the two irrigation regimes (after the test) to determine the effect of mechanization of the plot on the presence of mycorrhizae.

Statistical analysis

The results were analyzed by analyses of variance (ANOVA) and least significant difference test (LSD test) ($P<0.05$) using the statistical package Statgraphics Centurion XVI (2009). Correlations between the percentage of vesicle colonization and the number of spores were established with the statistical program PAST version 3.0 (Hammer *et al*., 2001).

Results

Percentage of vesicle colonization in roots

The results of mycorrhizal colonization showed only the presence of endomycorrhizal vesicles; no arbuscules were present in any of the samples. No significant differences were found between treatments for the presence of vesicles ($P>0.05$). However, the LSD test showed a slight tendency for a higher percentage of colonization in the roots in the 50% NPK + MC treatment under both irrigation conditions compared to the other treatments. The highest percentage (89.9%) corresponded to the 50% NPK + MC (WD) treatment, while the lowest (73.6%) corresponded to the 100% NPK + MC (WD) treatment (Fig. 1).

Spore quantification

The number of spores found after screening the soils is shown in Figure 2. The highest spore count (53.07%) was obtained in the 100% NPK + MC treatment under normal irrigation, while the lowest value (10%) was observed in the 0% NPK + MC and 100% non-inoculated NPK treatments (production control) under the same irrigation conditions. Significant differences were found between the eight treatments analyzed, with a greater number of spores in the soil

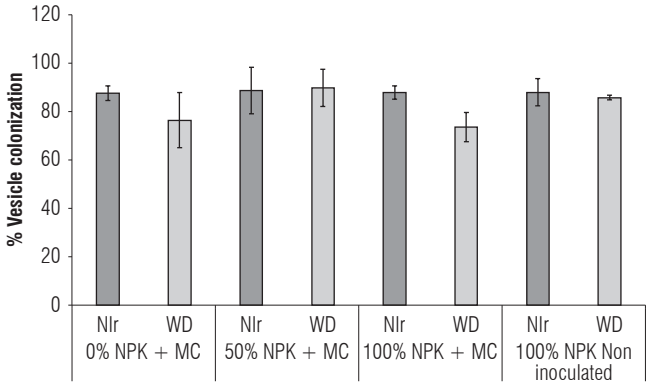

FIGURE 1. Percentage of vesicle colonization in *A. cepa* roots subjected to different treatments of inorganic NPK fertilization + MC (ME01+Leu2A (*Rhizobium tropici* + *Bradyrhizobium japonicum*) under water deficit conditions. NIr = normal irrigation 100% ETc, WD = water deficit 67% ETc. MC = microbial consortium. Error bars indicate the standard deviation.

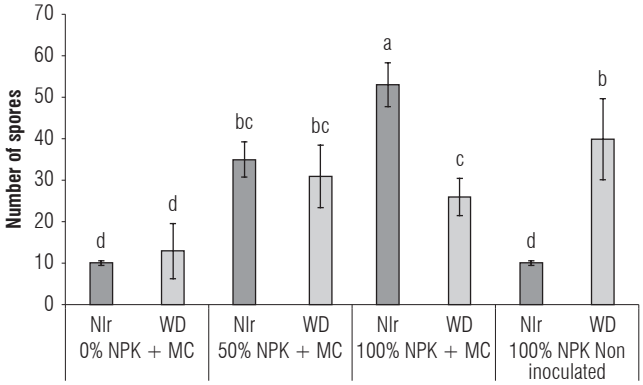

FIGURE 2. Number of spores per 100 g of rhizospheric soil of *A. cepa* for the different treatments of inorganic NPK fertilization and inoculation with biofertilizers under conditions of water deficit. MC = microbial consortium, NIr = normal irrigation, WD = water deficit. The percentage represents the applied dose of NPK fertilizer. Mean values (n=3). Different letters indicate statistically significant differences between treatments and conditions at a confidence level of 95% according to the LSD test. Error bars indicate the standard deviation.

under conditions of complete fertilization, inoculation with MC and normal irrigation.

No correlation was found between the percentages of root colonization and the number of mycorrhizal spores (P>0.05). Additionally, Figure 3 shows the spores isolated from the soil, as observed under the stereoscopic microscope (Figs. 3A-B), along with the vesicles observed on the onion roots (Fig. 3C). No arbuscules were observed in the root tissue.

Effects of mechanization on soil mycorrhizal populations

Figure 4 shows the number of mycorrhizal spores one month before the trial, when no mechanization had been applied to the plot, and after the trial (65 dat), at the end of the *A. cepa* bulbing stage. A decrease of 41.94% in the number of spores under NIr and 56.07% under WD conditions was detected after mechanization of the land, which implies an almost 50% reduction in the population of mycorrhizae in the soil due to the plowing prior to the trial.

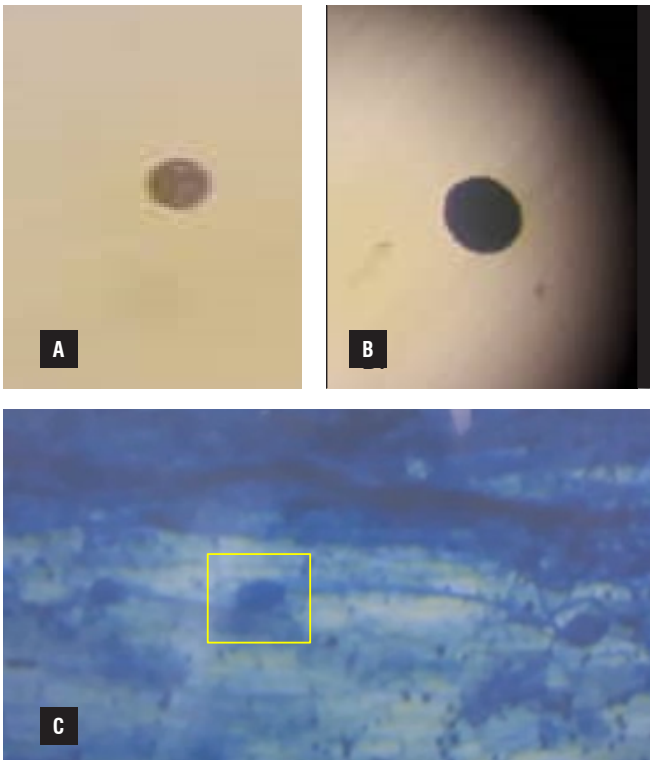

FIGURE 3. Images of mycorrhizal structures observed in the soil and roots of *A. cepa*. A and B) soil spores observed under a 500X stereoscopic microscope, C) vesicles in root tissue observed under a 400X microscope.

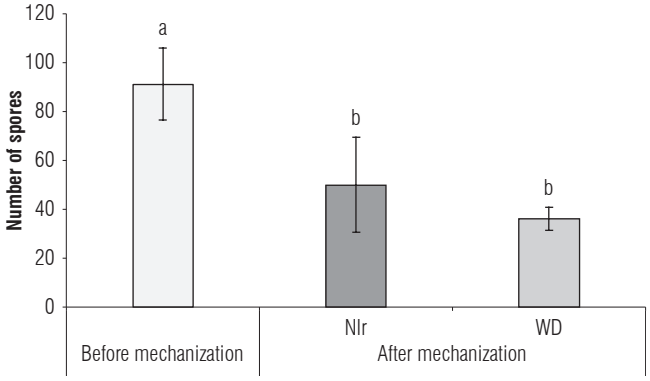

FIGURE 4. Number of spores per 100 g of non rhizospheric soil prior to the mechanization (reference values) and after mechanization (0% NPK Non inoculated) under irrigation conditions: Nir = normal irrigation, WD = water deficit. Mean values (n=3). Different letters indicate statistically significant differences at a 95% confidence level according to the LSD test. Error bars indicate the standard deviation.

Discussion

Tolerance to water stress is an important issue for crops, especially in arid environments. Onion cultivation is severely affected by periods of drought, which can reduce growth, yield and quality parameters (Bolandnazar *et al*., 2007). In this study, the WD condition used did not show a significant negative effect between treatments or on the presence of endomycorrhizal vesicles in onion plants. The absence of significant differences in the colonization percentage suggests that the positive effect on onion growth and development in the 50% NPK + CM treatments with respect to yield, CO_2 assimilation rate, leaf N content, etc., as shown by Blanco, Rada, Paolini *et al*. (2021) and Blanco, Rada, and Paolini (2023), was due solely to the presence of MC and not to a symbiosis between the beneficial bacteria in the MC and native mycorrhizae. According to Álvarez and Reyes (2018), effective mycorrhization during crop establishment depends on greater mycotrophy by AMF, which in our study did not occur for the F1 2000 hybrid onion. In another study with the pink Creole variety, mycorrhizal colonization with the applied mycorrhizal inoculum increased by more than 400% over the non-inoculated treatments, even though these latter treatments also showed smaller quantities of mycorrhizal hyphae. This suggests that mycorrhizal populations are naturally in the soil (Arandia *et al*., 2020) and can colonize this species, as shown in our study.

The highest number of spores occurred in the 100% NPK + MC treatment under the NIr condition, a treatment that had neither water nor nutrient deficit and was enriched with the inoculation of MC. The higher number of spores present in this treatment show that their germination could have been inhibited due to the full dose of fertilizers plus MC, which caused toxicity on the microbial metabolism, as demonstrated by Blanco (2021) during the determination of microbial activity variables in the soil in this same field trial.

According to our results, the native mycorrhizal population in the soil was considerably affected by field mechanization. Bowles *et al*. (2017) showed that no-plowing enhanced AMF activity and diversity by improving soil fertility, water storage and conservation, while reducing soil erosion. In contrast, long-term conventional plowing decreased AMF richness and induced a marked alteration in community composition by changing the functional quality of AMF, spore density, vesicles, or hyphal networks (Schalamuk *et al*., 2004; Zhang *et al*., 2015). The decrease in the number of spores in soil after field mechanization suggests, as reported in other studies, that soil mechanization negatively affects the native mycorrhizal population. Furthermore, the agronomic management of the plot between mechanization and the establishment of the trial could have inhibited the reestablishment of the native mycorrhizal population, since the soil was kept bare and herbicides were applied (Blanco, 2021). However, spores may be more resistant than other propagules and could remain in the soil longer throughout the crop cycle, compared to vesicles, whose main function is storage (Zangaro *et al*. 2012). According to Cuenca (2015), identifying a mycorrhizal species solely based on the morphology of the spores is insufficient; molecular information is needed for correct classification. Therefore, we choose not to suggest any genus for the spores observed in our study.

Several studies have reported the ability of AMF to enhance plant growth and yield under stress, promoting tolerance to adverse conditions through mechanisms such as improving water use efficiency and nutrient acquisition through the production of hormones and plant growth regulators, enhancing photosynthetic rate, regulating ionic balance, and producing antioxidants (Campanelli *et al*., 2013; Huey *et al*., 2020; Nasslahsen *et al*., 2022; Wahab *et al*., 2023). However, in our study, the interaction between native mycorrhizae and *A. cepa* was not significant in influencing production, as evidenced by the similarities found between the treatments with respect to the colonization. This indicates that there was no synergy between MC and native mycorrhizae, which also depends on the characteristics of the soil, especially its nutritional conditions. In our case, the soil was fertile, with high native biological activity (Blanco, 2021). The main effect of the synergism between mycorrhizae and PGPB is to enhance a generalized stimulation of the host's nutrition, although more local effects may occur at the root level, especially during the pre-colonization stage, when the two microorganisms interact as rhizospheric residents, or during the development of the tripartite symbiosis. The host genotype also influences this interaction (Spagnoletti *et al*., 2013).

Conclusions

The study showed that the effect of native mycorrhization of onion plants was not significant for the variables evaluated, but it confirmed that the positive effect of microorganisms on onion growth shown previously in the same field trial was due only to the action of the inoculated MC (*Rhizobium tropici* + *Bradyrhizobium japonicum*) and not to the additional action of native mycorrhizae. Soil mechanization significantly reduced the number of spores present in the soil, so it is recommended carrying out other land

conditioning procedures that are more environmentally friendly for the native microbiota.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author´s contributions

Conceptualization: ELB, FR, JP. Research: ELB, LA, YC. Methodology: ELB, LA, YC, MEP. Field data collection: ELB, YC. Laboratory analysis: ELB, LA, MEP. Data curation: ELB, LA, MEP. Data analysis: ELB. Writing – original draft: ELB, LA, YC. Writing – reviewing and editing: all authors. All authors have read and approved the final version of the manuscript.

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Determinants of the commercialization of urban agricultural products in Bogotá: A case study of the Mhuysqa indigenous cabildo of Bosa

Determinantes de la comercialización de productos de la agricultura urbana en Bogotá: estudio de caso del cabildo indígena Mhuysqa de Bosa

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ABSTRACT

Since the beginning of the 21st century, the Botanical Garden of Bogotá (Colombia) has been promoting urban agriculture in urban and peri-urban areas. The Mhuysqa (Muisca) indigenous cabildo of Bosa, among other communities, has been practicing this activity for more than a decade. Although their production is mainly for self-consumption, there are some surpluses that are sold. Because the commercialization of urban agriculture products has certain limitations, this research aims to identify and analyze its determinants, taking the Mhuysqa indigenous community as a case study. The data collection methodology is based on documentary research, observation of gardens, surveys of 33 gardeners and interviews with four experts. The analysis is based on descriptive statistics, content analysis and the use of non-formal logic. The results show that urban agriculture in the cabildo is multifunctional, and the surplus for sale is low (< 30%). The determinants of commercialization are: the establishment and fulfillment of objectives and goals; volumes, quality and frequency of supply; knowledge and specialized technical production capacities; quality of presentation and freshness of the product; costs of collection and transport; forms and means of payment; and fixed buyers for market development. A key determinant is the explicit and clear intention of the cabildo, as an organization, to market its surplus products from urban agriculture.

Key words: planning, production, product preparation, transportation, sale.

RESUMEN

Desde inicios del siglo XXI, el Jardín Botánico de Bogotá (Colombia) ha venido promoviendo la agricultura urbana en la ciudad y zonas periurbanas. El cabildo indígena Mhuysqa (Muisca) de Bosa, entre otras comunidades, practica esta actividad desde hace más de una década. Si bien su producción se destina principalmente para autoconsumo, hay unos excedentes que se venden. Debido a que la comercialización de los productos de la agricultura urbana presenta ciertas limitantes, esta investigación se propone identificar y analizar sus determinantes tomando como caso de estudio a la comunidad indígena Mhuysqa. La metodología de recolección de información se fundamenta en la investigación documental, observación de huertas, encuesta a 33 huerteros y entrevista a 4 expertos. El análisis es de estadística descriptiva, análisis de contenido y uso de lógica no formal. Los resultados muestran que la agricultura urbana del cabildo es multifuncional y el excedente para venta es bajo (< 30%). Por su parte, los determinantes de comercialización son: el establecimiento y cumplimiento de objetivos y metas; volúmenes, calidades y frecuencias de oferta; conocimiento y capacidades técnicas especializadas de producción; calidad de presentación y frescura del producto; costos de acopio y transporte; formas y medios de pago; y compradores fijos para desarrollo de mercados. Un determinante clave es la intención explícita y clara del cabildo, como organización, de comercializar sus productos excedentes de la agricultura urbana.

Palabras clave: planeación, producción, alistamiento de producto, transporte, venta.

Introduction

Urban agriculture in Bogotá

Currently, 56% of the world's population lives in urban areas, and by 2050, this figure is expected to reach 70% (Banco Mundial, 2024). At the same time, agricultural land per capita is decreasing drastically; in 1961, there was 0.36 arable ha per person, a figure that fell to 0.18 ha in 2021. In Colombia, the situation is even more worrying, with agricultural land per capita falling from 0.22 to 0.04 ha per person between 1961 and 2021 (Banco Mundial, 2024). This reduction affects sustainable food production and both urban and rural food security. In this context, urban agriculture (UA) emerges as a viable solution to

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grow vegetables, fruits and medicinal plants in small spaces (Centrone Stefani *et al*., 2018; Genovese *et al*., 2018). UA not only seeks to ensure local food security but also contributes to employment generation, environmental education and urban regeneration (Abegunde, 2012; Bojacá & Schrevens, 2010; Leandro, 2013; Orsini *et al*., 2014). Furthermore, by decreasing the number of intermediaries and optimizing the relationship between producers and consumers, it improves social inclusion, involving vulnerable communities (Jansma & Wertheim-Heck, 2021). UA also enhances resilience to climate change and promotes health and well-being through healthy habits and social cohesion, strengthening the local economy through commercial partnerships. However, it faces significant challenges, such as lack of urban planning, population growth, and extreme weather events, which require more effective marketing strategies for success (Abegunde, 2012; Agyeman & McEntee, 2014; Orsini *et al*., 2013; Weidner *et al*., 2019).

Urban agriculture in Bogotá, institutionally, has evolved since its beginnings in 1992 with the creation of the Botanical Garden of Bogotá "José Celestino Mutis", initially focused on ornamental plants in the urban environment. Over time, various policies have been implemented to promote food security and agricultural practices (Acuerdo 39 of 1992; Acuerdo 040 of 1993; Decreto 984 of 1998; Núñez & Cuesta, 2007). In 2004, the plan "Bogotá without indifference" promoted these initiatives, and subsequent development plans (2008-2012, 2012-2016, 2016-2020, and 2020-2024) have reinforced this approach, prioritizing food sovereignty, farmer networks, environmental sustainability, and economic reactivation. Strategies include the implementation of organic gardens and agroecological practices in urban spaces, seeking to mitigate climate change and foster social inclusion (Acuerdo 119 of 2004; Acuerdo 308 of 2008; Acuerdo 489 of 2012; Acuerdo 605 of 2015; Acuerdo 645 of 2016; Acuerdo 761 of 2020).

Urban agriculture in Bogotá is led by the District Secretariat of Environment and the Botanical Garden of Bogotá "José Celestino Mutis", which is responsible for technical advice and research (Horticultora60, 2016). Since 2004, the Botanical Garden has trained more than 60,000 people and supported more than 20,000 vegetable gardens, including community and home gardens. However, despite the institutionalization of this form of agriculture through District Agreement 605 in 2015, the city has seen a decrease of more than 20% in the number of urban and peri-urban farmers. In response, Project 7681 has been approved, which seeks to strengthen agriculture in Bogotá through the creation of planting spaces, orchard parks and agricultural parks, and improve productivity with technical assistance. It also proposes recovering ancestral species, implementing sustainable techniques, associating farmers and establishing agroecological routes, as well as connecting gardeners with peasant markets (Jardín Botánico de Bogotá, 2020).

Problem and research objective

Urban vegetable gardens generate food, medicines, aromatic herbs and condiments for self-consumption, as well as surpluses for commercialization. According to the Botanical Garden of Bogota, these vegetable gardens are classified as home, community, school, and institutional (Lara *et al*., 2022). The sale of surplus products is usually carried out in "peasant markets" and other agri-food markets (Parrado-Barbosa & Molina, 2014), which helps generate jobs and additional income for the families involved, promoting the development of urban agriculture in Bogotá and its social, economic and environmental benefits. However, marketing is a complex process, especially for urban agricultural products (Cattaneo & Lipshitz, 2008). Project 7681 (Strengthening Urban and Peri-Urban Agriculture in Urban Localities of Bogot**á**) has addressed this problem, establishing the goal of designing a promotion and marketing strategy for these products in conjunction with peasant markets. Within the framework of this project, and in order to meet Goal 11, among others, between December 2020 and September 2021, the "Specific cooperation agreement was signed between the Faculty of Agricultural Sciences of the Universidad Nacional de Colombia, Bogotá campus, and the Botanical Garden of Bogotá "José Celestino Mutis", whose objectives, among others, focused on strategies for the promotion and commercialization of urban agriculture products from the Cerros Orientales, Altos de la Estancia Polygon (Ciudad Bolivar), and the Mhuysqa indigenous cabildo (a self-governing authority) of the town of Bosa. This is where the contact with the cabildo originated.

The Mhuysqa indigenous community of Bosa has eight community gardens and 39 home (family) gardens. The production of these 47 gardens is mainly oriented toward self-consumption, although about five family gardens have been commercializing their surpluses. Based on this experience and considering the challenge of improving the commercialization of urban agriculture products, this research aims to identify and analyze the determinants of the commercialization of urban agriculture products, taking the Mhuysqa indigenous community as a case study.

Materials and methods

Geographical location of the study

Bogotá City District has 20 localities, one of which is Bosa. Bosa is the seventh (7th) locality, located in the southwest of Bogotá, with 823,041 inhabitants and an area of 2,466 ha. The indigenous cabildo Mhuysqa is the self-governing authority for Mhuysga community located there (Bogotá, 2024). The urban orchards are distributed throughout Bosa, although three of them lie outside its boundaries (Fig. 1). The locality's geographic characteristics are as follows: average altitude of 2,544 m a.s.l., flat topography with a very low slope, average annual rainfall 594 mm, moderately cold climate, average temperature 15°C, alluvial soils, and colluvial river deposits. The area is bordered to the north and west by the Bogotá and Tunjuelo rivers, respectively (Bogotá, 2024).

Information required

The information required relates to the following subtopics:

- General determinants of urban agriculture;
- Characteristics of urban agriculture in the indigenous cabildo Mhuysqa;
- Determinants of the commercialization of agricultural products;
- Determinants of the commercialization of the products of urban agriculture in the cabildo.

Sources of information

The sources of information are both primary and secondary. The primary sources include the representatives of 33 home gardens (of the 47 in the cabildo). Additionally, input was gathered from four experts in urban agriculture, three from the Botanical Garden of Bogotá and one authority from the Mhuysqa indigenous cabildo. The secondary sources consist of bibliographic documents and reports related to the research topic.

Methods of data collection

Literature review

A systematic search of specialized literature was carried out in the databases of the Universidad Nacional de Colombia and search engines such as Google, Google Scholar, and Semantic Scholar. The search terms included: "agricultura urbana", "urban agriculture", "mercadeo de productos agrarios", "marketing of agricultural products",

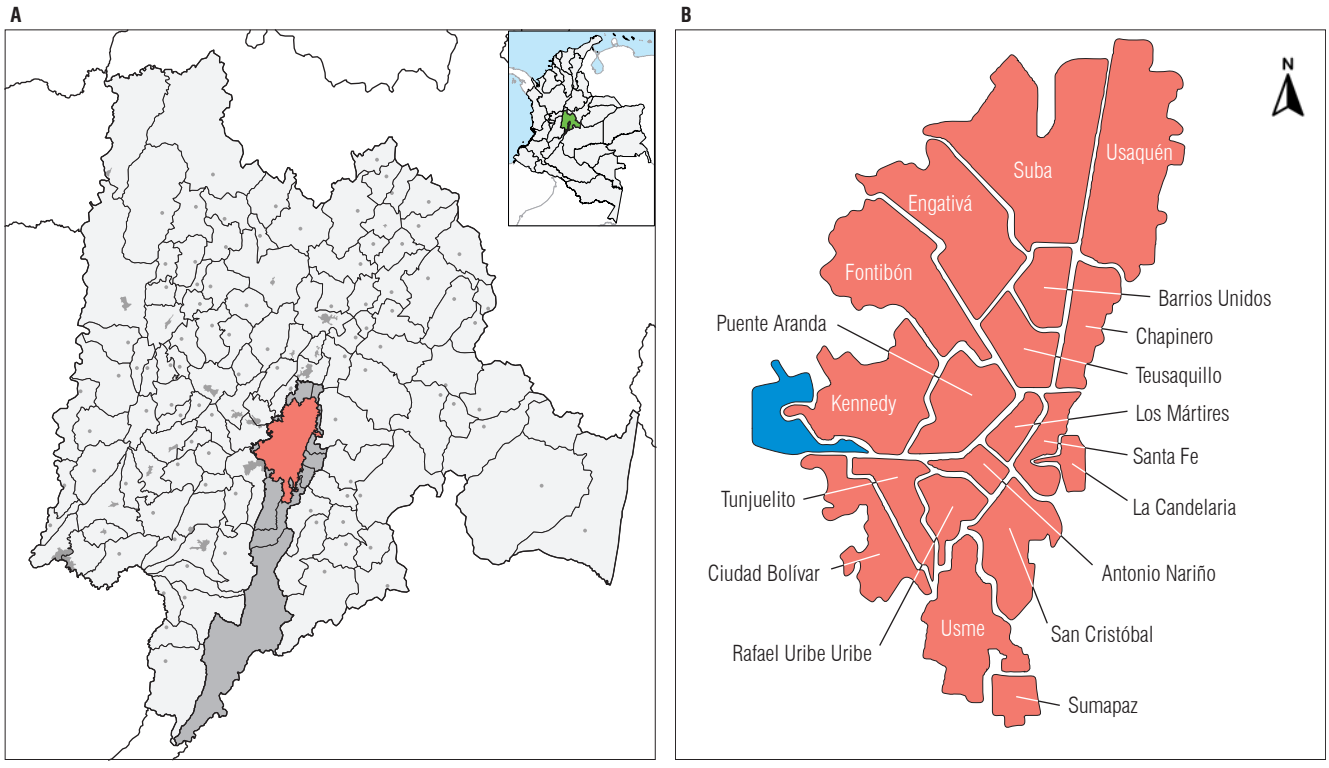

FIGURE 1. Department of Cundinamarca (green) (A), location of Bogota (red) (A), and location of the study area of the Mhuysqa indigenous cabildo of Bosa (blue) (B). Source: Wikipedia, 2024; Bogota, 2024; own elaboration.

"determinantes de mercadeo agropecuario", "determinants of agricultural marketing", "mercadeo y agricultura urbana", "marketing and urban agriculture", and variants of these keywords. In addition, books on the marketing of agricultural products and two (2) reports from the project "Urban and peri-urban agriculture with a differential approach in communities of Bosa, Ciudad Bolivar, and Cerros Orientales de Bogotá" were consulted.

Survey

Within the framework of the project "Urban and peri-urban agriculture with a differential approach in communities of Bosa, Ciudad Bolivar, and Cerros Orientales de Bogotá", 33 surveys were conducted among vegetable gardeners (four from community vegetable gardens and 29 from family vegetable gardens). The sample was selected by convenience from 47 vegetable gardeners participating in the project, including those who showed willingness to take the survey. The survey was conducted between May and August 2021 through a questionnaire that addressed the following topics: information about the vegetable gardeners, information about the vegetable garden, distribution of production, consumption of vegetable garden products, organization of the vegetable gardeners and constraints of urban agriculture.

Observation of vegetable gardens

At the time of conducting the surveys, observations of the vegetable gardens were made to obtain a complete picture of the vegetable garden. This method was used to corroborate and clarify the information provided by the vegetable gardeners.

Interviews

Interviews were conducted on topics similar to those of the survey, with a semi-structured questionnaire, with four experts in urban agriculture, three from the Botanical Garden of Bogotá (financiers of the project) and one authority of the Mhuysqa indigenous cabildo.

Methods of information analysis

The information from specialized documents (academic articles, books, reports) was organized into Tables 1 and 5 to support the topics: General determinants of urban agriculture and Determinants of the commercialization of agricultural products. The results of the surveys were systematized in Tables 2, 3 and 4, and the quantitative information was analyzed using descriptive statistics in Microsoft Excel. The information from the interviews was used to complement the results of the surveys, while the remaining analysis was conducted using logic tools.

Results and discussion

General determinants of urban agriculture

According to Indraprahasta (2013), in many countries, the development of urban agriculture has been institutionally promoted by the state. Cantor (2010) suggests that urban agriculture was primarily intended to provide food and income; however, he also recognizes that urban agriculture serves other purposes. Follmann *et al*. (2021), Kuusaana and Eledi (2015), and Marçal *et al*. (2021) also state that urban agriculture, like conventional agriculture, is multifunctional. Mackay (2018), Moustier and Renting (2015), and Thebo *et al*. (2014) clarify that the growth of the city has been displacing urban agriculture with other activities with higher returns. Finally, Follman *et al*. (2021) argue that the access of urban agriculture products to the market depends on several factors including technical, economic, social and political aspects. The following is a systematization of the most relevant factors of urban agriculture identified by these authors.

For urban agriculture to develop, certain conditions must be met across various areas, such as technical, economic, social, political and even environmental aspects. In general terms, Table 1 shows that the technical scope deals with

TABLE 1. Determinants of urban agriculture.

Scope	Determining factors	Sources
Technical	Size and location of the vegetable garden	Follman *et al.* (2021)
	Knowledge and access to technology	Odame *et al.* (2020)
	Access to and dependence on external inputs	Schmidt *et al.* (2015)
	Technical management of the vegetable gardens	Follman *et al.* (2021)
Economic	Volume and diversity of production	Cantor (2010), Degenhart (2016)
	Level of self-consumption	Cantor (2010), Chica Gómez (2022)
	Marketing channels	Ferrer *et al.* (2020)
	Types of transactions	Ferrer *et al.* (2020)
Social	Social profile of the vegetable gardeners and their families	Follman *et al.* (2021)
	Organization and governance	Cantor (2010), Chica Gomez (2022)
	Social networks	Ferrer *et al.* (2020)
Political	Public policies for social development	Cantor (2010)
	Public policies for urban agriculture	Rodríguez Pava & León Sicard (2018), Conpes (2019)
	Institutional programs for urban agriculture	Rodríguez Pava & León Sicard (2018)
	Development of marketing channels	Indraprahasta (2013)

Source: Authors based on the sources in the Table.

production, the economic scope with the market and the distribution (marketing) of production, the social scope with the vegetable gardeners and their organization, and the political scope with the intervention of the Colombian state in the promotion and development of urban agriculture. These determinants of urban agriculture will serve as a frame of reference for analyzing the determinants of urban agriculture in the case study, especially those related to the commercialization of its products.

Characteristics of urban agriculture in the Mhuysqa indigenous community

The following are the technical, economic, social, environmental and legal characteristics of urban agriculture in the Mhuysqa indigenous cabildo.

Technical aspects

The vegetable gardens stand out for their multifunctionality; beyond producing food and medicines, they serve as spaces for the education of children and young people, environmental care, recreation, and tourism. Another characteristic is the destination of production: urban vegetable gardens primarily produce for self-consumption, although part of the harvest is later dedicated to other purposes such as education, sales, environment, etc. It is worth mentioning that the vegetable gardeners possess accumulated knowledge and experience in agricultural production (Tab. 2).

Economic aspects

There are three relevant economic aspects in this case. First, there is an acceptable capacity for production based on the availability of production factors. Second, there is already a supply, although still small, that is marketable. Third, there are functional markets (peasant markets) and potential markets (La Canasta Program, neighborhood stores, etc.) for the products of urban agriculture in the cabildo (Tab. 3).

Social aspects

The characteristics of the cabildo vegetable gardeners are as follows: they are mostly women, adults, and older adults, mainly engaged in household chores and belonging to a low socioeconomic status. In general, vegetable gardeners have time and dedication to urban agriculture, which they also see as a source of income generation. The profile of vegetable gardeners is expected to change in the medium and long term, which implies a generational change that must be addressed.

TABLE 2. Technical aspects of urban agriculture in the Mhuysqa indigenous cabildo.

Variable	Characteristics
Type of vegetable garden	88% family and 12% community
Ownership of the vegetable garden	91% owned, 3% leased, 3% community property, 3% public
Age of the vegetable garden	18% is older than 30 years, 3% between 21 and 30 years, 9% between 11 and 20 years, 3% between 6 and 10 years, and 67% less than 5 years old
Size of the vegetable garden	70% has 23 m^2 on average, 12% has 85 m^2 on average, 6% has 147 m^2 on average, 6% has 180 m^2 on average, 3% has 250 m^2 on average, and 3% has 450 m^2 on average
Place of production	82% is produced in soil, 52% in pots, 45% in crates, 12% in used tires, and 12% in other (baskets, buckets). 61% of the vegetable gardens have 2 or more production sites
Protection of the vegetable garden	85% is in free exposure, 21% is surrounded by a fence, 6% has poly-shade (community vegetable garden), 3% has a plastic cover (community vegetable garden), and 3% has a greenhouse (community vegetable garden)
Source of water for production	76% is rainwater, 70% comes from the aqueduct, and 39% is harvested water. 70% of the vegetable gardens use 2 or more water sources
Source of plant material	73% is owned and 64% is purchased. 36% have both sources
Function of the vegetable gardens	100% production for self-consumption (food, medicines, ornamental crops), 45% as a means of training for children and youth, 42% production for sale, 36% for production of medicines, 15% for environmental practice, 12% as a point of agrotourism, and 9% as a place for recreation. 76% of them fulfill 2 or more functions
Production	97% edible (leafy vegetables, cold climate fruit trees, and tubers), 91% medicinal, 82% aromatic, 42% ornamental (flowers and succulents). 94% produce 2 or more product groups
Type of farming	It is mainly biological and/or agroecological (94% in nutrition, 52% in pest control, 90% in disease control, and 70% in weed control)
Agronomic knowledge and technical assistance	The cabildo has knowledge and technical production skills and, in addition, eventually receives technical assistance from the Botanical Garden of Bogotá and the Secretariat of Economic Development of Bogotá
Needs to improve the practice of urban agriculture	94% technical assistance, 76% containers, 73% seedlings, 67% tools, 64% biofertilizers, 61% seeds, 33% bio-controllers, 24% labor, 21% land, and 21% marketing

Source: Authors based on survey of 2021.

TABLE 3. Economic aspects of urban agriculture in the Mhuysqa indigenous cabildo.

Variable	Characteristics
Factors of production	Factors of production such as land, labor, inputs, services and capital goods are available to an acceptable extent (quantity and quality)
Supply of urban agriculture products	Less than 30% (low) of production is available for sale or distribution other than to the market gardener
Distribution and commercialization of UA products	73% for self-consumption, 33% for neighbors who belong to the community (barter and sale), 27% for the community vegetable garden (delivery), 24% for neighbors who do not belong to the community (sale), 12% for neighborhood stores, and 12% for the "canasta" program. A low percentage (5 out of 47 families) sell through "peasant markets" and one family has implemented electronic sales
Aspects to improve in marketing	33% increase the quantity of products for sale, 24% increase the frequency of production for sale, 15% aim to improve product presentation, 15% to improve packaging, 15% to increase the selling price to encourage supply, 3% to add value to products, 3% to improve logistics

Source: Authors based on survey of 2021.

TABLE 4. Social aspects of urban agriculture in the Mhuysqa indigenous cabildo.

Variable	Characteristics
Population of the cabildo	The cabildo is made up of 13 clans, 1,080 family groups for a total of 4,300 Indigenous "cabildantes", which include mestizos. According to the 2018 Census, 56% are women and 44% are men
Sex	61% women, 39% men
Age	42% adults over 60 years old, 55% adults between 27 and 59 years old, and 3% youth between 14 and 26 years old
Social class	82% social stratum 2 and 18% social stratum 1
Occupation	61% housework, 15% employed, 12% agriculture, and 12% self-employed
Sources of family income	Mainly construction work, recycling, and handicraft production
Housing	Mostly made of brick and cement, with a few of mud and straw. Most are owned, the rest are rented

Source: Authors based on survey of 2021.

Environmental aspects

The main sources of water for urban agriculture are rainfall and aqueducts, with the latter used as the main source for irrigation due to the limited amount of stored rainwater. The soils in the vegetable gardens are classified as good (Class II) and acceptable (Class III). Regarding pollution, agricultural production generates some waste, such as leaves and plant and crop residues, and polluting materials such as plastics and agglomerates.

Legal aspects

Approximately half of the land where the vegetable gardens are located is owned and the other half is leased. The community gardens are located on district land, operating under a permit.

Determinants of the commercialization of agricultural products

Defining the commercialization of agricultural products

Based on various authors, the marketing of agricultural products is defined as a series of processes and activities that move agricultural goods from the place of production to the place of consumption. The sequence of processes includes planning based on market information (market research), market-driven production, product preparation, transportation and sale (Kohls & Uhl, 2002; Lohosha & Semchuk, 2021; Ramkishen, 2005). This definition serves as a starting point in the section on the determinants of commercialization of urban agriculture products.

Determinants of the marketing of agricultural products: rural and urban

The bibliographic reference sample (Tab. 5) provides an approximate picture of supply and distribution at the level of small rural and urban agricultural producers. Due to their socioeconomic condition, the limitations in production factors are evident, especially in terms of land, water and financing. With regard to distribution, distance to market and access to transportation are the limitations, although for urban agriculture these are less significant. The most striking determinants in both cases are social, *i.e.*, human resources, including age, the number of people in the family unit (available labor), education (related to the ability to continue learning) and agricultural training (knowledge of agricultural production). The authors in Table 5 assume that producers, especially rural producers, have the plan and the intention to market their products; however, this is not always the case in urban agriculture. This fact is crucial for the analysis of the commercialization of urban agriculture products.

TABLE 5. Determinants of the commercialization of urban and rural agricultural products.

Authors	Supply	Commercialization	Other
Nigus *et al.* (2024)	Land	Market information, market access	Age, education, occupation, household size, farming experience
Grebitus (2021)			Confidence, attitude, knowledge, household size, age, sex, income
Ater *et al.* (2021)	Land, irrigation		Age, farming background, associativity, earnings
Tafesse *et al.* (2020)	Size of production unit	Access to transport service	Education, age
Hagos Belay *et al.* (2020)	Quantity of production, size of production unit, quantity destined for sale	Distance to market	Educational level, family size, extensionist visits
Musitini *et al.* (2019)	Quantity of production	Transport availability	Associativity
Melese *et al.* (2018)	Productivity, productive unit size, financial capital, volume of production	Access and distance to market	Age, education, access to extension service
Muricho (2015)	Size of production unit, access to credit, access to cell phone	Access to transportation, transportation costs	
Akinlade & Balogun (2013)	Size of production unit, input costs		Age, associativity, social capital
Tufa *et al.* (2013)	Access to irrigation and land, size of production unit	Distance to market	Education, size of household

Source: Authors based on the sources in the Table.

Determinants of the commercialization of cabildo urban agriculture products

Based on the definition of commercialization of agricultural products, which includes planning, production, product preparation, storage and transportation, sale and market development; and on the information on urban agriculture in the cabildo, the analysis of marketing is proposed in six stages: planning, production, product preparation, storage and transportation, sale and market development.

Planning

Planning is very important for the successful execution of any activity. It does not necessarily have to be formal, but the following elements must be clearly established: an objective, strategies, resources, and time. Although there is an intention to commercialize urban agriculture products in the cabildo, it is not a clearly established and mandatory objective. During the project, five out of 47 families (11%) have been identified with the intention and action to sell their surpluses (Tab. 3). Since there is no established marketing plan, there is no clarity about the organization of the product to be sold or the human resources to carry it out. Commercialization has taken place in peasant markets more on the initiative of some members of the cabildo and the District. In addition, the cabildo does not have a determined financial resource for the commercialization process, evidencing individual rather than organizational initiative.

Production

For this research, the small amount of agricultural production destined for sale is considered a determinant, which in the case of the cabildo is approximately 25% of the total. This production comes mainly from five of the 47 vegetable gardens (11%). The agricultural production of the cabildo at the time of the survey has the necessary production factors (land, water, inputs, and labor). It also has the knowledge and experience for agricultural production, although there is a need for more specialized knowledge for a more efficient and commercial urban agriculture (Tab. 2). Technical advice can eventually be provided by the Bogotá District or the Botanical Garden of Bogotá. Since there is no established supply information, it is necessary to plan production in terms of quantity and frequency of supply according to the target market.

Product preparation

There are two situations in this process: when the producer supplies an intermediary, and when the producer sells directly, for example, at peasant markets. The first case is more complicated for the producer, because the volume, quality and presentation, and the specific time of delivery must be arranged. The cabildo does not have enough experience as a supplier and, therefore, needs some preparation (discipline and commitment) if it decides to take on this role. In the second case, the cabildo does have experience, having already sold its products to neighbors who do not belong to the cabildo and in peasant markets organized by

the District and the Botanical Garden of Bogotá (5 out of 47 families). In this case, the preparation involves smaller volume, quality considerations and flexible presentation.

Collection and transport

During participation in peasant markets organized by the Botanical Garden of Bogotá, the cabildo has been observed requesting assistance with the collection and transportation of their merchandise from the production sites to the point of sale. This indicates that the costs of collection and transportation are not included in the sale price of the products. In the future, the goal is for the cabildo to self-finance the collection and transportation while maintaining the level of profits (profitability) generated by the production and commercialization of UA products.

Sales

Sales were conducted on a cash-on-delivery basis. The most common form of payment was cash, although there was discussion about the use of low-cost mobile deposit applications such as "Daviplata" and "Nequi", but these had not yet been adopted by 2021. Electronic sales are a potential channel that has yet to be explored or exploited. Moreover, due to the small volumes of supply handled by the cabildo, the vegetable gardeners are not currently interested in post-delivery payments (more than two weeks).

Market development

Although potential markets are known (Tab. 3), such as the cabildo itself, the "Canasta" program (subsidized by the district), non-indigenous neighborhoods, neighborhood stores and peasant markets (organized by the district), there are no fixed markets with a clear development objective. Without a production and marketing plan, it becomes difficult to secure fixed buyers. In the commercialization and development of markets, individual efforts again outweigh community efforts.

Conclusions

Urban agriculture in the Mhuysqa indigenous cabildo is multifunctional, mainly oriented to agricultural production for self-consumption and the generation of social services. The surplus of agricultural supply destined for commercialization is relatively low, approximately 25% of the total. From a process perspective, the determinants of the commercialization of urban agricultural products are: strengthening the cooperative work in the cabildo, setting and meeting commercialization objectives and goals (in planning); achieving adequate volumes, quality and frequency of supply, as well as knowledge and specialized technical skills (in production); ensuring the quality of presentation and freshness of the product (in product preparation); managing storage and transportation costs (in storage and transportation); defining forms and means of payment as well as exploring electronic sales (in sales); and securing regular buyers (in market development). Finally, it is important to acknowledge the limitations of the research in terms of the vegetable gardener own vision about the commercialization of urban agriculture products. Likewise, an information gap is identified in the detail (sub-factors) of the determinants of the commercialization of urban agriculture products.

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Conflict of interest statement

The authors declare that there are no conflicts of interests regarding the publication of this article.

Author´s contributions

DCMQ contributed significantly to data collection, systematization and draft writing of the manuscript; JCBF structured the article, provided and systematized information and collaborated in the draft writing and translating the manuscript; CFCM collaborated with the revision and correction of the final version of the manuscript. All authors have read and approved the final version of the manuscript.

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SCIENTIFIC NOTE

Effect of bacterial xylanase on the automated production of barley flour-enriched sandwich bread

Efecto de la xilanasa bacteriana en la producción automatizada de pan tipo molde enriquecido con harina de cebada

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ABSTRACT

The aim of this research was to determine the effect of bacterial xylanase on the automated baking of barley flour-enriched sandwich bread. For this purpose, xylanase (0.02% to 0.04%) was added to the basic formulation of sandwich bread enriched with 10% whole barley flour. The resulting bread slices were subjected to image analysis to determine colorimetric properties, cell count, and sensory evaluation using a 9-point hedonic scale. A 0.03% xylanase addition produced a higher number of cells compared to other treatments. Regarding the colorimetric properties, no significant differences were observed, and the bread with 0.03% xylanase addition received the highest preference score in the sensory evaluation. In conclusion, bacterial xylanase has positive effects on bread, improving its overall quality.

Key words: cell count, chromatic properties.

RESUMEN

El objetivo del presente estudio fue determinar el efecto de la xilanasa bacteriana en el horneado automatizado de pan tipo molde enriquecido con harina de cebada. Para tal efecto se realizaron adiciones de xilanasa (0,02 al 0,04%) a la formulación básica de un pan de molde enriquecida con 10% de harina de cebada integral. A los panes obtenidos se les realizaron análisis de imágenes para determinar las propiedades cromáticas y el número de células, y se evaluó sensorialmente cada muestra en una escala hedónica de 9 puntos. El 0,03% de adición de xilanasa presentó mayor número de células en contraste con los demás tratamientos; respecto a las propiedades cromáticas, no hubo diferencias significativas y el pan con adición de 0,03% de xilanasa presentó la mayor preferencia en la evaluación sensorial. En conclusión, la xilanasa bacteriana genera efectos positivos en el pan, mejorando su calidad.

Palabras clave: conteo de células, propiedades cromáticas.

Introduction

Barley is one of the most important crops in Peru, grown in the central highlands, southern highlands, and the Altiplano, thus becoming part of the Andean population's diet (MIDAGRI, 2024). It is also consumed in Europe, certain regions of Africa (north and south of the Sahara), central and southwestern Asia, as well as in the Andean regions of Ecuador and Bolivia. Industrially, it is used for animal feed, in the brewing industry, and as a coffee substitute (Esquisabel, 2022). There are improved varieties in Peru, such as INIA 411 and INIA 418, developed by the National Institute of Agrarian Innovation (INIA). These and other varieties are consumed in the form of grain and/or flour at the household level (INIA, 2018).

The marketing of barley as food in Peru is still for domestic consumption. In this country, industries import forage and malt barley from Argentina for use in the brewing and animal industries (Esquisabel, 2022; SENASA, 2021). Therefore, it is important to diversify the use of this product, since barley contains soluble and insoluble fiber, proteins, carbohydrates, minerals, and vitamins (Baltazar, 2024; Gupta *et al*., 2010). It also has a naturally high content of β-glucan, a polysaccharide comprising glucose residues made of 1,3-beta-d-glucopyranose (30% bonds) and 1,4-beta-d-glucopyranose (70% bonds) (Pontonio *et al*., 2020).

For bread production, wheat flour is the most important component due to its gluten content and ease of processing.

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However, other cereals like barley and rye, which contain high amounts of the amino acid proline (Sánchez & Esteban, 2022), can be utilized as flours in the bread industry. In ancient Egypt and in the Middle Ages, barley bread was made, but its consumption decreased as it was considered less smooth and digestible (Hidalgo López *et al*., 2023). For this reason, it is desired to incorporate enzymes to improve the technological and functional characteristics of barley bread.

Currently, various methods, including biotechnology, have been used to optimize and improve the quality of bread, with special attention to enzymes. For example, this included the use of pentosanase and glucose oxidase in a bread with sprouted wheat, where these enzymes reduced the formation time and increased the stability of the mass, thus improving the technological functionality of the mass (Tran *et al*., 2024). The use of amylolytic enzymes such as exoamylases and debranching enzymes improves the texture and freshness of bread (Zhao *et al.,* 2022). The combination of α-amylase and lipases in a steamed potato bread reduced its toughness and improved the volume of the bread (Ma *et al*., 2022). The cocktail of enzymes α-amylases, xylanases and cellulases intensified the brown color of the bread crust and improved the rheology of the dough and properties of the bread (Hmad *et al*., 2024). The use of an enzyme cocktail can produce breads of better quality, but, in this research, emphasis was placed on one of these enzymes. Endo-1,4-xylanases (xylanases; EC 3.2.1.8) are hydrolytic enzymes that catalyze the degradation of xylans by randomly breaking the β-(1-4) glycosidic bonds of xylose chains present in the hemicellulose of plant cell walls, producing oligosaccharides of different sizes, both soluble and insoluble. The action of these enzymes in bread is to solubilize insoluble arabinoxylan to yield soluble, high molecular weight arabinoxylans. This action removes insoluble arabinoxylan that interferes with gluten network formation in the dough, making the dough more elastic and increasing its viscosity (Kim & Yoo, 2020; Sheikholeslami *et al*., 2021). Thus, they contribute to the baking industry by providing technological benefits (Obando Garzón, 2013), such as improving the dough's rheological properties, specific volume of the bread, and crumb firmness, resulting in a softer texture (Butt, 2008). Xylanolytic activity during the baking process begins during kneading, modifying the dough's viscoelastic characteristics, and continues during fermentation and the initial minutes of baking until denaturation occurs due to the high baking temperatures (Caballero *et al*., 2007).

The aim of this study was to determine the effect of bacterial xylanase on the automated production of barley flour-enriched sandwich bread.

Materials and methods

Raw materials

For the bread-making process, Nicolini brand commercial wheat bread flour and barley flour with a particle size of 300 μm were acquired, along with other ingredients such as sea salt, Famosa brand vegetable shortening, Fleischmann's instant yeast, and sugar. As for the bacterial xylanase enzyme, it was purchased from Polifood Perú, food-grade with 80% purity.

Bread formulation

For the bread formulation, a standard bread recipe was used with the addition of 10% barley. Additionally, experiments were conducted with three levels of bacterial xylanase addition. The formulations for each experiment and the control sample can be observed in Table 1.

TABLE 1. Formulations used for bread making.

Ingredients	Levels of xylanase addition			
	Control	0.02%	0.03%	0.04%
Wheat flour (%)	90	90	90	90
Barley flour (%)	10	10	10	10
Sugar (%)	8	8	8	8
Salt (%)	1	1	1	1
Vegetable shortening (%)	9	9	9	9
Yeast (%)	2	2	2	2
Powdered milk (%)	5	5	5	5

Making bread

To prepare the bread, all ingredients were placed into a Blackline® BM-6301 bread maker (Blanik, Chile) programmed for basic bread setting with dark crust baking.

Image acquisition system

The image acquisition system in this study (Fig. 1) had the following specifications: (1) the walls had a dimension of 30 cm and a matte black internal color, (2) equilateral white light bulbs with a 10 cm edge, (3) a 13-megapixel image resolution camera, (4) a computer with an Intel Core i3 processor with 4 GB of RAM. The collected samples were recorded through images in *.jpg format.

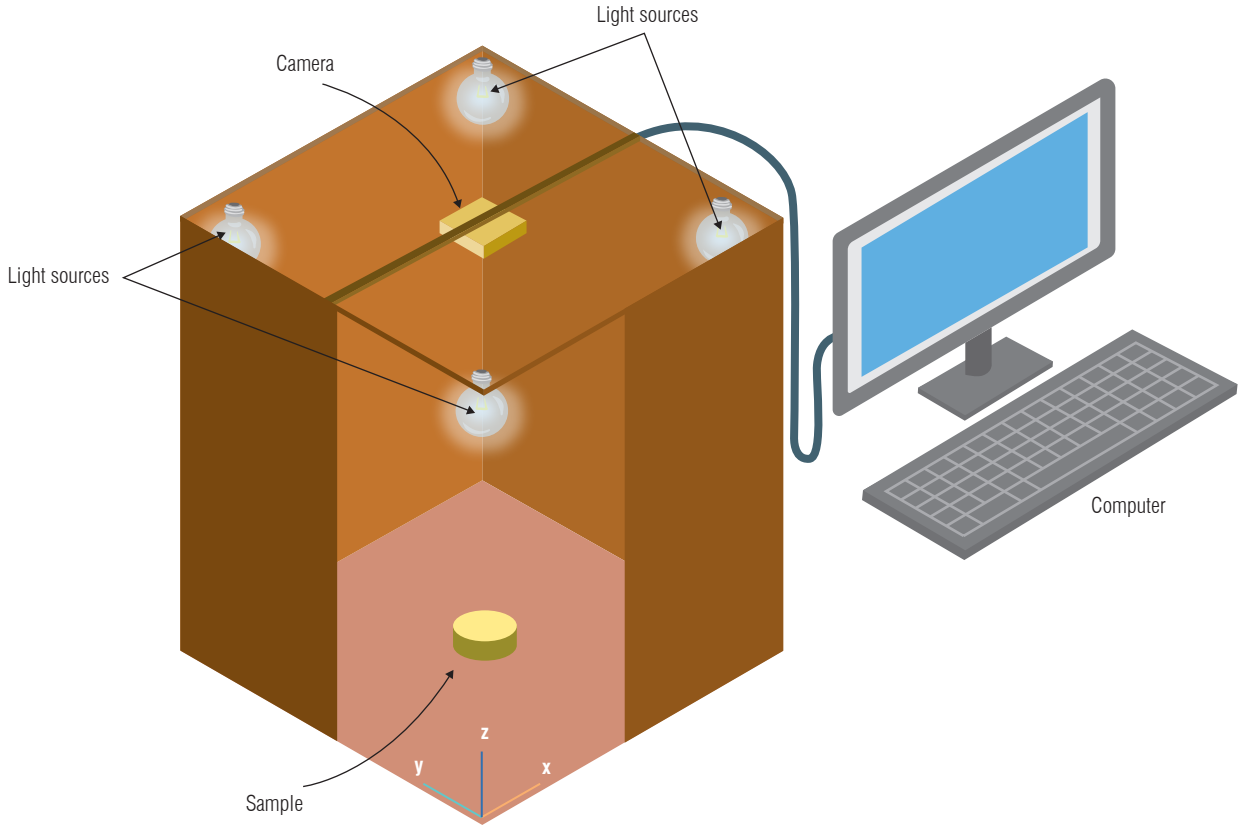

FIGURE 1. Image acquisition system (reproduced from Chambi-Rodriguez *et al*. (2023), with permission by Agronomía Colombiana).

Cell analysis and chromatic properties

The experiments involved segmenting samples into sections (slices) of 1 cm thick; subsequently, each section was subjected to the image acquisition system (Fig. 1). The resulting images were analyzed using ImageJ software to determine the number and size of cells present in each section. To assess chromatic properties in the crust and crumb, including luminosity (L*) and redness level (a*/b* ratio, where a* denotes the red-green hue and b* denotes the yellow-blue hue), RGB information was captured from regions of interest (ROI). Subsequently, chromatic information was calculated using CIELab coordinates derived from these data. These analyses were conducted in three replicates to ensure result consistency. The transformation from RGB to CIELab involves a mathematical procedure that facilitates color representation in a three-dimensional space based on human perception (Castro *et al*., 2017). The formulas used in this process were as follows:

$$L* = 116 * f\left(\frac{Y}{Y_n}\right) - 16 \quad (1)$$

$$a* = 500 * \left[f\left(\frac{x}{x_n}\right) - f\left(\frac{Y}{Y_n}\right)\right] \quad (2)$$

$$b* = 200 * \left[f\left(\frac{Y}{Y_n}\right) - f\left(\frac{Z}{Z_n}\right)\right] \quad (3)$$

In these equations, L* represents the luminosity, while a* and b* denote the chromaticity coordinates. Y stands for the relative luminance of the sample in RGB, and Yn represents the relative luminance of a reference white color, such as D65. Additionally, X, Y, and Z represent the trichromatic coordinates of the sample in RGB, and X_n, Y_n, and Z_n represent the trichromatic coordinates of a reference white color, also exemplified by D65. Furthermore, f(t) refers to a nonlinear function employed to adjust the human eye's response to various wavelengths (Chambi *et al*., 2023).

Using these coordinates, the purity (c*) was derived through the following equation:

$$c* = \sqrt{(a*)^2 + (b*)^2} \quad (4)$$

Sensory analysis

The control sample and the breads made with the addition of xylanase were evaluated by 50 untrained judges of both sexes aged between 18 and 30 years. The assessed properties

included odor, color, taste, texture, and overall appearance. A 9-point hedonic scale was employed for this evaluation (1 = Extremely dislike to 9 = Extremely like). The results were displayed on a radar chart (Garcia *et al*., 2022).

Statistical analysis

A one-way analysis of variance (ANOVA) was applied at a significance level of $P<0.05$, adding bacterial xylanase at different concentrations (0.02%, 0.03%, or 0.04%) followed by a comparison of means (Tukey´s test).

Results and discussion

Cell analysis and chromatic properties

In Figure 2, it can be observed that the number of cells in the samples with 0.03% xylanose was higher than in the other samples. This is followed by the sample with 0.04% xylanose. The control and the 0.02% xylanase addition samples have the lowest numbers of cells. These are considered statistically equal at a significance level of 0.05.

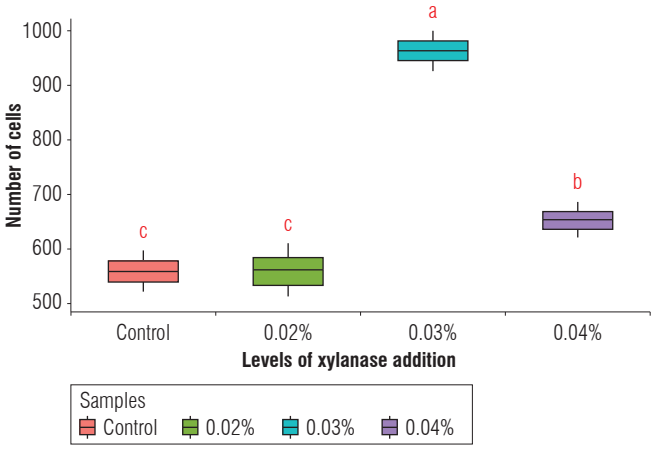

FIGURE 2. Boxplot of the number of cells in the 1 cm-thick bread sample with added xylanase enzyme. Different letters indicate significant differences according to the Tukey´s test ($P<0.05$).

The results obtained for the chromatic coordinates (L*, a*, and b*) of bread containing 10% barley and bacterial xylanase at different concentrations (0.02%, 0.03%, and 0.04%) indicated no significant differences in the luminosity, red-green hue, or yellow-blue hue of the bread crumb compared to the control bread. All values remained within ranges similar to those of the control bread, suggesting that the addition of bacterial xylanase did not significantly influence the chromatic characteristics of the evaluated bread crumb.

In terms of the bread crust, a similar trend was observed. The chromatic coordinates for the crust (L*, a*, and b*) remained close to control values across different xylanase concentrations. Specifically, the luminosity (L*) of the crust decreased slightly with the increase in xylanase concentration, indicating a darker crust, but the differences were not substantial enough to be considered significant. The red-green hue (a*) and yellow-blue hue (b*) values also remained within the same range as in the control samples, suggesting no significant impact on the crust's color. Therefore, the addition of bacterial xylanase at the evaluated concentrations did not cause any meaningful changes in the chromatic properties of the bread crust either.

Sensory analysis

Figure 3 presents the sensory evaluation of barley-containing bread samples with different levels of xylanase addition. Five aspects were assessed: odor, color, flavor,

TABLE 2. Chromatic properties of bread with xylanase addition.

Levels of xylanase addition	Chromatic properties			
	L*	a*	b*	c*
		Crust		
Control	39.70 ± 4.47	14.09 ± 1.61	33.127 ± 0.21	35.99 ± 6.89
0.02%	27.61 ± 6.64	14.08 ± 1.65	33.81 ± 1.86	36.64 ± 6.89
0.03%	34.15 ± 5.65	34.81 ± 1.63	33.95 ± 1.74	48.63 ± 6.89
0.04%	36.36 ± 3.43	12.59 ± 0.90	30.39 ± 0.76	32.89 ± 6.89
		Crumb		
Control	45.45 ± 2.69	0.86 ± 0.20	11.86 ± 0.76	11.89 ± 1.25
0.02%	38.25 ± 5.53	1.31 ± 0.25	14.35 ± 1.33	14.41 ± 1.25
0.03%	39.46 ± 3.09	0.91 ± 0.23	11.72 ± 1.02	11.76 ± 1.25
0.04%	36.54 ± 6.19	0.85 ± 0.33	12.12 ± 1.50	12.15 ± 1.25

Note: All data are expressed as means ± SD (n=3).

texture, and overall appearance, using a 9-point hedonic scale. It is observed that the odor tended to increase slightly with xylanase addition, reaching its peak in the sample with 0.03% xylanase. The color remained intense in all samples, with a slight decrease in samples with 0.02% and 0.04% xylanase. Regarding the flavor, a gradual increase was observed in samples with xylanase addition, being the highest in the sample with 0.03% xylanase. However, the texture appeared to decrease slightly with xylanase addition, with the lowest score in the sample with 0.04% xylanase. The overall appearance tended to improve with xylanase addition, reaching its highest score in the sample with 0.03% xylanase. In conclusion, xylanase addition variably affected the sensory characteristics of bread, enhancing its flavor and overall appearance at certain addition levels, but potentially negatively impacting the texture at higher concentrations.

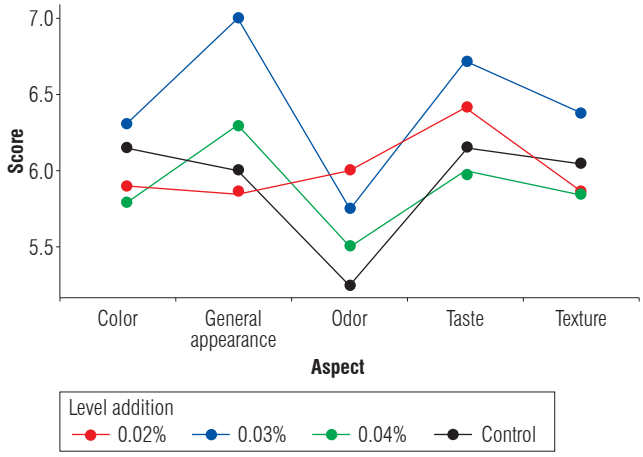

FIGURE 3. Sensory attribute scoring of barley-containing bread samples with different levels of xylanase addition (0.02%, 0.03%, and 0.04%).

Discussion

Regarding the cell size (Fig. 2), the results obtained are supported by previous research, such as that conducted by Vega Castro *et al.* (2015) on the influence of enzymes on bread production. They observed that samples treated with xylanase had an increase in volume as well as greater uniformity in cell size. Furthermore, according to Moreno-Araiza *et al.* (2018), at an addition concentration of 0.02%, the samples showed higher volume and a greater number of cells compared to those treated with a concentration of 0.04%. In the study by Wang *et al.* (2023) on wheat sprout-incorporated bread, xylanase addition resulted in higher specific volume and lower bread hardness. Similarly, in the study by Belyavskaya *et al.* (2022) on rye and flaxseed bread, xylanase application resulted in increasing specific volume and improving crumb resilience. This finding is consistent with data obtained in our study. However, our study demonstrated better performance with an addition of xylanase at 0.03%, where a significant increase in the number of cells was observed. According to Liu *et al.* (2022), the volume increase, consequently leading to an increase in cell number and bread quality, is attributed to the action of xylanase on arabinoxylans present in the flour.

Regarding the chromatic properties (Tab. 2) of the samples analyzed, no marked differences were found. However, previous studies by Altuna *et al.* (2015) identified discrepancies among samples, attributing this phenomenon to the degradation of hemicellulose to xylose, a reducing sugar that exhibits greater reactivity than hexoses and disaccharides.

Finally, the samples subjected to sensory evaluation presented significant differences for at least one sample with the concentration of 0.03% showing the highest level of acceptance. However, some studies did not detect significant differences (Altuna *et al.*, 2015). The color of bread is due to Maillard reactions; the more reducing sugars there are, the darker the color will be. This effect is seen when amylases are introduced, while xylanases produce it but at a lower scale (Kim & Yoo, 2020).

Conclusions

The addition of 0.03% xylanase resulted in the highest cell count, followed by 0.04%, while the control and 0.02% showed the lowest count, with statistically similar values. The chromatic properties (L*, a*, b*) did not significantly differ between control and the bread samples with xylanase at 0.02%, 0.03%, and 0.04%. Sensory evaluation highlighted improved taste and appearance of the bread with 0.03% xylanase, but a potential decline in texture at higher concentrations. These results emphasize the importance of precise xylanase usage to enhance bread quality.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

ADCR: conceptualization, methodology, formal analysis, research, writing of original draft, editing. LVCT: writing of original draft, editing. PSS: data curation. AMTJ: writing of original draft, editing, research, validation. All authors reviewed the final version of the manuscript.

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The journal's official language is English. Regarding measurement units, the metric system (SI) should be consistently used through the manuscript, unless the need is seen to apply any specific units that are of frequent use by the scientific community. Multiplication followed by negative superscript (e.g., kg ha^{-1}) can only be used with SI units. The slash (/) is a mathematical operation symbol that indicates "divided by". Anyway, in sciences it is used as a substitute for the word "per", and it is used to indicate rates. Use the slash to connect SI to non-SI units (e.g., 10°C/h or 10 L/pot).

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Title and authors

The title in English, as well as its corresponding Spanish translation, shall not exceed 15 words. The scientific names of plants and animals shall be italicized and lowercased, except for the first letter of the genus (and of the species author), which must be uppercased.

The authors (including first and second names) shall be listed in order of their contribution to the research and preparation of the manuscript, in completely justified text format (filling the whole line, or, if necessary, the next

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The Abstract should be written in English with Spanish translation for the "Resumen". Both texts should contain brief (no longer than 200 words in a single paragraph) and accurate descriptions of the paper's premise, justification, methods, results and significance. Both language versions shall be mandatorily provided with a list of (maximum six) key words that have not appeared in the title or abstract, and included in the Agrovoc thesaurus by Agris (FAO).

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Acknowledgments

When considered necessary, the authors may acknowledge the researchers or entities that contributed - conceptually, financially or practically - to the research: specialists, commercial organizations, governmental or private entities, and associations of professionals or technicians.

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