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CONTENIDO

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Isolation of antimicrobial lactic acid bacteria with potential use as a protective culture for 'Queso Fresco'

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Abstract

Lactic acid bacteria (LAB) isolated from various sources are used in the food industry for different purposes depending on their characteristics; being its antimicrobial capacity one of the most important. The objective of this work was to determine the antagonism of LAB's isolated from milk and its derivatives against *Salmonella*, *Staphylococcus* and *Listeria*, contaminants of 'queso fresco' produced in the south of the state of Chiapas, Mexico, as a strategy for the development of protective crops for this artisan cheese. A total of 54 samples of dairy products (whey, milk and cheeses) from the Istmo-Costa region were analyzed. 52 strains of LAB were isolated, which were analyzed to investigate their antibacterial capacity by the methods of the drop on the surface and diffusion in agar. From the first method, 36 strains evaluated showed signs of being antagonistic to pathogens: nine against *Staphylococcus aureus*, seven against *Salmonella* sp. and five against *Listeria monocytogenes*. In the agar diffusion test, 31 strains evaluated showed antagonistic capacity: eight against *S. aureus*, 12 against *Salmonella* sp. and 11 against *L. monocytogenes*. These results encourage one to think that LAB's isolated from dairy products may function as protective cultures from pathogens that contaminate 'queso fresco'. Additional testing is required to validate this hypothesis.

Keywords:
Dairy products
Inhibition
Listeria monocytogenes
Salmonella sp.
Staphylococcus aureus

Aislamiento de bacterias lácticas antimicrobianas con potencial uso como cultivo protector para Queso Fresco

Resumen

Las bacterias ácido lácticas (BAL) aisladas de diversas fuentes son utilizadas en la industria de los alimentos con distintos propósitos, dependiendo de sus características; siendo su capacidad antimicrobiana una de las más importantes. El objetivo de este trabajo fue determinar el antagonismo antimicrobiano de las BAL aisladas de leche y sus derivados contra *Salmonella*, *Staphylococcus* y *Listeria*, contaminantes del queso fresco producido en el sur del estado de Chiapas, México, como estrategia para el desarrollo de cultivos protectores para este queso artesanal. Se analizaron un total de 54 muestras de productos lácteos (suero, leche y quesos) de la región Istmo-Costa. Se aislaron 52 cepas de BAL, las cuales fueron analizadas para investigar su capacidad antibacteriana por los métodos de la gota sobre la superficie y difusión en agar. Del primer método, 36 cepas evaluadas demostraron antagonismo contra los patógenos: nueve contra *Staphylococcus aureus*, siete contra *Salmonella* sp. y cinco contra *Listeria monocytogenes*. En la prueba de difusión en agar, 31 cepas evaluadas mostraron capacidad antagónica: ocho contra *S. aureus*, 12 contra *Salmonella* sp. y 11 contra *L. monocytogenes*. Estos resultados hacen pensar que las BAL aisladas de productos lácteos pueden funcionar como cultivos protectores de patógenos que contaminan el queso fresco. Pruebas adicionales son requeridas para validar tal hipótesis.

Palabras clave:
Productos lácteos
Inhibición
Listeria monocytogenes
Salmonella sp.
Staphylococcus aureus

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1. Introduction

As is the case in many regions of the world, artisan cheeses are widely consumed food products in Mexico. Of these, the preference for 'queso fresco' stands out, which represent one of the most important types, with a production of 89 557 tons per year (SIAP, 2020). These cheeses are made almost exclusively from raw milk (unpasteurized), which freshly milked has a low bacterial presence, however, in subsequent processes, there is the possibility of contamination from various sources (Willis et al., 2022). The pasteurization of milk is the first option to reduce the microbiological load and it is also a regulatory requirement (NOM-243-SSA1-2010), however, it is still not used among small and medium producers, since during pasteurization they are also destroyed desirable bacteria, responsible for the specific sensory characteristics, which detracts from the acceptability of the products by consumers (González-Córdova et al., 2016).

Biopreservation, on the other hand, is a method that offers various conditions to extend the shelf life and increase the safety of fresh cheeses, through the implementation of either native or exogenous microbiota (protective cultures) and/or its microbial products (Todorov, 2019). Different studies have used this procedure, mainly using bacteria belonging to the natural microbiota of various products, such as lactic acid bacteria (LAB) isolated from dairy, meat, fish and vegetable products (Aragon-Alegro et al., 2021; Martín et al., 2022). This procedure makes use of the antibacterial properties, attributed to the final products of LAB metabolism such as lactic acid, acetic acid, diacetaldehyde, reuterin and bacteriocins, among the main ones (Londoño et al., 2015). In addition, it has been shown that this preservation option offers better results, when LABs obtained from similar environments are applied to where they are to be applied (Punia-Bangar et al., 2022). Fuentes et al. (2017) isolated 32 strains of BAL from 'doble crema' cheese and 'quesillo' (Oaxaca cheese), 13 strains showed antagonistic capacity against *Listeria monocytogenes* and against *Salmonella typhimurium*. Also, Peralta (2014) obtained 75 strains of BAL, of which 10 strains, presented antagonistic capacity against *L. monocytogenes* and *Escherichia coli*.

In the Istmo-Costa region, in the state of Chiapas, in southern Mexico, various artisan cheeses are produced with unpasteurized milk, which can be potentially contaminated with human pathogens as occurs in cheeses from other regions of the country (Guzmán -Hernández et al., 2016a; Ibarra-Sánchez et al., 2017). To date, the information on undesirable or pathogenic microorganisms that can be found in Chiapas cheeses is scarce but given the similarity in the characteristics of the cheeses and the production procedures, it is highly probable to find bacteria such as *Salmonella, Staphylococcus* or *Listeria*, as reported in 'queso fresco' made in the Mexican state of Tabasco (Guzmán-Hernández et al., 2016a; 2016b).

This work had two main purposes, to verify the presence of pathogens in 'queso fresco' made and marketed in the coastal strip of the state of Chiapas; as well as isolate lactic acid bacteria from milk and dairy derivatives that show antimicrobial activity specifically against *Salmonella, Staphylococcus* and *Listeria*; bacteria considered contaminants in fresh cheeses; as a strategy for the development of protective cultures.

2. Materials and Methods

2.1. Isolation of lactic acid bacteria

As raw material for the isolation of lactic acid bacteria (LAB), six types of samples were collected in triplicate: 1) freshly milked milk, 2) freshly whey obtained from the cheese factory, 3) 'doble crema' cheese, 4) quesillo (Oaxaca cheese), 5) 'queso fresco' and 6) Cotija cheese; all samples from the Istmo-Costa region. For the milk and whey samples, 500 mL were collected in sterile plastic containers, and, for the cheese samples, 500 g of cheese were purchased and placed in sterilized hermetic bags. This procedure was repeated three more times for the cheese samples, and each sample was treated as independent, making a total of 54 samples that were at all times transferred on ice to the laboratory for immediate analysis.

From each cheese samples, 10 g of each were weighed and placed in a sterile blender containing 90 mL of sterile peptone water (SPW). After low-speed liquefaction, the suspensions were placed in an Erlenmeyer flask. For the liquids (milk and whey), 10 mL of each were measured and they were added to 90 mL of SPW to proceed to homogenize the solution.

Subsequently, 100 µL of each suspension were taken and seeded in triplicate by casting into Petri dishes containing de Man Rogosa and Sharpe (MRS) agar. The plates were incubated at 37 °C under anaerobic conditions in anaerobic chamber. With the colonies grown, the isolation process was started by taking with a handle the colonies that were morphologically different in the edges (circular or irregular, wavy or smooth), the surface (convex or flat) and the color (white, yellowish reddish, opaque, creamy and shiny). For consecutive reseeding, the selective medium from which the strain was obtained was used and they were placed under anaerobic conditions for 24-48 h until a single colony morphology per plate was observed. Subsequently, each of the strains underwent the catalase and peroxidase tests. The strains that turned out to be catalase and peroxidase negative were smeared, fixed with heat and stained by the Gram method to verify the microscopic morphology and staining with the help of an Axiolab® optical microscope with an image analyzer, selecting those that resulted Gram positive and presented bacilli form (Vázquez-Velázquez et al., 2018).

2.2. Isolation of pathogenic bacteria

As raw material for the isolation of pathogenic bacteria, four samples of 'queso fresco' were collected in triplicate from four cheese factories in the Istmo-Costa region. All samples (repetitions) were treated independently, obtaining a total of 12 samples, which were later transferred on ice to the laboratory for immediate analysis.

For the isolation of *L. monocytogenes*, 25 g of 'queso fresco' were weighed in a sterile bag, the sample was macerated and 100 mL of enrichment broth with a *Listeria* supplement were added. The samples were homogenized, placed in bottles with a hermetic closing lid, and incubated at 35 °C for 48 h. Subsequently, 0.5 mL of the samples were seeded in Oxford selective medium, the seeded plates were incubated for 48 h at 37 °C. Colonies with characteristic *Listeria* morphology underwent Gram staining, mobility test, catalase and hemolysis (Guzmán-Hernández et al., 2016a).

For the isolation of *Salmonella* spp., the method of Guzmán-Hernández et al. (2016a) with slight modifications was followed. 25 g of 'queso fresco' previously macerated under sterile conditions were added to 225 mL of peptone water for homogenization. The samples were transferred to bottles with a hermetic closing cap and incubated at 37 °C for 24 h. After incubation, 1 mL of the sample was transferred to 10 mL of selenite-cysteine broth, and 1 mL to 10 mL of tetrathionate broth. Both medias were incubated at 37 °C. After 24 h, 10 µL of each medium was taken and spread onto the Salmonella-Shigella agar, Xylose Lysine Deoxycholate (XLD) agar, MacConkey agar, and incubated at 37 °C for 24 h. Three to five colonies with characteristic of *Salmonella* morphology were selected from each medium for their biochemical confirmation with the Triple Sugar Iron (TSI), Lysine Iron agar (LIA) and urease production tests.

For *Staphylococcus aureus* isolation, 10 g of 'queso fresco' previously macerated in sterile conditions, were added to 90 mL of phosphate buffer and homogenized. Decimal serial dilutions were made from the homogenate. 0.1 mL of each dilution were taken and spread in Petri dishes containing Baird-Parker agar (BPA) supplemented with potassium tellurite and egg yolk emulsion. The plates were incubated at 37 °C for 48 h. Those black colonies with transparent halo were selected, to later perform the Gram staining tests, coagulase, thermonuclease and mannitol for the identification of *S. aureus* (Guzmán-Hernández et al. 2016a).

2.3. Antagonistic capacity of LABs

For the drop-on-surface method, the LAB isolates were transferred to tubes containing 5 mL of MRS broth and incubated at 37 ºC overnight, then they were spotted (20 µL) on MRS agar plates under anaerobic conditions for 12 h at 37 ºC. The pathogenic bacteria (*Salmonella* sp., *Listeria monocytogenes* and *Staphylococcus aureus*) were individually incubated in tubes with 5 mL of brain-heart infusion (BHI) broth (Oxoid) at 37 ºC for 12 h under anaerobic conditions. Then, 100 µL of the suspension was transferred to 10 mL of fresh BHI broth. The mixture was completed with 0.75% bacteriological agar and placed in a water bath at 45 ºC. Each mixture containing cells of the pathogenic bacteria was poured (overlaid) onto the LAB-cultured plates. After completing semi-solidification of the upper layer, the plates were incubated for 24 h at 37 °C under anaerobic conditions. The antagonistic activity of LABs against pathogenic bacteria was confirmed by the formation of inhibition halos; halos were measured, and LABs were chosen for the modified plate diffusion method (Lima et al., 2007).

For the plate diffusion method, LAB´s strains were grown in MRS broth at 37 ºC until reaching the stationary phase (12-16 h). Subsequently, the media were centrifuged at 6 860 *xg* for 10 min. CFS were used in the antimicrobial assay that was performed by the plate diffusion method. 50 µL of CFS was placed in a 6 mm diameter well perforated on BHI agar, which was previously inoculated with 10 µL of a culture of the pathogenic strains. For this, *L. monocytogenes*, *Salmonella* sp. or *Staphylococcus aureus* were grown in BHI broth up to an optical density of 0.102 read at 600 nm, which corresponds to an approximate 10^6 CFU.mL^{-1}. The plates were incubated at 37 ºC for 24 h (Lima et al., 2007), the growth of the LAB was monitored and the diameter of the zone of inhibition (mm) around the wells (including the well), it was measured with a Vernier caliper (three times).

2.4. Data analysis

The data obtained from the inhibition tests were subjected to analysis of variance and subsequent multiple comparison of ranges by Fischer's least significant difference (LSD) procedure ($\alpha = 0.05$). This analysis was performed using Statgraphics Centurion XV v. 15.2.06 software.

3. Results and Discussion

3.1. Lactic acid bacteria isolated

One hundred and five bacterial isolates were obtained from freshly milked milk, fresh whey and cotija, 'fresco', 'quesillo' and 'doble crema' cheeses. Of these isolates, only 52 presented characteristic LAB morphologies. 32 had a point shape, 12 circular and 8 irregulars; all showed round edge and flat surface. All strains being catalase and peroxidase negative and Gram positive. The isolates were coded with a letter that signifies the type of cheese or milk, sample and a sequential number (Table 1).

The total number of strains obtained in our study is lower than that reported by Luiz et al. (2017) with a similar product (Minas cheese) from Brazil. This difference may be due to the number of samples (84) and the seven ripening times (cheese) that were considered for that study. Only fresh milk, whey and cheeses were analyzed in our study.

3.2. Pathogens isolated

From 'queso fresco' from four different sources (12 samples in total), 20 isolates were obtained in the selection culture media. Of these isolates, 10 strains were positive for the pathogenic microorganisms of interest. Three positive isolates for *Staphylococcus aureus*, four with characteristics of *Salmonella* spp. and three isolated from *Listeria monocytogenes* (Table 2). The pathogenic strain selected (of each type) for the confrontations against LAB, was the one that exhibit the best response to growth and came from the sample with the highest prevalence of pathogens.

Table 1. Morphological characteristics of lactic acid bacteria isolated from dairy products from the coastal region of Chiapas, Mexico.

Strain	Elevation	Form	Surface	Strain	Elevation	Form	Surface
CM1.C1	Round	Punctiform	Flat	CM3.C1	Round	Punctiform	Flat
CM1.C2	Round	Circular	Flat	CM3.C2	Round	Punctiform	Flat
CM1.C3	Round	Punctiform	Flat	CM3.C3	Round	Punctiform	Flat
DCM1.C1	Round	Punctiform	Flat	DCM3.C1	Round	Punctiform	Flat
DCM1.C2	Round	Punctiform	Flat	DCM3.C2	Round	Punctiform	Flat
DCM1.C3	Round	Punctiform	Flat	DMC3.C3	Round	Punctiform	Flat
FM1.C1	Round	Punctiform	Raised	FM3.C1.1	Round	Punctiform	Flat
FM1.C3	Round	Circular	Flat	FM3.C1.2	Round	Punctiform	Flat
FM1.C4	Round	Irregular	Flat	FM3.C2	Round	Punctiform	Flat
FM1.C5	Round	Irregular	Flat	FM3.C3	Round	Punctiform	Raised
FM1.C6	Round	Punctiform	Flat	CM4.C1	Round	Circular	Flat
FM1.C10	Round	Circular	Flat	CM4.C3	Round	Punctiform	Flat
QM1.C3	Round	Circular	Flat	QM3.C1	Round	Punctiform	Flat
QM1.C5	Round	Punctiform	Flat	QM3.C2	Round	Punctiform	Flat
CM2.C1	Round	Irregular	Flat	QM3.C3	Round	Punctiform	Flat
CM2.C3	Round	Punctiform	Flat	CM4.C2	Round	Punctiform	Flat
CM2.C5	Round	Punctiform	Flat	DCM4.C1	Round	Circular	Flat
DCM2.C1	Round	Punctiform	Flat	DCM4.C2	Round	Irregular	Raised
DCM2.C2	Round	Punctiform	Flat	DCM4.C3	Round	Irregular	Flat
DCM2.C3	Round	Punctiform	Flat	FM4.C1	Round	Irregular	Flat
FM2.C2	Round	Punctiform	Flat	FM4.C2	Round	Circular	Flat
FM2.C3	Round	Punctiform	Flat	QM4.C1	Round	Circular	Flat
FM2.C4	Round	Circular	Flat	QM4.C2	Round	Punctiform	Flat
QM2.C1	Round	Irregular	Flat	QM4.C3	Round	Circular	Flat
QM2.C2	Round	Circular	Flat	LM3.C3	Round	Punctiform	Flat
QM2.C3	Round	Punctiform	Flat	SM1.C2	Round	Irregular	Flat

In the strain code, C: Cotija cheese; F: 'queso fresco'; DC: 'doble crema' cheese**;** Q: 'quesillo'; L: milk; S: whey; M: sample

Table 2. Prevalence of pathogenic bacteria in 'queso fresco' samples collected in the coastal region of Chiapas, Mexico.

Cheese	Sample	*Staphylococcus aureus*	*Salmonella* sp.	*Listeria monocytogenes*
Q1	1	+	+	+
	2	-	+	+
	3	-	+	-
Q2	1	+	-	-
	2	+	+	+
	3	+	+	+
Q3	1	-	+	+
	2	-	+	+
	3	-	-	+
Q4	1	+	+	-
	2	+	-	-
	3	-	+	-

+: presence, -: absence

All the cheeses analyzed (Q1-Q4) in this study were positive for the presence of *Salmonella* spp., while 75% of the samples were positive for both *S. aureus* and *L. monocytogenes*. González-Montiel and Franco-Fernández (2015) report that, from 12 cheese samples of the Cañada Oaxaqueña, all tested positive for *S. aureus* and *Salmonella* spp., the results being very similar to ours. Guzmán-Hernández et al. (2016a), report that of 52 cheese samples from the Gulf of Mexico, 19 presented *S. aureus*, one cheese was positive for *L. monocytogenes* and two for *Salmonella* spp. In another study carried out with 52 samples of cheese from Tabasco (Mexico), only two samples were positive for *Salmonella* spp. (Guzmán-Hernández et al., 2016b). Our results were discordant in the number of strains obtained of *L. monocytogenes* and *S. aureus* with these authors, this may be since these samples are from different geographical region (different environmental conditions and manufacturing practices), as well as the types of cheese analyzed, but they coincide in the presence of pathogens. The mere presence of pathogens in cheeses already represents a serious problem for the health of consumers and may be due to the lack of hygienic-sanitary conditions that usually appear from the beginning of the process in the handling of milking to the preservation of the final product (cheese) at the points of sale (Guzmán-Hernández et al., 2016b).

3.3. Antagonistic capacity of LABs against pathogenic bacteria

From 52 strains of LAB, antagonism tests were carried out by direct confrontation between the LAB and the pathogen by the method of the drop on the surface. From this test, 36 strains (69.23%) with antagonistic capacity were obtained; of which 24 had activity against a single genus of pathogen (14 against *S. aureus*, nine against *Salmonella* sp. and one against *L. monocytogenes*). 11 LABs showed activity against two genera of pathogens (six against *S. aureus* and *Salmonella* sp., one against *S. aureus* and *L. monocytogenes* and four against *Salmonella* sp. and *L. monocytogenes*). Among the

BAL strains, the FM4.C1.2 strain stands out, which exhibited antagonism against the three pathogens tested in this study, through the absence of pathogen growth (Table 3).

Rivera de la Cruz et al. (2017) obtained 11 strains that formed inhibition halos against *Salmonella enterica* var. *Typhimurium*, which agrees with our results. Similarly, in another work by Ferrari et al. (2016) reported three different strains of LAB with the ability to stop the growth (inhibition halos) of *Salmonella Typhi*, *S. aureus* and *L. monocytogenes*, respectively.

Table 3. Lactic acid bacteria strains isolated from dairy products with antibacterial capacity.

LAB strain	Inhibiting bacteria	LAB strain	Inhibiting bacteria
CM1.C1	*Staphylococcus aureus*	CM4.C2	*Salmonella* sp.
CM1.C2.1	*Staphylococcus aureus*	DCM2.C3	*Salmonella* sp.
CM3.C2	*Staphylococcus aureus*	DCM3.C1.2	*Salmonella* sp.
CM4.C2	*Staphylococcus aureus*	DCM3.C2.1	*Salmonella* sp.
DCM1.C1	*Staphylococcus aureus*	FM1.C1	*Salmonella* sp.
DCM1.C5.1	*Staphylococcus aureus*	FM1.C4	*Salmonella* sp.
DCM2.C1	*Staphylococcus aureus*	FM1.C10.2	*Salmonella* sp.
DCM2.C3	*Staphylococcus aureus*	FM2.C3	*Salmonella* sp.
DCM3.C1.2	*Staphylococcus aureus*	FM3.C1.1	*Salmonella* sp.
DCM3.C3	*Staphylococcus aureus*	FM4.C1.1	*Salmonella* sp.
DCM4.C1.1	*Staphylococcus aureus*	FM4.C1.2	*Salmonella* sp.
FM1.C5	*Staphylococcus aureus*	QM1.C3	*Salmonella* sp.
FM2.C3	*Staphylococcus aureus*	QM1.C5	*Salmonella* sp.
FM3.C1.2	*Staphylococcus aureus*	QM2.C2	*Salmonella* sp.
FM4.C1.2	*Staphylococcus aureus*	QM4.C1	*Salmonella* sp.
QM1.C5	*Staphylococcus aureus*	QM4.C3	*Salmonella* sp.
QM2.C1	*Staphylococcus aureus*	CM1.C1	*Listeria monocytogenes*
QM2.C4	*Staphylococcus aureus*	CM3.C3.2	*Listeria monocytogenes*
QM2.C5	*Staphylococcus aureus*	DCM3.C2.1	*Listeria monocytogenes*
QM3.C2.1	*Staphylococcus aureus*	DCM4.C2.1	*Listeria monocytogenes*
QM3.C2.2	*Staphylococcus aureus*	FM1.C1	*Listeria monocytogenes*
CM1.C.2.1	*Salmonella* sp.	FM3.C1.1	*Listeria monocytogenes*
CM3.C2	*Salmonella* sp.	FM4.C1.2	*Listeria monocytogenes*

In the strain code, C: Cotija cheese; F: 'queso fresco'; DC: 'doble crema' cheese**;** Q: 'quesillo'; L: milk; S: whey; M: sample

The CFS of the same 52 strains of BAL were challenged against pathogens by the plate diffusion method. In this test, 21 CFS (40.28%) with antagonistic capacity were obtained. 13 CFS showed activity against a single genus of pathogen (seven against *Salmonella* sp., four against *L. monocytogenes* and two against *S. aureus*). Six CFS showed activity against two genera of pathogens (three against *S. aureus* and *L. monocytogenes*, two against *Salmonella* sp. and *L. monocytogenes* and one against *S. aureus* and *Salmonella* sp.).

The CFS of the strains CM3.C2 and FM4.C1.2, inhibited the three pathogens tested in this study, with inhibition halos ranging from 9 mm to 25.5 mm. The CFS of the strains CM1.C1 and QM1.C5 also stood out, which inhibited two of the three pathogens with the highest inhibition halos. The strain CM1.C1 inhibited to *L. monocytogenes* (8 mm diameter) and *S. aureus* (25 mm diameter); while the QM1.C5 strain inhibited to *Salmonella* sp. (25 mm diameter) and *L. monocytogenes* with 14 mm of diameter (Table 4).

The amount of CFS with antibacterial capacity obtained in our work was greater than that obtained by Arrioja-Bretón et al. (2020), who report that 12 CFS were antagonistic to *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *Salmonella Typhimurium* and *L. monocytogenes*. However, the results regarding the diameter of inhibition were similar, since the authors report values of 28.35, 20.45, 27.71 and 25.83 mm, respectively. In another work reported by De Almeida et al. (2015) obtained six CFS with antagonistic capacity against *E. coli*, *S. aureus*, *Salmonella* spp. and *L. monocytogenes*. Asurmendi et al. (2015) obtained 21 CFS antagonistic to *L. monocytogenes*, the strain B87 being notable with a 24.5 mm inhibition diameter. These results demonstrate that the CFS of the isolates in our study are efficient both in number and in the size of the inhibition diameter.

From the results obtained in both antagonism tests, the FM4.C1.2 strain stands out above the others, since in both tests the strain inhibited the growth of the three pathogens, in addition, the inhibition values of thus CFS strain against two pathogens are among those with the highest antagonistic activity with inhibition diameters of 22.50 mm for *S. aureus* and 25.50 mm for *Salmonella* sp. These results may be due to the production of different organic acids (lactic, acetic, benzoic, fatty acids); which can impede cellular activity, interfere with the conservation of the membrane potential, decrease the cellular pH and thus prevent a variety of essential metabolic functions for the pathogen (Mani-Lopez et al., 2012; Omemu and Faniran, 2011). It can also be attributed to CO_2, acetaldehyde, diacetyl, hydrogen peroxide, and low molecular weight molecules such as methylhydiatonin, mevalanolactone, reuterin, and bacteriocins (De Paula et al., 2014; Moračanin et al., 2012).

Table 4. Diameter (mm) of the inhibition zones generated by the lactic acid bacteria supernatants studied against pathogenic microorganisms.

LAB strain	*Staphylococcus aureus*	*Salmonella* sp.	*Listeria monocytogenes*
CM3.C2	20.00±7.07 ab	10.00±7.07 ab	9.75±7.42 ab
FM4.C1.2	22.50±3.54 a	25.50±6.36 a	9.00±1.41 ab
CM1.C1	25.00±0.00 a	-	8.00±0.00 ab
DCM2.C3	12.50±3.54 b	-	10.00±0.00 ab
DCM3.C1.2	12.50±3.54 b	8.50±7.78 ab	-
FM1.C3	18.50±4.95 ab	-	12.50±3.54 ab
QM1.C5	-	25.00±4.24 a	14.00±2.83 ab
QM1.C5.1	-	11.00±1.41 ab	15.00±1.41 a
CM1.C3	-	-	7.25±3.89 b
CM4.C2	-	20.00±0.00 ab	-
DCM1.C1	-	26.50±4.53 a	-
DCM3.C1.1	-	2.50±0.71 b	-
DCM3.C3	22.50±3.54 a	-	-
FM1.C6.1	-	-	12.00±2.83 ab
FM2.C2	-	25.00±0.85 a	-
FM3.C1	-	-	13.00±2.83 ab
FM4.C1.1	-	-	11.00±1.41 ab
QM1.C3	12.50±3.54 b	-	-
QM2.C6	-	15.00±1.41 ab	-
QM4.C1	-	19.00±1.41 ab	-
QM4.C3	-	26.00±7.07 a	-

–: No inhibition was observed. [a-b] Equal letters per column denote significant equality (Fischer's least significant difference, $\alpha = 0.05$)

A phenomenon observed in this study is the fact that one CFS inhibited the three pathogens while the strain from which the CFS originated in direct confrontation inhibited two pathogens. This may be due to the fact that the production of active substances could vary depending on the growth conditions (agar for the strain and broth to obtain the CFS), such as oxygen concentration, pH, temperature, water activity and the nutrients; and, in this way, modify the population density, affecting the expression of genes (*quorum sensing*) that encode the production of fatty acids and bacteriocins (Beristain-Bauza et al., 2016; Sip et al., 2012).

4. Conclusion

From cheese samples it was possible to isolate lactic acid bacteria (LAB) with antagonistic capacity against pathogenic bacteria. The cell-free supernatants of some LAB were equally efficient in controlling the growth of pathogens. The strains encoded as FM4.C1.2 and CM3.C2 are potential pathogen controllers since they inhibited the three evaluated pathogens. It is convenient to evaluate the effect of the strains or their supernatants in situ (in cheeses) to validate the antagonistic effect.

Conflicto de intereses

Los autores declaran no tener ningún conflicto de intereses

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ARTÍCULO CORTO

An easy hardening off method for micropropagated *Guarianthe skinneri* (Orchidaceae) plantlets

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Abstract

This study reports an easy protocol for hardening off plantlets of the tropical orchid *Guarianthe skinneri*, which is a culturally relevant plant species in several countries of the Central American region. In order to increase the *post vitro* survival rate of micropropagated plants, we included a "preparation step" before transferring the plants from the laboratory to the greenhouse. This preparation step (two weeks of duration in Dalla Rosa and Laneri or KO7 medium) consisted in the ventilation of the culture vessel. Results showed that, in comparison with the control treatment, ventilation of the culture vessel (aperture of 88 mm^2) yielded the greatest survival (90%) and best appearance (3.4 on a hedonic scale of 0.0 to 4.0) of plants. Results are explained with base in the hydric relations of the plants with their microenvironment.

Keywords:
Acclimatization
in vitro propagation
Micropropagation
Orchids
Water balance

Un método simple para endurecer plántulas micropropagadas de *Guarianthe skinneri* (Orchidaceae)

Resumen

Este estudio reporta un protocolo simple para el endurecimiento de plántulas de la orquídea tropical *Guarianthe skinneri*, la cual es una especie vegetal de importancia cultural en varios países de la región centroamericana. Con el fin de incrementar la tasa de supervivencia *post vitro* de las plantas micropropagadas, incluimos un "paso de preparación" antes de transferir las plantas del laboratorio al invernadero. Este paso de preparación (de dos semanas de duración en medio Dalla Rosa y Laneri o KO7) consistió en la ventilación del recipiente de cultivo. Los resultados mostraron que, en comparación con el tratamiento control, la ventilación del recipiente de cultivo (apertura de 88 mm^2) produjo mayor supervivencia (90%) y mejor apariencia (3.4 en una escala hedónica de 0.0 a 4.0) de las plantas. Los resultados se explican con base en las relaciones hídricas de las plantas con su microambiente.

Palabras clave:
Aclimatación
Propagación *in vitro*
Micropropagación
Orquídeas
Balance hídrico

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1. Introduction

Orchids have been one of the most appreciated and admired groups of ornamental plants for many centuries by different civilizations, due to the great beauty of their flowers (Ticktin et al., 2023). They are mainly used as potted ornamentals and for cut flowers. *Guarianthe (Cattleya)* is one of the most popular genera of orchids in the international market, such as *Cattleya skinneri* (Pant et al., 2020), which has been reclassified as *Guarianthe skinneri* (Dressler and Higgins, 2003). This species is culturally important in several countries of the Central American region; for example, in Costa Rica was declared the national flower in 1939 (Abarca, 2020), while in Southern Mexico, it is commonly known as "Candelaria" and is the symbol of a religious festival (Ovando et al., 2023).

In order to increase the rate of propagation of this species, many laboratories have undertaken processes for its micropropagation with successful results (Hernández-Domínguez et al., 2024; Leyva-Ovalle et al., 2020). However, a frequent problem with orchid in vitro propagation is the lack of ability of *ex vitro* plants to adequately acclimatizing in field conditions (Hartmann et al., 1997; Ovando et al., 2005), particularly in hostile, warm, humid, and propitious areas for the development of pathogens as in the humid tropics. *Ex vitro* orchids have been reported to survive from 30% to 50%, due to the morphological and physiological changes of the plant in the *in vitro* stage (Hew and Yong, 1997; Poniewozik et al., 2021). Therefore, it is necessary to develop an effective protocol to "harden off" orchid plants before the acclimatization phase to prepare morpho-physiologically the plants.

This study proposed the establishment of a "preparation phase" that includes the modification of culture conditions in the last *in vitro* stage, such as the micro-ventilation of the container in order to determine their effects on the quantity and quality of *G. skinneri* vitroplants.

2. Materials and Methods

2.1. Plant material

Six-month-old plantlets from an *in vitro* propagation system were used, which were in the multiplication stage. Plants had a size ranging between 1.5 cm and 2.0 cm in height. Before the experiment, plants were sub-cultivated three times in media with 1.0 mg L^{-1} of the cytokinin 6-benciladenine. Plants had four to five leaves and six to eight roots.

2.2. Culture medium

The culture medium used was KO7 (Dalla Rosa and Laneri, 1977), whose composition is: $Ca(NO_3)_2.4H_2O$ (1 g L^{-1}), $MgSO_4.7H_2O$ (0.25 g L^{-1}), KH_2PO_4 (0.25 g L^{-1}), $(NH_4)_2SO_4$ (0.5 g L^{-1}), $MnSO_4.4H_2O$ (7.5 mg L^{-1}), $FeSO_4.7H_2O$ (27.85 mg L^{-1}), NaEDTA.$2H_2O$ (37.25 mg L^{-1}). The medium was supplemented with coconut water (150 mL L^{-1}), sucrose (20 g L^{-1}), agar (8 g L^{-1}) and 0.5 g L^{-1} of activated charcoal; the last one to avoid oxidation of the culture medium and roots of the plants. The pH of the culture medium was adjusted to 5.5, deposited in 100 mL flasks (baby food jars) with screw plastic cap in 20 mL volumes and autoclaved at 121 °C for 15 minutes.

2.3. Effect of the ventilation of the culture container

A complete random experiment was designed to evaluate the effect of the ventilation of the culture vessel, with four treatments and three replicates, 12 experimental units (one plant per culture container). The bottles used were "baby food jars" with capacity of 110 mL and with a mouth area of 1590 mm^2. Ventilation was done by circular perforation of the bottle caps and prior to autoclaving the windows were covered with Whatman No. 40 filter paper. Treatments were Control (without ventilation), T1 (44 mm^2), T2 (88 mm^2) and T3 (132 mm^2).

The "preparation" period had a duration of two weeks, under a luminous intensity of 660 lux with a photoperiod of 12 hours and room temperature of 28 °C, after which the plantlets went to the greenhouse for their acclimatization for 30 days, where the conditions were average temperature of 30 °C and relative humidity of 70%.

2.4. Variables and data analysis

After the preparation period (two weeks), water content of the plant and of humidity of the remainder culture medium was determined. After the greenhouse period (30 days), the plant survival, appearance of plantlets (on a hedonic scale of 0 to 4), leaf and cuticle thickness, and number and size of stomata were evaluated. Data were processed by analysis of variance and means separation by the Tukey method (α 0.05).

3. Results and Discussion

The influence of the ventilation of the culture container for two weeks, followed by 30 days of acclimatization, on the survival of the tropical orchid *Guarianthe skinneri* is showed in Figure 1. It is remarkable that while the water content in the culture medium diminished (Figure 1A), the plants accumulated most water in their tissues (Figure 1B).

Plants response to water stress by dramatically complex mechanisms, including drought avoidance via enhancing capacity of getting water and drought tolerance mainly via improving osmotic adjustment ability and increasing cell wall elasticity to maintain tissue turgidity (Taticharoen et al., 2023; Xu et al., 2010). In this study, plants grown with ventilation may have experienced rapid adjustments in their water relations.

Several authors have pointed out that plants grown *in vitro* exhibit physiological and anatomical modifications depending on culture conditions, especially, the leaves undergo temporary modifications that prevent them from adequate adaptation (Buyun et al., 2021; George and Sherrington, 1984; Hazarika, 2006). In our study, the treatment of 44 mm^2 of ventilation area promoted the greatest thickening of the leaves and of cuticle thickness (Table 1,

ANOVA P <0.05), being the last variable one of the most important, since the cuticle is a waxy layer that protects the top of the leaves from solar radiation. Similar results were found by Ramos et al. (2001), who proved that ventilation of the culture vessel and the addition of the phytohormone abscisic acid (ABA) to the culture medium favors leaf development (greater thickness, fewer stomas, and more chloroplasts), allowing a better survival of vitroplants of *Tagetes erecta* when these are transferred to the field. Figure 2 shows a comparison between the thickness of the leaves in the treatment of 44 mm^2 and the control without ventilation.

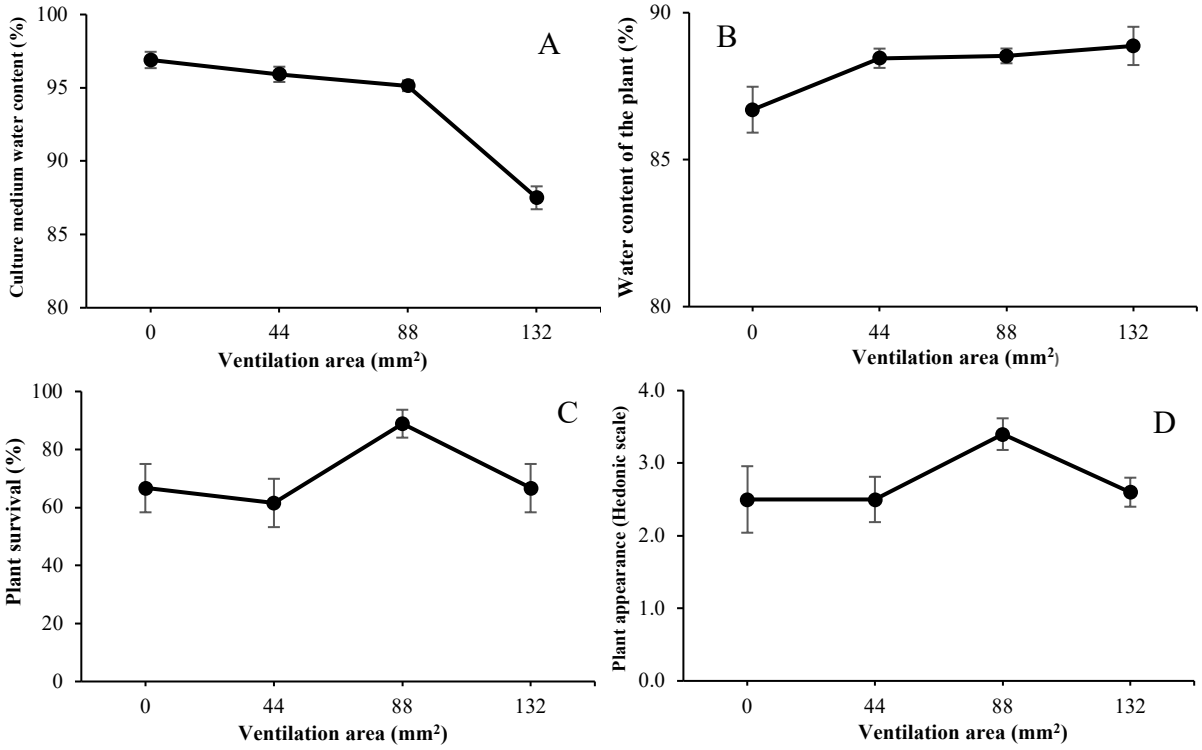

Figure 1. Influence of the ventilation of the culture container on the micropropagation of the tropical orchid *Guarianthe skinneri*. After two weeks of laboratory incubation: A) Effect on the water content of the remainder culture medium; B) Effect on the plant water content. After 30 days in greenhouse: C) Effect on the survival of the plants; D) Effect on the appearance of the plants.

Table 1. Foliar anatomical variables of *ex vitro* plantlets of the tropical orchid *Guarianthe skinneri* grown in culture containers with ventilation.

Ventilation area (mm^2)	Leaf thickness (µm)	Cuticle thickness (µm)	Leaf stomata/mm^2	
			Adaxial	Abaxial
0	446.4 ± 70.0ab	4.6 ± 0.4c	14.6 ± 2.1a	60.4 ± 11.1b
44	509.0 ± 64.6a	12.2 ± 1.5a	3.9 ± 1.1b	89.3 ± 15.2a
88	410.4 ± 26.3b	5.1 ± 1.8bc	3.2 ± 1.4b	119.5 ± 17.3a
132	383.2 ± 15.9bc	7.4 ± 2.0b	3.9 ± 1.8b	108.3 ± 9.1a

The data with different letters indicate Tukey statistical difference (α 0.05).

The stomata number per square millimeter showed that the control plants (microenvironment with high relative humidity) have more stomata on the adaxial surface of the leaf in comparison to the rest of treatments, which, in nursery conditions, leads to a faster dehydration of the plantlet. It is possible that ventilation of the containers reduces the relative humidity in them, allowing better stomatal functioning, increases the production of epicuticular waxes, and allows the increase of CO_2 (Paz-Silva et al., 2004).

However, the treatment with better results was that of 88 mm^2 of ventilation had a survival rate of almost 90% and an appearance value of 3.4 (Figures 1C, 1D). These results contrast with that mentioned by several authors, who cite 30% to 50% of *ex vitro* survival rates for orchids in hostile, hot, humid climates, and favorable for the development of phytopathogenic microorganisms (Ovando et al., 2005). Although the present study was carried out in the dry season, the conditions were of excessive heat and with relatively high ambient humidity. Figure 3 shows plants grown in containers with 88 mm^2 of ventilation and plants grown in closed containers.

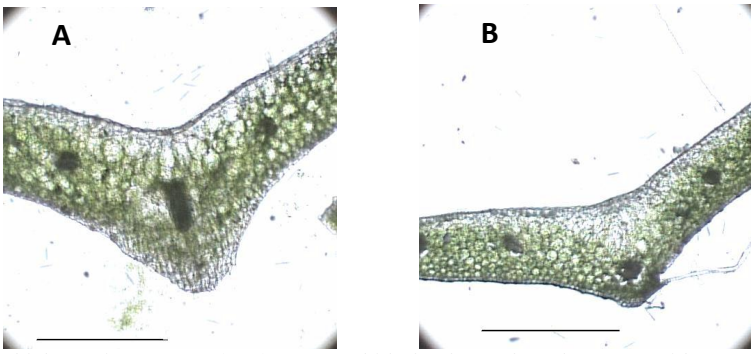

Figure 2. Comparison of leaf thickness in *Guarianthe skinneri* orchid plantlets cultured *in vitro* with vessel ventilation (44 mm^2, A) and the control without ventilation (B). The bar represents 500 µm.

Figure 3. *Guarianthe skinneri* orchid plants grown in closed containers and with 88 mm^2 of ventilation.

Based on the results obtained, the micropropagation process of the tropical epiphytic orchid *G. skinneri* is improved when a "preparation phase" of the plantlets is used for *ex vitro* conditions; this stage lasts for two weeks and consists in the decrease of the relative humidity of the container (through ventilation of 88 mm^2). This finding is similar to that of Fuentes et al. (2001), who found that the ventilation of the culture container and reduction of saccharose together with increased light intensity minimized the problems of low survival and slow growth of vitroplants of several species. Ventilation helped plants to have better stomatal control and reduced leaf water loss, while reducing saccharose to half the normal concentration produced higher rates of photosynthesis.

4. Conclusion

The hardening off method designed in this work consisted in the culture of six-month-old plants in KO7 medium supplemented with coconut water, sucrose, and activated charcoal, for two weeks. In the best treatment, the culture containers had ventilation of 88 mm^2 and was incubated under a luminous intensity of 660 lux with a photoperiod of 12 hours and room temperature of 28 °C. After the transference of the plants to greenhouse conditions (average temperature of 30 °C and relative humidity of 70%) the plant survival was about 90%.

Conflict of interest

The authors declare that they have no conflict of interest

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ARTÍCULO CORTO

Does marking affect the mating performance of sterile males of *Anastrepha ludens* (Diptera: Tephritidae)?

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Abstract

In fruit flies three methods of marking are commonly used for the evaluation of the sexual performance of adults in field cages. These methods are marking with label, marking with acrylic paint, and marking by dyed food intake. Some of these techniques can have a negative effect on the sexual performance. Our objective here was to determine the marking effect and color on mating performance of sterile males of *Anastrepha ludens* (Loew) (Diptera: Tephritidae*)*. The results of the study did not indicate significant differences on mating performance between marked and unmarked sterile males. In the evaluation of marking colors, there were also no significant differences on mating behavior. In conclusion, any of the three methods and colors of marking evaluated do not affect the selection choices of wild females and therefore the sexual performance of sterile males of *Anastrepha ludens*. The advantages and disadvantages of their application are discussed.

Keywords:
Identification technique
Mexican fruit fly
Sexual behavior
Sterile insect technique

Palabras clave:
Técnica de marcaje
Mosca mexicana de la fruta
Comportamiento sexual
Técnica del insecto estéril

¿Afecta el marcaje el desempeño de apareamiento de machos estériles de *Anastrepha ludens* (Diptera: Tephritidae?

Resumen

En moscas de la fruta se utilizan comúnmente tres métodos de marcaje para la evaluación del desempeño sexual de adultos en jaulas de campo. Estos métodos son el marcaje con etiqueta, el marcaje con pintura acrílica y el marcaje mediante la ingesta de alimento teñido. Algunas de estas técnicas pueden tener un efecto negativo en el desempeño sexual. Nuestro objetivo aquí fue determinar el efecto del marcaje y el color en el desempeño de apareamiento de machos estériles de *Anastrepha ludens* (Loew) (Diptera: Tephritidae). Los resultados del estudio no indicaron diferencias significativas en el desempeño de apareamiento entre machos estériles marcados y no marcados. En la evaluación de los colores de marcaje, tampoco hubo diferencias significativas en el comportamiento de apareamiento. En conclusión, ninguno de los tres métodos y colores de marcaje evaluados afecta las opciones de selección de hembras silvestres y, por lo tanto, el desempeño sexual de machos estériles de *Anastrepha ludens*. Se discuten las ventajas y desventajas de su aplicación.

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1. Introduction

The sterile insect technique (SIT) is an environmentally friendly method that is being used in various parts of the world to suppress insect pests (Dyck et al., 2005). The SIT involves the rearing, irradiation, and release of a massive number of sterile insects in the field (Knipling, 1955). Estimating the level of efficacy of the SIT requires a protocol that allows discrimination between released and wild insects (Enkerlin et al., 1996). A fluorescent dye is commonly used to mark fruit flies (Day-Glo fluorescent, neon red, Cleveland, Ohio), and this powder is internalized by the insect during the process of eclosion. Dye adhering to the pupae and dye on surrounding pupae is contacted by the ptilinum at adult eclosion and the material is retracted into the head of the fly along with this structure. Dye particles that adhere to other body parts are usually disposed of by the continual "cleaning" activity of the adult (Schroeder and Mitchell, 1981). This technique is only efficient in identifying insects during the release and capture process, and it is not recommended for the observation of sexual behavior. According to Hagler and Jackson (2001), the selection of a specific marker depends on the type of study that the researcher is planning, which involves the identification of insects with and without movement.

Currently, the estimation of sexual behavior is evaluated under field conditions in cages, which are more complex than laboratory tests because they evaluate a broad combination of non-controllable parameters (Chambers et al., 1983). This approach involves the development of marking techniques that allow for individual identification and monitoring of released insects. In fruit flies three methods of marking are commonly used for the evaluation of the sexual performance in field cages: marking with label, marking with acrylic paint and marking by dyed food intake.

In the genus *Anastrepha* and *Ceratitis*, insects have been marked using a numbered label (Meza et al., 2005) or printed letter (McInnis et al., 2002) stuck to the side of thorax that allows evaluation of the sexual competition of individual adults. In the genus *Ceratitis* and *Bractocera*, the application of acrylic paint to the thorax of laboratory and wild insects in the evaluation of sexual behavior has been reported (McInnis et al., 2002; Shelly et al., 2000). In the genus *Rhagoletis*, *Ceratitis* and *Drosophila* a new technique of identification using a dye added to the diet of adults was applied (Arévalo et al., 2009; Ramirez-Santos et al., 2017; Verspoor et al. 2015).

Although the use of these techniques has been reported in numerous studies, no detailed analysis has been conducted on the effect of these marking and color methods on sexual performance of the flies.

Our objective here was to determine the marking effect and color on mating performance of sterile males of *Anastrepha ludens (*Loew). Our hypothesis was that marking and color on insects does not affect mating performance of *Anastrepha ludens* sterile males. We conducted this study in field cages and compare three commonly used methods of marking on fruit flies.

Our results should provide reliable evidence for the potential application of these techniques in quality tests of flies used for SIT programs. The advantages and disadvantages of the methods' application are also discussed.

2. Materials and Methods

2.1. Biological material

Wild and sterile adults of *A. ludens* were used in this study. Sterile insects were obtained from irradiated pupae as a product of mass rearing of *A. ludens* in the Metapa de Domínguez Moscafrut Facility, Chiapas, Mexico (14°49'49.2"N, 92°11'44.8"W, and altitude 102 m above sea level). For the sterilization process, the pupae were irradiated under hypoxia conditions 48 h before peak adult emergence to a radiation dose of 80 Gy in a Gamma Beam 127 irradiator with a Cobalt-60 gamma source (series no. 226, dry storage source; Nordion International, Kanata, ON, Canada).

Wild insects were obtained by the recovery of larvae from bitter orange (*Citrus aurantium*, L.) collected in the region of Soconusco, Chiapas, Mexico. Once the larvae reached maturity, they were placed in containers with wet vermiculite to promote pupation and remained in those containers until they emerged as adults (Orozco et al., 2013).

2.2. Insect management

The pupae of both strains were placed in wooden cages (30x30x30 cm) covered at the ends with tulle fabric (2 mm) until the emergence of the adults. Upon emergence, females and males were isolated in separate cages (to avoid any contact of pheromones before testing) and maintained under a photoperiod of 12 h of light (from 7:00 to 19:00 h at a light intensity of 550 ± 50 lux) and 12 h of darkness, a temperature of 25 ± 1 °C, and relative humidity of 65 ± 5%. The adults were fed *ad libitum* with diet formulated with water and a mixture of sugar and hydrolyzed protein (3:1 ratio), yeast hydrolysate enzymatic, BP Biochemicals, LLC. In all of the experiments, sexually mature, virgin wild and sterile adults of 18 and 10 days of age after emergence, respectively, were used.

2.3. Study site

Tests were performed in a mango orchard (*Mangifera indica* cv. Ataulfo) close to the Moscafrut facility, which is located at 14°49′33.9″N, 92°11′46.2″W at 97 m above sea level in Metapa de Dominguez, Chiapas, Mexico. Field cages of 3 m in diameter by 2 m in height were used and were supported by a metal structure (Calkins and Webb, 1983). The field cage covered a citrus (*Citrus x aurantium* L.) of approximately 1.8 m in height.

The tests were performed under the following environmental conditions: 25-29 ºC, 70-80% relative humidity, and 3,000-0 Lux. Observations were made from 16:00 to 17:00 h, which is considered to be the period of maximum sexual activity for this species (Aluja et al., 2000).

2.4. Insect marking

Two days before the test, sterile insects were marked according to the following methods: 1) Mark with label. The flies were marked with a small paper tag (2 mm diameter) and glued on the fly thorax for individual identification (Meza et al., 2005). The tag was glued using a toothpick and white school glue of the mark Resistol^{MR} 850 of manufacturing in Mexico. 2) Mark with acrylic paint. The flies were marked with a small amount of acrylic paint (white, green, yellow, blue or red) applied on the surface of the thorax. The paint was added with a toothpick and placed gently on the insect. The methodology was like described in the manual of the FAO/IAEA/USDA (2014). 3) Mark by dyed food intake. The flies were marked by the intake of food coloring (DEIMAN®), which was added to the water (1 mL of coloring in 50 mL of water) two days prior to the test. This dye enters the intestines of the fly, and the abdomens were observably colored one day after exposure. 4). Unmarked. This treatment corresponds to the control, and unmarked sterile flies were used.

2.5. Sexual performance of males with different marking colors

Tests were conducted between marked sterile males (with different colors) and wild females (unmarked). In this study the three marking methods mentioned above were used. In each field cage, 10 males marked of each color (blue, green, red, white, yellow) and 50 wild females (unmarked) (1:1:1:1:1:5 ratio) were released. The males were released 30 min before the females to allow them to establish territories. The number of mating (which in this case served to evaluate the selection of wild females), was recorded in each cage.

Each field cage was considered as an experimental unit. Three different production batches were evaluated, with four replicates for each batch. In total, 12 replicates were performed for each one of the marking methods.

2.6. Sexual performance of marked and unmarked males

In each field cage, 20 marked males, 20 unmarked males and 20 wild females (unmarked) (1:1:1 ratio) were released. The males were release as described above. Tests were conducted for each one of the marking methods. The number of mating's was recorded in each cage. Each field cage was considered as an experimental unit. Three different production batches were evaluated. Ten replicates were performed for each one of the marking methods.

2.7. Data analysis

Data normality and the homogeneity of variance were determined using the Shapiro test and the Bartlett test, respectively. The data of sexual performance of males with different marking colors were analyzed using an analysis of variance (ANOVA) followed by a comparison of means with Tukey's test ($P<0.05$) (Zar, 1999). The data of sexual performance of marked and unmarked males were analyzed by a paired Student *t* test. The data were analyzed using the statistical software Minitab 16 (2010) from Minitab Inc. (www.minitab.com).

3. Results

3.1. Sexual performance of males with different marking colors

The evaluation of sterile males marked with different colors indicated no significant differences in the selection of the wild females in the different marking methods evaluated ($F_{4,55}=1.07$, $P=0.38$ Mark by dyed food intake; $F_{4,55}=0.74$, $P=0.57$ Mark with label; and $F_{4,55}=1.95$, $P=0.12$ Mark with acrylic paint) (Figure 1).

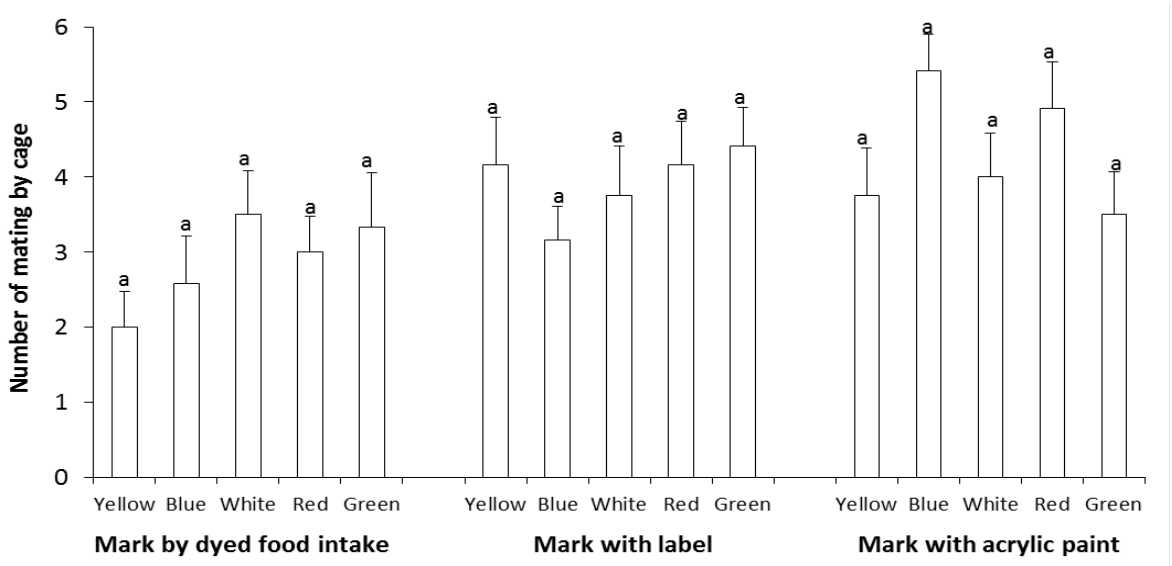

Figure 1. Sexual performance of sterile males marked with different colors under different marking methods. Bars marked with the same letter in each marking method are not significantly different ($P>0.05$).

3.2. Sexual performance of marked and unmarked male

The results of the study did not indicate significant differences between marked and unmarked males among the different marking method evaluated (t_9=0.61, P=0.56 Mark by dyed food intake; t_9=2.08, P=0.067 Mark with label; and t_9=0.53, P=0.606 Mark with acrylic paint).

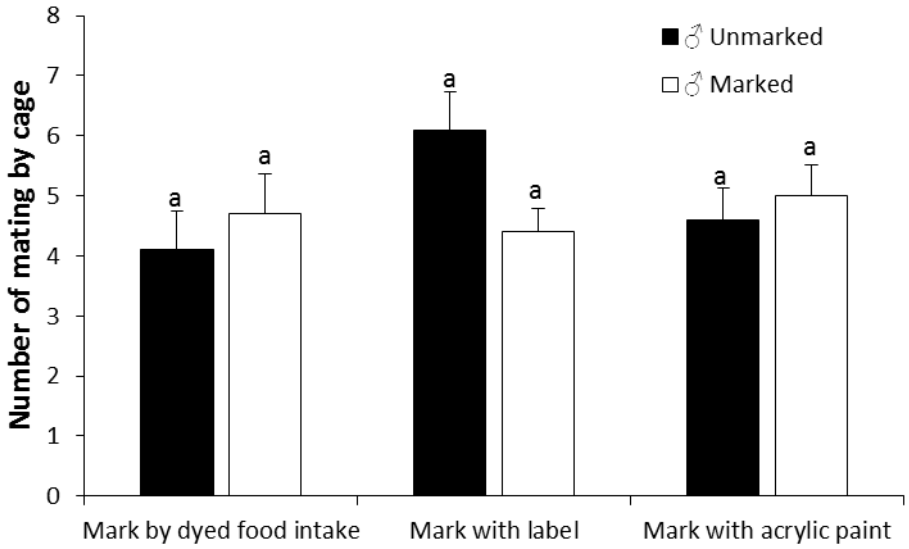

Figure 2. Sexual performance of marked and unmarked sterile males using different marking methods. Bars marked with the same letter in each marking method are not significantly different (P>0.05).

4. Discussion

Our results confirmed our hypothesis that color and marking on insects does not affect mating performance of *Anastrepha ludens* sterile males. These results were similar to those documented in *Ceratitis capitata* (Wiedemann), where the marking color does not affect the performance of the flies in tests of sexual behavior (Holbrook et al. 1970; Liedo et al., 2002). Three reasons can explain this observed behavior. The first is that the color in the thorax and / or abdomen of the flies is not perceptible to wild females. Second, the colors used are not a cause of rejection for wild females and the third, color is not a factor of discrimination of wild females. Although the latter is unlikely, for *A. ludens* a preference for the colors green, yellow and orange by females is reported (Robacker et al., 1990).

In *Anastrepha ludens* it is inferred that the type of marking does not interfere with the sexual activity of the flies (Meza et al., 2005; Meza-Hernández and Diaz-Fleischer, 2006). This study confirms these results and is similar to those documented in other species. In *C. capitata*, there are no differences between marked and unmarked males in mating tests (Guerfali et al., 2011). In the ectoparasite *Dinarmus basalis*, there is also documented no effect of marking on sexual performance tests (Lacoume et al., 2007). According to the results, the three marking methods evaluated are viable to evaluate the sexual behavior of the flies.

According to Hagler and Jackson (2001), to evaluate the movement of insects in their natural habitat, it is essential to develop a wide variety of markers that permit the evaluation of different insect behaviors, such as population dynamics, dispersion, territoriality, feeding behavior, trophic-level interactions, and other ecological interactions of insects, and the ideal marker must persist without inhibiting the normal biology of the insect. In this study, the three marking methods evaluated meet these premises.

Although there are no significant effects of marking on the sexual performance of insects, not all of the methods allow for the individual assessment of certain behavioral aspects, such as male calling, leks, and remating, as is the case for acrylic paint and dyed food intake. The greatest advantage of using the Marking method with label is that has low cost, is long lasting, and can be used to individually identify insects. This method has been successfully applied in species such as *A. ludens* (Liedo et al., 2010; Meza-Hernández and Díaz-Fleischer, 2006), *A. obliqua* (Quintero-Fong et al., 2011; Telles-Romero et al., 2011), and *C. capitata* (McInnis et al., 2002). However, one of the disadvantages is that individual application is tedious and time-consuming. Additionally, there is a risk that the label will detach from the thorax during handling or exposure to water during the rainy season.

Despite the limitation of differentiating individual insects, the marking method with acrylic paint is practical and reliable, and the marking is retained in the insect for a sufficient period. This method is generally the most used for the evaluation of the sexual behavior of fruit flies (FAO/IAEA/USDA, 2014). However, the risk of using toxic paint restricts the use of many solvents (Southwood, 1978).

The Marking method by dyed food intake offers many advantages compared to other methods, primarily in that adding the dye to the diet requires a minimum amount of

additional work. Secondly, the procedure avoids the handling of insects. Finally, the detection of the marking inside the insects is easy. However, it is possible that some dyes are not visible, have a retention period that is too short, or are harmful to the insects. In addition, there is the limitation of not being able to differentiate between individual insects. In *C. capitata* this method was documented to evaluate the sexual competence of a genetically modified strain (Ramirez-Santos et al., 2017).

In conclusion, in *A. ludens* the marking does not affect the sexual performance of sterile males or the selection of wild females. Any of the three methods and colors evaluated can be used to mark flies in sexual behavior tests.

Conflict of interests

The authors declare that they have no conflict of interest.

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REVISIÓN BREVE

Insectos polinizadores en el género *Vanilla*. Caso de estudio *Vanilla planifolia* (Jacks ex. Andrews)

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Resumen

Vanilla planifolia Jacks ex. Andrews, es una planta de importancia comercial de la cual se obtiene el extracto de Vainilla. Sin embargo, debido a la baja tasa de polinización por insectos se han realizado diversas técnicas de polinización manual desde hace 180 años, lo que ha ocasionado una pérdida de variabilidad genética que pone en riesgo a las plantaciones comerciales. Con base en lo anterior, el objetivo del presente trabajo fue realizar un análisis de la información disponible acerca de los insectos polinizadores que se han asociado a *Vanilla planifolia*. En varios estudios se ha reportado la participación de abejas del género *Euglossa* como polinizadores de *Vanilla planifolia*, y algunos otros visitantes recurrentes que podrían ser polinizadores potenciales.

Palabras clave:
Abejas
Euglossini
Recompensa floral
Vainilla

Pollinating insects in the genus *Vanilla*. Case study: *Vanilla planifolia* (Jacks ex. Andrews)

Abstract

Vanilla planifolia Jacks ex. Andrews, is a plant of commercial importance from which the Vainilla extract is obtained. However, due to the low rate of pollination by insects, manual pollination techniques have been carried out for 180 years, which has caused a loss of genetic variability that puts commercial plantations at risk. Based on the above, the objective of this work was to carry out an analysis of the available information about pollinating insects that have been associated with *Vanilla planifolia*. In several studies, the participation of bees of the genus *Euglossa* as pollinators of *Vanilla planifolia*, and some other recurring visitors that could be potential pollinators, have been reported.

Keywords:
Bees
Euglossini
Floral reward
Vanilla

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1. Introducción

Vanilla es un género pantropical de la familia Orchidaceae que agrupa cerca de 100 especies (Pérez-Castro, 2013). Estas plantas se presentan en muchas áreas tropicales como hemiepífitas, monopodiales, con flores relativamente grandes en tonos verdes y amarillos. Dichas flores son efímeras, pues por lo general duran menos de un día (Díaz-Bautista et al., 2018). La vainilla (*Vanilla planifolia* Jacks ex. Andrews) es una de las especies agrícolas de mayor valor comercial, ya que de ella se obtiene la popular esencia de vainillina, utilizada ampliamente en diversas industrias (Rodríguez-Deméneghi et al., 2023). Los frutos de *Vanilla* son vainas carnosas y cilíndricas de hasta 25 cm de largo, las cuales, tras pasar por procesos de curado, son comercializadas (Chambers et al., 2019).

Se ha reportado que solo tres especies de vainilla se cultivan de forma comercial, dentro de las características del género *Vanilla*, se ha descrito que estas son plantas de crecimiento hemiepífito o trepador, es decir, crecen sobre un árbol. Asimismo, se ha señalado que requieren condiciones específicas de temperatura, luz, un rango de altitud determinado, elevada humedad, entre otros factores (Soto-Arenas y Dressler, 2010). Además, su polinización es compleja y en la mayoría de los casos, se realiza de forma manual debido a que las diversas especies de vainilla carecen de recompensas florares. Por lo tanto, utilizan su similitud morfológica con otras especies de orquídeas y sus aromas para atraer a los polinizadores. Diferentes autores han identificado abejas de los géneros *Euglossa* y *Eulaema* como posibles dispersores de polen en *Vanilla* spp., sin embargo, aún no se ha logrado determinar las especies específicas. Con base en lo anterior, el objetivo de este trabajo fue realizar un análisis de la información disponible acerca de los insectos polinizadores asociados a *Vanilla planifolia*.

2. Vainilla, usos y aplicaciones

2.1. Usos de la vainilla

V. planifolia es una orquídea que produce frutos aromáticos comestibles y se ha convertido en el aromatizante más popular a nivel mundial. Ampliamente utilizada en el arte culinario, particularmente en la repostería y la elaboración de helados, así como en diversas industrias como la refresquera, vinícola, tabacalera y de perfumería, entre otras. Por este motivo, la vainilla se considera uno de los productos agrícolas con mayor valor comercial en el mundo (De la Cruz-Medina et al., 2009). El glucósido presente en las vainas de vainilla se convierte a glucosa durante el proceso de curado, y posteriormente se obtiene vanillina que representa apenas el 2% de su peso seco (Gallage et al., 2018). Investigaciones recientes han demostrado que la vainillina posee efectos antinflamatorios, antitumorales, antioxidantes (Kim et al., 2019). Asimismo, se ha descubierto que la vainillina posee una potente propiedad antimicrobiana, lo que permite su uso como conservante natural de alimentos (Anuradha et al., 2013; Kim et al., 2019).

2.2. Países productores

V. planifolia se encuentra en las regiones tropicales de América; sin embargo, su principal área de producción está localizada en Totonacapan, una región integrada por 20 municipios del estado de Veracruz y 19 del estado de Puebla (Luis-Rojas et al*.,* 2020), donde se dónde se produce cerca del 95% de vainilla mexicana (Retes-Mantilla et al*.*, 2015). Los principales importadores de vainilla son Estados Unidos, Francia y Alemania (FAO, 2018; Retes-Mantilla et al., 2015). A pesar de que México es el centro de origen y diversidad de la vainilla y cuenta con agroecosistemas idóneos para su crecimiento y desarrollo, no es el principal productor a nivel mundial (Bory et al*.*, 2008b; FAO, 2018; Lubinsky et al., 2008). Los mayores productores de vainilla incluyen países tropicales como Indonesia, Papúa Nueva Guinea, China y Madagascar (Quintana y Aguilar, 2020), siendo este último responsable de más de la mitad de la producción mundial (Wilde et al., 2019).

2.3. Volumen de producción

La producción mundial de vainilla se estima entre 5, 000 y 10, 000 ton anuales, de las cuales aproximadamente el 95% provienen de *V. planifolia*, siendo Madagascar e Indonesia los principales productores (Díez-Gómez, 2015). En el año 2018, la producción global alcanzó las 7, 575 ton, de las cuales México contribuyó con solo 495 ton (FAO, 2018). Entre 2003 y 2019, la producción nacional de vainilla aumentó de 257 ton a 521 ton, influenciada principalmente por las limitaciones en la producción de Madagascar derivadas de desastres naturales (SIAP, 2020).

Un factor crítico en la producción de vainilla es el bajo rendimiento de las vainas, lo que limita su aprovechamiento. Se estima que, para obtener 1 kg de extracto de vainilla natural, son necesarios aproximadamente 500 kg de vainas frescas, equivalentes a la polinización de 40, 000 flores. Este rendimiento tan bajo implica que únicamente el 1% de la producción mundial de vainilla procede directamente de las vainas, lo cual explica su elevado costo en el mercado (Hansen et al., 2009).

A pesar de la importancia económica y sensorial de la vainilla, la producción mundial de extracto natural asciende apenas a unas 100 ton. Este déficit se suple con sustitutos sintéticos elaborados a partir de eugenol, cumarina y subproductos de fermentación aromática, los cuales dominan el mercado global (Ciriminna et al., 2019; Retes-Mantilla et al., 2015).

2.4. Morfología de la planta

Vanilla es un género que comprende más de 120 especies y pertenece a la familia Orchidaceae (Chaipanich et al., 2020; Wilde et al., 2019). En muchas áreas tropicales, las especies del género *Vanilla* se desarrollan como lianas y hemiepífitas. Este género es ampliamente conocido por el cultivo de *V. planifolia* (Lubinsky et al., 2008); sin embargo, las especies no comercializadas representan fuentes valiosas de rasgos útiles para el mejoramiento en cuanto a resistencia a

enfermedades, calidad aromática y autopolinización (Anuradha et al., 2013).

Por lo general, la floración en las plantas de vainilla ocurre durante el tercer o cuarto año después de la siembra. En cuanto a las condiciones climáticas necesarias para su cultivo, se requieren zonas con clima cálido-húmedo, una temperatura media anual de entre 21 °C y 32 °C, y una humedad relativa del 80% o más (Quintana y Aguilar, 2020). *V. planifolia* crece preferentemente a aproximadamente 750 m.s.n.m., en regiones con sustrato kárstico que reciben más de 2, 500 mm de precipitación anual (Flores-Jiménez et al., 2017; Quintana y Aguilar, 2020). También prospera en suelos ricos en humus y nutrientes como nitrógeno, fósforo y potasio, con un pH entre 6 y 7. Se requiere sombra parcial proporcionada por arbustos o árboles que puedan servir como soporte, en caso contrario, la planta produce enredaderas con hojas más pequeñas, tallos delgados y una floración y fructificación reducidas (De la Cruz et al., 2014).

Anuradha et al. (2013) reportaron que *V. planifolia* puede alcanzar una altura de hasta 150 cm, lo que facilita la polinización manual y la recolección; sin embargo, en condiciones óptimas, puede crecer hasta 15 m. Esta especie presenta un tallo carnoso, suculento, flexible y quebradizo. Los tallos, que son largos, cilíndricos y monopodiales, pueden ser simples o ramificados. Tienen un diámetro de entre 1 y 2 cm, son de color verde oscuro, poseen estomas y son fotosintéticos. Según lo descrito por Soto-Arenas y Dressler (2010), los entrenudos miden entre 5 y 15 cm de largo. Presenta hojas grandes, planas, lisas, gruesas y carnosas, con forma oblongo-elíptica a lanceolada y color verde brillante, estas hojas crecen de forma alternada y tienen un tamaño de entre 8 y 25 cm de largo y de 2 a 8 cm de ancho. La punta de las hojas es aguda o acuminada, mientras que la base es ligeramente redondeada; el pecíolo es corto, grueso y presenta un canal en la parte superior (Anuradha et al., 2013; De la Cruz et al., 2014).

Las plantas de vainilla requieren entre dos y tres años para florecer después de la plantación de los esquejes. Las inflorescencias emergen como protuberancias de color verde claro en las axilas de las hojas, generalmente ubicadas en la parte superior de la planta, y miden entre 5 y 8 cm de largo (Anuradha et al., 2013). Las flores de vainilla son efímeras y, por lo general, su duración no excede un día o apenas unas pocas horas (Anuradha et al., 2013; Chaipanich et al., 2020). En cuanto a su morfología, se ha descrito como diversa, lo que sugiere que varios vectores bióticos podrían intervenir en la polinización de esta especie. Las abejas han sido identificadas como sus polinizadores naturales, atraídas por flores cuya apariencia es similar a otras especies de orquídeas que ofrecen recompensas florales. Las flores de vainilla presentan una tonalidad amarillo verdoso pálido, son grandes, cerosas y fragantes, con un diámetro aproximado de 10 cm y un pedúnculo muy corto.

Los sépalos y pétalos son similares en color y forma, aunque los pétalos son ligeramente más pequeños y forman parte del perianto; las flores poseen tres sépalos oblongo-lanceolados, de entre 4 y 7 cm de largo, y de uno a 1.5 cm de ancho. El pétalo inferior está modificado en un labelo con forma de trompeta, más corto que los demás, con dimensiones de entre 4 y 5 cm de largo y entre 1.5 y 3 cm en su punto más ancho (Figura 1). La punta trilobulada del labelo tiene una tonalidad más oscura y presenta bordes irregularmente dentados. El ginostemo también conocido como columna mide 3 a 5 cm de largo y está adherido al labelo en la mayor parte de su longitud. Es piloso en la superficie interna, llevando en su punta el único estambre que contiene las 2 masas de polen o polinias cubiertas por un estigma en forma de gorro. El estigma de forma cóncava y textura pegajosa, está separado del estambre por el delgado rostelo en forma de colgajo (Díaz-Bautista et al., 2018).

La fruta es una cápsula reconocida como vaina, que mide de 10 a 25 cm de largo y de 5 a 15 mm de diámetro y es reconocida como vaina. Alcanza su tamaño final entre 10 y 15 semanas después de la polinización, los cambios en la concentración de los compuestos químicos precursores del sabor comienzan a ser significativos cuando la coloración de la fruta cambia a amarillenta, por esta razón los productores enfrentan dificultades para predecir el momento óptimo de cosecha, un factor crítico para garantizar la calidad aromática de la vainilla curada (Quintana y Aguilar, 2020; Reyes-López et al., 2014).

Figura 1. Estructura floral de *Vanilla* spp.

3. Problemáticas de cultivo

De la *Vanilla* se desconoce en gran medida su variación genética, así como sus interacciones específicas con polinizadores y hongos micorrízicos, su biología reproductiva especializada, requerimientos nutricionales,

manejo del cultivo, control de plagas y enfermedades, entre otros. El desconocimiento de todos estos tópicos impide realizar acciones de conservación, potencializar las características de calidad para mejorar los beneficios a los usuarios de ese recurso genético (Herrera-Cabrera et al. 2012), identificar y seleccionar clones de *V. planifolia* con características fitoquímicas específicas que se expresan en el aroma (Salazar-Rojas et al., 2012), o que, de forma indirecta, mejoran el contenido de metabolitos secundarios relacionados con la defensa contra plagas y enfermedades.

Una de las principales limitantes del cultivo de vainilla para su aprovechamiento comercial es el crecimiento tardío que presentan las plantas, las cuales requieren entre tres y cuatro años para florecer. Este cultivo enfrenta diversos problemas en su reproducción, como baja viabilidad de las semillas, floración efímera, desconocimiento de las interacciones micorrícicas específicas y ausencia de polinizadores. Soto-Arenas (2006) señaló que las condiciones climáticas del trópico exponen a las plantas a enfermedades que pueden diezmar poblaciones completas debido a la baja variabilidad genética de la especie. Es importante destacar que las plantas cultivadas en sistemas comerciales requieren polinización manual para garantizar la producción, lo que se atribuye a la escasa presencia de insectos polinizadores (Chambers et al., 2019). No obstante, existen poblaciones silvestres de otras especies del género *Vainilla* que han sido menos estudiadas y que podrían ofrecer información valiosa sobre los polinizadores naturales; esta información podría ser de utilidad para llevar a cabo acciones de conservación y reproducción de los insectos (Chaipanich et al., 2020; Gigant et al., 2014; Lubinsky et al., 2006; Soto-Arenas y Dressler, 2010).

4. Polinización en *Vanilla* spp.

4.1. Alogamia

Las plantas con flores han desarrollado una amplia variedad de estrategias de polinización que garantizan una transferencia eficiente de polen, promoviendo así su seguridad reproductiva. En la familia Orchidaceae, la mayoría de las especies dependen de vectores bióticos, como insectos y roedores, para lograr su polinización; reportando una notable diversidad de insectos polinizadores, incluidos lepidópteros, dípteros, himenópteros y ortópteros, así como aves, que desempeñan un papel crucial en el proceso de polinización de estas orquídeas (Bory et al., 2008a; Chaipanich et al., 2020; Costa, 2009; Dos Anjos et al., 2016; Mărgăoan et al., 2019). Pansarin y Pansarin (2014) señalaron que la mayoría de las especies de orquídeas son polinizadas por abejas, las cuales son atraídas mediante diversos recursos o engañadas a través de diferentes estrategias florales. La recompensa floral más común producida por las orquídeas es el néctar, el cual es recolectado por numerosos grupos de himenópteros. Además del néctar, se han documentado otras recompensas florales como el polen, tricomas comestibles, cera, aceites comestibles y fragancias florales (Costa, 2009). Sin embargo no se conocen especies de *Vanilla* que produzcan recompensas florales (Chaipanich et al., 2020), en su lugar, estas plantas emplean aromas específicos y aprovechan su similitud morfológica con otras especies de orquídeas para atraer a los polinizadores. Varias especies de vainilla en los trópicos americanos son polinizadas por abejas del grupo Euglossini (Figura 2), conocidas por recolectar fragancias de orquídeas que no producen néctar (Chaipanich et al., 2020; Gigant et al., 2011). *V. pompona, V. hameri y V. cribbiana* producen grandes cantidades de limoneno y son polinizadas por abejas del género *Eulaema*. Por otro lado, se ha reportado que *V. insignis, V. odorata y V. planifolia* son polinizadas tanto por abejas del género *Euglossa* como por *Eulaema,* y producen pequeñas cantidades de 1-8 cineol (Soto-Arenas, 1999).

Figura 2. Abeja del grupo Euglossini posada sobre una vaina de *V. planifolia*. Foto tomada por Damaris Luna Hernández en parcela "La Ceiba" en municipio de Tuzántan, Chiapas, México.

Otras especies de insectos tales como *Eulaema meriana* y algunas abejas meliponas recolectan polen, pero no logran polinizarlas debido a la poca accesibilidad al labelo (Lubinsky et al., 2006). Además, otros insectos no polinizadores también interactúan con diversas especies del género *Vanilla*, por ejemplo, en *V. siamensis* se ha observado la presencia de hormigas que buscan los nectarios. Hasta el siglo XIX se creía que el género *Vanilla* era polinizado por *Melipona beecheii* Bennet y posteriormente por especies del género *Euglossa* spp. y *Eulaema* spp. (Lubinsky et al., 2006). Se ha reportado que las especies de abejas más pequeñas, *Euglossa,* son polinizadores de *V. planifolia y V. trigonocarpa* Hoehne, mientras que las especies de abejas más grandes *Eulaema* polinizan *V. bahiana* Hoehne (Brasil), *V. pompona* Schiede *y V. insignis* Ames (Chaipanich et al., 2020).

En Brasil, se ha reportado poblaciones de *V. edwallii* Hoehne polinizadas por abejas del género *Epicharis (Apidae: Centridini)*. Esta especie carece de recompensa floral, pero los machos de *Epicharis* son atraídos por sus flores a través de su fragancia (Pansarin et al. 2012), en *V. siamensis* se ha encontrado polinización por abejas del género *Thrinchostoma* las cuales se ven atraídas por el olor

(Chaipanich et al., 2020). Gigant et al. (2016) reportan que hembras de *Allodapula variegata, A. rufogastra* y *Anthophorini* han sido encontradas con polinias en las patas traseras, provocando movimiento de polen en *V. roscheri* (Gigant et al., 2014) mientras que en *V. mexicana* se reporta como polinizadores potenciales a las abejas carpinteras del *género Xylocopa* spp. (Gigant et al., 2016).

4.2. Autopolinización

Algunas especies de vainilla producen frutos por autopolinización espontánea, ésta mecanismo de autogamia se ha documentado para *V. griffithii* Rchb.f., *V. palmarum Lindl., V. planifolia* Jacks. ex Andrews y *V. savannarum* Britt. (Pridgeon et al., 2003). En la Amazonía peruana, se ha identificado una población de Vanilla bicolor Lindl., cuyas flores son exclusivamente autógamas, produciendo frutos únicamente a través de cleistogamia (Van Dam et al., 2010).

4.3. Polinización manual

Debido a la baja fructificación observada en las poblaciones naturales, las plantas cultivadas de *V. planifolia* son polinizadas manualmente para aumentar la producción de frutos, los cuales son una fuente natural de vainillina. Ante la ausencia de polinizadores naturales necesarios en las regiones del océano Índico, el profesor Charles Morren y Edmund Albius, un agricultor, desarrollaron un método de polinización manual como alternativa a la polinización natural (Berenstein, 2016; Lubinsky et al., 2008), este proceso se realizó utilizando una vara de bambú o un instrumento del tamaño de un palillo (Chaipanich et al., 2020). Las flores se sostienen en una mano y el polen se frota contra el estigma, este proceso estimula el ovario. Actualmente se contempla que un trabajador calificado, en promedio, puede polinizar unas 1, 000 flores al día.

En caso de que ocurra la fertilización, los pólenes se adhieren al racimo y aumentan de tamaño, mientras que las flores no fertilizadas se marchitan en un plazo de 24 h. Posteriormente, entre las próximas seis a ocho semanas, las vainas se desarrollan a partir del ovario fertilizado y alcanzan su madurez completa en un período de seis a siete meses (Anuradha et al., 2013).

Anuradha et al. (2013) reportaron como alternativa la inducción de fructificación con hormonas. Sin embargo, se considera menos efectiva, ya que estudios demostraron que la aplicación de ácido 2, 4-diclorofenoxi acético, ácido 2-metoxi-6-dicloro benzoico, ácido indolacético y ácido indol butírico, dio como resultado, vainas partenocarpicas de menor peso, longitud y diámetro en comparación con las vainas polinizadas a mano.

5. Conclusión

Las plantas de *Vanilla planifolia* tienen numerosos visitantes, pero estos no contribuyen a su polinización debido a que el tamaño del labelo les impide acceder al polen. Aunque se han documentado insectos polinizadores en el género *Vanilla*, los estudios no concluyen en afirmar que estos se encarguen del entrecruzamiento de material genético y ayuden a la preservación de las especies de vainilla, ya que su participación es mínima en estado natural, y no se refleja significativamente en la formación de frutos. En otras especies *de Vanilla*, en estado silvestre la formación de fruto es muy baja (alrededor del 20%). En numerosos estudios concluyentes se menciona a las abejas del género *Euglosa* como responsables de la polinización de varios tipos de orquídeas incluyendo *Vanilla planifolia*. Sin embargo, considerando las limitaciones de la polinización natural, la polinización manual se mantiene como la alternativa más viable a nivel comercial para garantizar la producción de vainas.

Conflicto de intereses

Los autores declaran no tener ningún conflicto de intereses.

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ARTÍCULO CORTO

Chitosan-based edible coatings extend shelf life and preserve antioxidant properties of 'Gran Enano' banana under tropical conditions

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Abstract

The shelf life of bananas is significantly reduced under tropical conditions due to rapid ripening, weight loss, and degradation of functional compounds. This study evaluated the effect of two chitosan concentrations (1.5% and 2%) as edible coatings on the physicochemical and antioxidant properties of 'Gran Enano' bananas stored at tropical ambient temperatures (30 ± 2 °C; 70 ± 10% RH) for 12 days. Weight loss, peel color, respiration rate, total soluble solids (TSS) and total phenolic compounds, and antioxidant activity (DPPH and ABTS methods) were monitored every two days. The results showed that the chitosan coatings modified some parameters indicative of the ripening process, with lower TSS and CO2 production values, as well as higher values of the L^* and b^* indices, indicative of color, being found at the end of storage. Antioxidant activity and phenolic content peaked around day 7, decreasing thereafter, although both chitosan treatments maintained significantly higher levels than the control. The 2% chitosan treatment increased antioxidant activity on day 1 (DPPH and ABTS), but at the end of storage, the values were like the control. These results demonstrate that the chitosan concentrations evaluated in edible films do not negatively alter the characteristics of the ripening process and, on the contrary, allow for extended shelf life.

Keywords:
ABTS
DPPH
Respiration
Ripening
Shelf life

Recubrimientos de quitosán prolongan la vida de anaquel y conservan antioxidantes del banano 'Gran Enano' en condiciones ambientales

Resumen

La vida de anaquel del banano se ve significativamente reducida bajo condiciones tropicales debido al rápido proceso de maduración, pérdida de peso y degradación de compuestos funcionales. Este estudio evaluó el efecto de dos concentraciones que quitosán (1.5% y 2%) como recubrimientos comestibles sobre las propiedades fisicoquímicas y antioxidantes del banano 'Gran Enano' almacenado a temperatura tropical ambiente (30 ± 2 °C; 70 ± 10% RH) durante 12 días. Se monitorearon la pérdida de peso, el color de la cáscara, la tasa de respiración, el contenido de sólidos solubles totales (SST) y de compuestos fenólicos totales, así como la actividad antioxidante (métodos de DPPH y ABTS) cada dos días. Los resultados mostraron que los recubrimientos de quitosán modificaron algunos parámetros indicativos del proceso de maduración, encontrándose al final del almacenamiento menores valores de SST, CO2 producido, así como mayores valores de los índices L^* y b^*, indicativos de color. La actividad antioxidante y el contenido fenólico alcanzaron su punto máximo hacia el día 7, disminuyendo posteriormente, aunque ambos tratamientos con quitosano conservaron niveles significativamente superiores al control. El tratamiento con 2% de quitosán incrementó la actividad antioxidante al día 1 (métodos DPPH y ABTS), pero al final del almacenamiento los valores fueron similares al control. Estos resultan demuestran que las concentraciones de quitosán evaluadas en las películas comestibles no modifican negativamente las características del proceso de maduración y por el contrario permiten alargar la vida de anaquel.

Palabras clave:
ABTS
DPPH
Respiración
Maduración
Vida de anaquel

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1. Introduction

Chitosan is a natural biopolymer that has garnered increasing attention over recent decades due to its wide range of applications in the food, pharmaceutical, biomedical, and agricultural industries (Bhowmik et al., 2024). Derived from the deacetylation of chitin—the second most abundant natural polymer—chitosan exhibits several desirable functional properties, including film-forming ability, biodegradability, biocompatibility, and antimicrobial and antioxidant activities. These characteristics make it a promising candidate for the development of edible coatings designed to extend the postharvest shelf life of perishable commodities, particularly fruits and vegetables (Riseh et al., 2023).

The film-forming capacity of chitosan enables the elaboration of thin, transparent, and non-toxic coatings that act as semipermeable barriers, modulating gas exchange (O_2 and CO_2) and thereby delaying ripening and senescence. Moreover, chitosan coatings can inhibit the growth of various pathogenic microorganisms and reduce oxidative spoilage due to their intrinsic antimicrobial and antioxidant properties (Subramani and Manian, 2024). As such, chitosan represents a sustainable and consumer-friendly alternative to synthetic preservatives. Chitosan-based coatings have been extensively studied in both climacteric and non-climacteric fruits, including strawberries, apples, mangoes, guavas, and papayas. Several authors (Hajji et al., 208; Priyadarshi et al., 2024) demonstrated that a 1-1.5% chitosan coating significantly reduced fungal growth, weight loss, and preserved firmness in strawberries during storage. Similarly, a 2% coating extended the shelf life of papaya and reduced anthracnose symptoms (Vilaplana et al., 2020), while studies in pears reported reduced weight loss and respiration rate, along with higher retention of firmness, total soluble solids, titratable acidity, and polyphenol content (Adhikary et al., 2022).

Several studies have confirmed that the efficacy of chitosan coatings is strongly influenced by concentration of the polymer. In apricots, Algarni et al. (2022) found that 1.5% chitosan nanoparticles coating extended shelf life up to 30 days at 5 °C, compared with a 1% concentration. In cherries, Zam (2019) observed that 2% chitosan coatings enriched with olive leaf extract preserved higher levels of phenolic compounds and antioxidant activity than lower concentrations. In tomatoes, coatings of chitosan combined with grapefruit extract effectively prevented weight loss during storage (Won et al., 2018). In papaya, Escamilla-García et al. (2018) reported that a 1% chitosan coating improved firmness, reduced weight loss, and maintained antioxidant activity. Similarly, in cashews nuts, higher chitosan concentrations were more effective at reducing lipid oxidation, as evidenced by lower peroxides and TBARS levels (Azimzadeh and Jahadi, 2024). Vasile and Baican (2021) concluded that increasing chitosan concentrations enhances functional properties such as mechanical strength, selective gas permeability, and antimicrobial and antioxidant effects. However, they caution that excessively high concentrations may produce overly dense films that negatively impact fruit quality.

Banana (*Musa* spp.), a highly perishable climacteric fruit of considerable economic and nutritional importance, is particularly susceptible to rapid postharvest deterioration. Its high respiratory rate and sensitivity to ethylene accelerate ripening, leading to uneven coloration, firmness loss, and increased susceptibility to microbial decay. Several studies have demonstrated the effectiveness of chitosan coatings in mitigating these issues. Hossain and Iqbal (2016) reported that a 1% shrimp-derived chitosan coating significantly reduced weight loss and disease incidence, extending banana shelf life by 3 to 4 days. Suseno et al. (2014) found that a 2% chitosan formulation more effectively preserved vitamin C and reducing weight loss, while Sikder and Islam (2019) observed similar benefits with a 1% concentration. These findings suggest that the performance of chitosan coatings is influenced by several factors, including concentration, degree of deacetylation, molecular weight, application method (e.g., dipping, spraying, or brushing), and storage conditions. Overly concentrated solutions may lead to the formation of dense films that restrict fruit respiration and negatively affect sensory quality. Therefore, optimizing the chitosan concentration for each specific fruit cultivar is essential to ensure postharvest effectiveness and product acceptability.

Although the application of chitosan coatings in bananas has been broadly explored, limited research is available on the 'Gran Enano' (*Musa* AAA) cultivar. Furthermore, the postharvest behavior of this variety when treated with chitosan solutions at concentrations above the commonly used 1.5%—and the corresponding effects on its antioxidant properties—remains largely unknown. Because of this, the objective of this study was to evaluate the effect of edible coatings formulated with different concentrations of chitosan (1.5% and 2%) on the postharvest shelf life and antioxidant properties of 'Gran Enano' bananas.

2. Materials and Methods

2.1. Materials, reagents, and treatments

Banana fruits (*Musa* sp.) cv. 'Gran Enano' at physiological maturity were obtained from a local packing facility in Tapachula, Chiapas, Mexico. A total of 150 fruits from the same harvest lot were selected based on uniform size, absence of defects, and good sanitary condition (50 fruits per treatment). Three treatments were applied: T1 = control (no treatment), T2 = coating with 1.5% chitosan solution (Q-1.5%), and T3 = coating with 2% chitosan solution (Q-2%). Chitosan (85% deacetylated, MW = 340.33), glacial acetic acid (Meyer®), and Tween 20 (Sigma-Aldrich®) were used. All reagents were of analytical grade.

2.2. Preparation of chitosan solutions

Chitosan coating solutions were prepared by dissolving 1.5% or 2% (w/v) chitosan in distilled water previously acidified

with 1.5% (v/v) acetic acid to reach a pH of 4. The mixtures were stirred on a magnetic plate for 24 h and used immediately after preparation (Monzón-Ortega et al., 2018).

2.3. Application of coatings

Banana fruits were washed with distilled water and immersed in a 100-ppm sodium hypochlorite solution for 1 min. They were then individually separated from the cluster using a sterile scalpel and randomly assigned to one of the three treatments. The coating solutions were manually applied using a polyurethane foam brush. After application, fruits were dried at room temperature under continuous airflow. All treated fruits were stored under ambient conditions (30 ± 2 °C; 70 ± 10% relative humidity) in a closed storage room for 9 days (Cruz-Ortiz et al., 2021).

2.4. Postharvest physiology of the fruits

One day after coating application (day 1) and subsequently every 48 h, the following parameters were measured: weight loss (Adventurer™ Pro, model AV264C), external color (MiniScanEZ colorimeter), and total soluble solids (TSS) using a digital refractometer (ATAGO, model PAL-1. CO_2 production was also measured by placing individual fruits in 3 L sealed containers for 2 h. The concentration of CO_2 was then determined using an IAQ-CALC probe (TSI®) and reported as mg CO_2 kg^{-1} h^{-1} (Monzón-Ortega et al., 2018). Weight loss and external color were measured in all fruits per treatment, while the other parameters were determined using five fruits per sampling point, with three measurements per fruit (15 measurements per sampling), performed at the apical, middle, and peduncular regions (Cruz-Ortiz et al., 2021).

2.5. Antioxidant properties of the fruits

2.5.1. Total polyphenols content

A total of 100 mg of banana pulp was macerated in 1 mL of 80% (v/v) methanol (Merck, USA) and stirred at 200 rpm for 24 h at room temperature. The mixture was centrifuged at 2,626 × g for 25 min, and the supernatant was collected and stored at −25 °C. The pellet underwent a second extraction under identical conditions. Both extracts were pooled and stored at −25 °C until analysis. Total polyphenol content was determined using the colorimetric Folin–Ciocalteu method as described by Vázquez-Olivo et al. (2019). Briefly, 20 µL of extract were mixed with 80 µL of 80% (v/v) methanol, 500 µL of Folin–Ciocalteu reagent (1:10 dilution; Sigma-Aldrich, USA), and 400 µL of sodium carbonate solution (Na_2CO_3; Meyer, Mexico). After incubation in the dark for 60 min, absorbance was measured at 765 nm using a microplate reader (MR-96A, Mindray, China). A calibration curve (0–10 mg L^{-1}) was generated using gallic acid (Merck, USA), and results were expressed as mg of gallic acid equivalents per gram of fresh pulp (mg GAE g^{-1}).

2.5.2. DPPH radical scavenging activity

The free radical scavenging activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), following the method described by Farzana et al. (2023), with slight modifications. In brief, 4 mL of a 0.1 mM methanolic DPPH solution were mixed with 1 mL of sample extract. The mixture was vortexed vigorously for 1 min and then incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using a UV-Vis spectrophotometer. Scavenging activity was calculated as follows: DPPH scavenging activity (%) = $(A_{control} - A_{sample})/A_{control} \times 100$.

2.5.3. ABTS radical scavenging activity

The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assay was conducted following the method by Nenadis et al. (2004), with adjustments for microplate absorbance measurements. The $ABTS^{\bullet+}$ radical cation was generated by mixing 5 mL of a 7 mM ABTS solution with 88 µL of 140 mM potassium persulfate ($K_2S_2O_8$; Meyer, Mexico), and incubated in the dark at 25 °C for 12 h. The mixture was diluted with 7% ethanol to obtain an absorbance of 0.80 ± 0.03 at 734 nm, measured using a microplate reader (MR-96A, Mindray, China). For calibration, 280 µL of the $ABTS^{\bullet+}$ solution were mixed with 20 µL of Trolox standard solutions (0–800 µM), and absorbance was recorded after 6 min. The same protocol was followed for banana extracts, using 20 µL of sample extract. Results were expressed as micromoles of Trolox equivalents per gram of fresh pulp (µmol TE g^{-1}).

2.6. Data analysis

All data from the physicochemical variables were subjected to analysis of variance (ANOVA), followed by mean comparison using Tukey's test ($\alpha = 0.05$). Statistical analyses were conducted using XLSTAT © v2012 software.

3. Results and Discussion

3.1. Weight loss

As expected, weight loss increased progressively in all treatments (Figure 1) over time due to transpiration and respiration processes. However, the rate and extent of weight loss were modulated by the presence and concentration of the chitosan coatings. Control fruits exhibited a final weight loss of approximately 23% by day 9, while Q-2% coated fruits showed the lowest loss (~20%). Notably, fruits treated with Q-1.5% displayed slightly higher weight loss than the control from day 3 onwards, which may be attributed to differences in coating uniformity or permeability at lower concentrations. Although these results show a trend and possible effect, no significant differences ($P>0.05$) were found between treatments.

The best performance of the 2% chitosan coating in reducing moisture loss suggests a more effective formation of a semipermeable barrier that limits water vapor diffusion. This agrees with findings in other climacteric fruits, such as tomatoes and apricots, where thicker chitosan layers provided better protection against dehydration (Algarni et al., 2022; Won et al., 2018). Moreover, the reduced water loss in Q-2% may help delay fruit softening and senescence, indirectly contributing to the overall maintenance of

postharvest quality. The results reinforce the importance of optimizing polymer concentration to balance gas and moisture permeability. Although 1.5% is frequently reported as effective, the current data suggest that under tropical storage conditions, a 2% formulation offers greater efficacy in limiting water loss in bananas. Nevertheless, further investigation into film thickness, structural integrity, and microstructural interactions would be necessary to confirm these mechanisms.

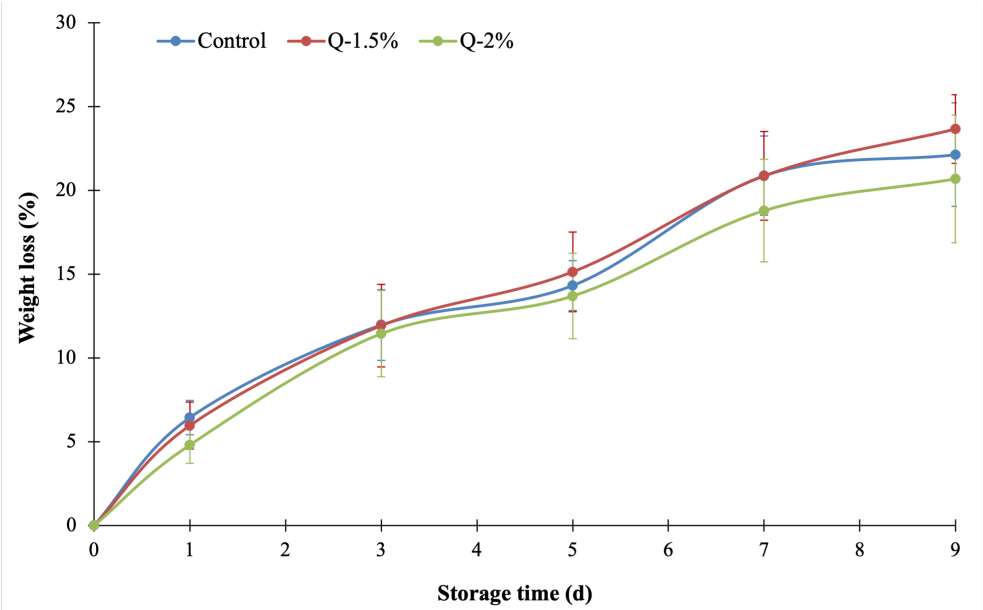

Figure 1. Weight loss of banana with and without chitosan coating during storage under tropical ambient conditions.

3.2. Peel color

*L** (lightness) values indicate the brightness of the peel, where higher values reflect a lighter color. At day 0, control bananas showed the highest *L** (55.73), while Q-1.5% and Q-2% started at lower values (46.78 and 50.49, respectively) (Figure 2). Over time, a marked decline was observed in all treatments, but the extent of the decrease varied significantly only the day 9. This day, *L** dropped sharply in the control (35.8), indicating higher browning and senescence. In contrast, Q-2% maintained a substantially higher *L** (44.67), suggesting that the coating delayed peel darkening. Q-1.5% showed the lowest *L** at day 9 (32.98), indicating less effective preservation of surface brightness. These results suggest that the 2% chitosan coating offered a more efficient barrier against oxidative and enzymatic browning, likely due to reduced oxygen penetration and water loss. Similar trends have been reported in papaya and strawberry, where chitosan-coated fruits retained better peel lightness (Priyadarshi et al., 2024; Vilaplana et al., 2020).

The *a** values reflect the transition from green (negative values) to red (positive values), closely associated with chlorophyll degradation and carotenoid accumulation during ripening. Initially, all fruits exhibited negative values (−10.21 to −8.67), typical of unripe bananas. A rapid increase in *a** values occurred in all treatments as storage progressed, with the control reaching 6.63 by day 9, indicating advanced ripening. Coated fruits followed a similar trajectory, with Q-1.5% peaking at 8.05 (day 7) and Q-2% reaching 6.73 at day 9. Interestingly, Q-2% exhibited a delayed transition to positive *a** values, remaining at −1.79 even at day 3, suggesting a slower breakdown of chlorophyll pigments. This delay in color change further supports the hypothesis that higher chitosan concentrations may reduce ethylene diffusion and respiratory activity, effectively slowing the ripening process (Sikder and Islam, 2019; Suseno et al., 2014).

The *b** component represents the intensity of yellow coloration. At the beginning, control fruits had the highest *b** value (36.79), while Q-1.5% showed the lowest (25.68), and Q-2% was intermediate (32.48). A general decrease in *b** values occurred over time, especially in the control, which reached 13.56 by day 9—a sign of senescence and pigment breakdown. Remarkably, Q-2% retained much of its yellow hue (24.48), whereas Q-1.5% declined more sharply (10.33). The preservation of *b** in Q-2% suggests a protective effect against the oxidative degradation of carotenoids. This may be attributed to both reduced oxidative stress and maintenance of membrane integrity, as suggested the results in antioxidants.

These results reinforce the idea that 2% chitosan coatings are more effective in delaying visible ripening and browning in ‘Gran Enano’ bananas under ambient tropical conditions, in agreement with previous studies in mangoes, tomatoes, and bananas (Hossain and Iqbal, 2016; Silva et al., 2020).

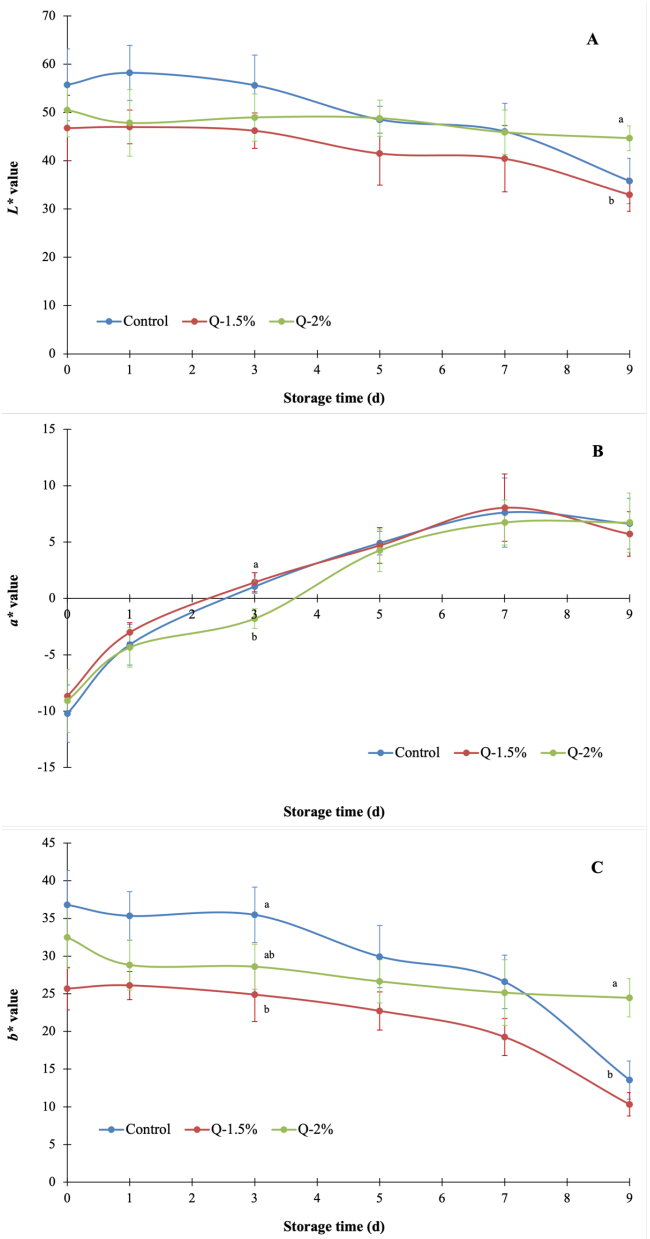

Figure 2. Color parameters of banana peel with and without chitosan coating during storage under tropical ambient conditions.

3.3. Respiration rate

The Figure 3 shows the evolution of respiration rates in 'Gran Enano' bananas. At the start of storage (day 0), all treatments displayed similar respiration rates, ranging from 23.5 to 36.3 mg CO_2 kg^{-1} h^{-1}, with Q-2% showing a slightly higher initial rate (36.3 mg CO_2 kg^{-1} h^{-1}), possibly due to temporary stress following coating application. By day 1, respiration decreased sharply in all treatments, reaching values between 6.2 and 13.0 mg CO_2 kg^{-1} h^{-1}, which may reflect transient metabolic suppression post-treatment and storage acclimatization.

A pronounced climacteric peak was observed on day 5 in the control treatment, with CO_2 production surging to 202.1 mg CO_2 kg^{-1} h^{-1}, indicating the onset of full ripening. In contrast, both chitosan treatments displayed delayed and attenuated peaks: Q-1.5% peaked later (day 9, 188.9 mg CO_2 kg^{-1} h^{-1}), while Q-2% peaked earlier but at a lower magnitude (day 7, 185.6 mg CO_2 kg^{-1} h^{-1}). Q-1.5% exhibited an early modest increase at day 5 (65.4 mg CO_2 kg^{-1} h^{-1}), followed by a sharp rise at day 9, suggesting a stronger delaying effect but less control over the eventual respiratory surge.

These trends confirm that chitosan coatings modulate respiratory activity by acting as a semipermeable barrier that restricts gas exchange and alters the internal atmosphere surrounding the fruit. This effect reduces ethylene action and oxygen availability, thereby slowing down metabolic reactions associated with ripening and senescence (Subramani and Manian, 2024). Among treatments, Q-2% demonstrated the most consistent suppression of respiration through day 9, with lower or more moderated peaks compared to the control and Q-1.5%. This finding aligns with the delay in color change and lower weight loss previously observed, reinforcing the effectiveness of the 2% chitosan concentration in extending postharvest life. The delayed climacteric peak in Q-1.5% and Q-2% also supports previous findings in bananas and papayas, where chitosan coatings postponed peak respiration and associated biochemical changes (Escamilla-García et al., 2018; Suseno et al., 2014).

3.4. Total soluble solids content

Total soluble solids (TSS) reflect the metabolic conversion of starch into simpler carbohydrates during the ripening. In untreated bananas (control), TSS levels exhibited a rapid and pronounced increase from 5.2 °Brix at day 0 to 21.7 °Brix at day 9 (Figure 4), with the most abrupt rise observed between days 3 and 5 (from 7.5 to 19.4 °Brix), corresponding to the peak of ethylene-mediated ripening and starch degradation (Zhu et al., 2021). In bananas coated with 1.5%, the TSS values increased more gradually, reaching 10.3 °Brix by day 5—nearly half of the value observed in the control at the same time point—suggesting a transient delay in starch hydrolysis. However, a substantial rise occurred between days 5 and 7 (from 10.3 to 19.5 °Brix), indicating that the delaying effect of the coating was temporary and that the fruit eventually resumed normal ripening. These results are consistent with reports in strawberries and papayas, where 1–1.5% chitosan coatings delayed—but did not fully suppress—ripening-related metabolic processes (Hajji et al., 2018; Vilaplana et al., 2020). Notably, the 2% chitosan coating demonstrated a more effective and prolonged delay in TSS accumulation. Throughout the first five days of storage, TSS values remained below 8 °Brix, reflecting a more substantial inhibition of respiratory and enzymatic activity.

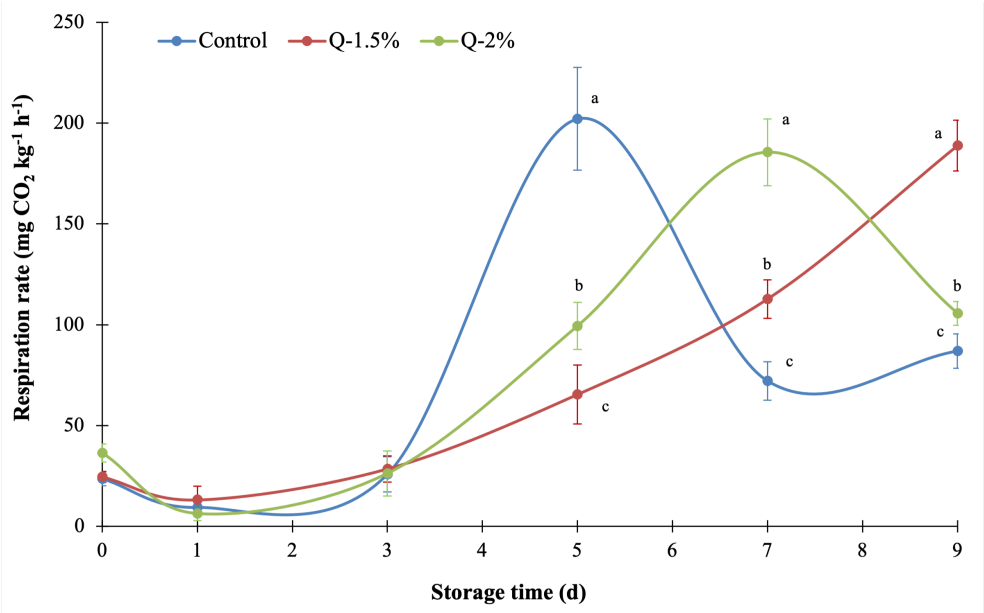

Figure 3. CO_2 production rate of banana with and without chitosan coating during storage under tropical ambient conditions.

This is likely due to the increased thickness and gas barrier properties of the higher-concentration chitosan film, which may have reduced oxygen permeability and suppressed ethylene biosynthesis, as previously documented (Algarni et al., 2022; Vasile and Baican, 2021). By day 9, however, TSS in this group increased sharply to 22.1 °Brix, surpassing the control. This sudden rise suggests that although the chitosan barrier was initially effective, ripening resumed—possibly due to saturation of the film's permeability threshold. The observed dual pattern in the 2% chitosan group (initial suppression followed by a rebound in TSS) aligns with findings in other climacteric fruits where higher chitosan concentrations extended the pre-climacteric phase, yet ultimately allowed normal ripening to resume once internal metabolic signals overcame the diffusion barrier (Subramani and Manian, 2024; Zam, 2019). Overall, these findings confirm that chitosan coatings, particularly at 2%, can effectively delay the onset of ripening in 'Gran Enano' bananas by modulating soluble solids accumulation. However, the effectiveness is time-limited, and extended storage may lead to a compensatory increase in TSS.

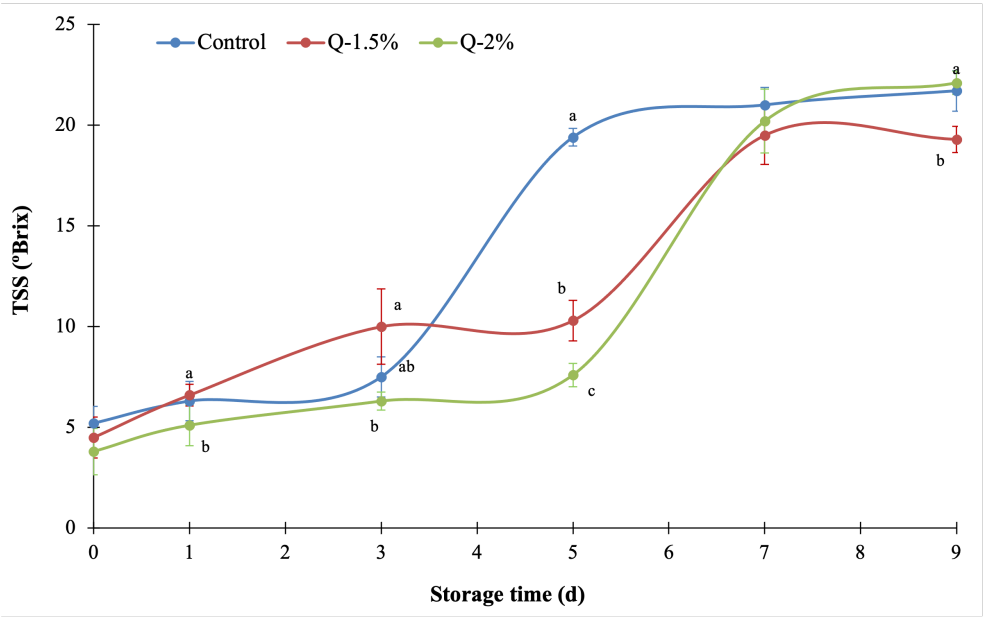

Figure 4. Total soluble solids content of banana with and without chitosan coating during storage under tropical ambient conditions.

3.5. Total phenolic content and antioxidant activity

At the beginning of storage (day 0), no significant differences (P>0.05) were observed among treatments for TPC or antioxidant capacity (Table 1), indicating that the coating process itself did not immediately alter the initial biochemical status of the fruit. However, variations became evident as storage progressed.

The TPC in control fruits showed fluctuations, peaking significantly on day 5 (5.1 ± 0.4 mg GAE 100 g^{-1}), likely due to stress-induced phenolic synthesis in response to abiotic conditions. In contrast, TPC in coated fruits (Q-1.5% and Q-2%) remained relatively stable throughout storage, ranging from 3.2 to 3.9 mg GAE 100 g^{-1}, with no significant (P>0.05) increases. This suggests that chitosan coatings may reduce oxidative or physiological stress, thereby stabilizing the phenolic profile. These findings align with previous observations in mangoes and papayas, where chitosan coatings were found to suppress stress-related phenolic accumulation (Salgado-Cruz et al., 2021; Vilvert et al., 2023).

Regarding DPPH radical scavenging activity, the control treatment showed variable values across storage, with a decline on day 1 (20.7 ± 1.8%) followed by an increased-on days 3 and 9. Interestingly, fruits coated with 2% chitosan exhibited a significantly higher DPPH activity (25.0 ± 1.9%) than the control and Q-1.5% treatments as early as day 1 (P<0.05). This trend continued throughout storage, with Q-2% consistently maintaining or improving radical scavenging capacity, suggesting that higher chitosan concentrations may enhance or preserve antioxidant systems. Similar behavior was reported in cherries and tomatoes where enriched chitosan matrices improved ROS-scavenging capacity (Zam et al., 2019; Won et al., 2018).

Table 1. Phenolic compounds content and antioxidant activity of banana fruits coated with chitosan and stored under tropical environmental conditions.

t	TPC (mg GAE 100 g^{-1})			DPPH (%)			ABTS (µmol TE g^{-1})		
	Control	Q-1.5%	Q-2%	Control	Q-1.5%	Q-2%	Control	Q-1.5%	Q-2%
0	4.0 ± 0.2	3.8 ± 0.5	3.7 ± 0.3	22.9 ± 3.4	22.6 ± 2.9	21.8 ± 3.8	65.9 ± 3.4	65.5 ± 4.2	66.6 ± 2.8
1	4.0 ± 0.2	3.4 ± 0.4	3.5 ± 0.5	20.7 ± 1.8	22.0 ± 1.0	25.0 ± 1.9*	68.5 ± 1.1	68.4 ± 0.9	66.7 ± 0.8*
3	3.4 ± 0.8	3.3 ± 0.2	3.5 ± 0.6	24.8 ± 3.8	24.1 ± 3.4	24.8 ± 4.5	67.2 ± 0.9*	65.4 ± 3.8	64.1 ± 3.5
5	5.1 ± 0.4*	3.3 ± 0.3	3.2 ± 0.2	20.1 ± 1.3*	23.0 ± 0.9	25.0 ± 1.4	64.0 ± 2.8	64.9 ± 4.2	65.6 ± 3.6
7	3.5 ± 0.7	3.9 ± 0.5	3.6 ± 0.3	23.3 ± 4.5	22.1 ± 2.8	25.3 ± 5.1	61.2 ± 5.6	60.6 ± 3.9	61.1 ± 4.5
9	3.4 ± 0.6	3.4 ± 0.4	3.6 ± 0.5	25.7 ± 2.9	27.2 ± 3.9	27.4 ± 3.6	60.9 ± 4.8	60.6 ± 4.2	60.2 ± 2.9

t = time of storage at room conditions (days); TPC = Total phenolics compounds. * Values with an asterisk are different from the other treatments (P<0.05) for the same sampling.

ABTS radical scavenging activity showed no major differences among treatments during the early storage period (days 0–5), with values ranging from 64–68 µmol TE g^{-1}. However, a significant increase was observed in Q-1.5% and Q-2% treatments on day 1 (66.7 and 66.7 µmol TE g^{-1}, respectively), compared to the control (68.5 µmol TE g^{-1}). Although the differences were minor, these findings suggest a protective effect of chitosan in maintaining redox stability during the early stages of ripening. By day 9, all treatments exhibited a decline in antioxidant parameters, with TPC and both scavenging activities decreasing slightly. However, Q-2% maintained the highest antioxidant levels, indicating a better protective effect against oxidative degradation during extended storage. This agrees with findings from Azimzadeh et al. (2018), where higher concentrations of chitosan reduced lipid peroxidation in cashew nuts.

Overall, the data indicate that chitosan coatings, particularly at 2%, can preserve antioxidant potential and phenolic stability during postharvest storage. This effect may be attributed to the semipermeable barrier formed by the coating, which reduces oxidative stress, moisture loss, and enzymatic activity, thereby delaying senescence.

4. Conclusion

Chitosan-based edible coatings proved to be an effective postharvest treatment to extend the shelf life and maintain the physicochemical and antioxidant quality of 'Gran Enano' bananas under tropical conditions. The application of 2% chitosan was particularly effective in reducing respiration rate, delaying the TSS accumulation, and preserving total phenolic and antioxidant activity throughout storage.

Conflict of interest

The authors declare that they have no conflict of interest

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